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The Sko1p Repressor and Gcn4p Activator Antagonistically Modulate Stress-Regulated Transcription in *Saccharomyces cerevisiae*

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In the transcriptional response of *Saccharomyces cerevisiae* to stress, both activators and repressors are implicated. Here we demonstrate that the ion homeostasis determinant, *HAL1*, is regulated by two antagonistically operating bZIP transcription factors, the Sko1p repressor and the Gcn4p activator. A single CRE-like sequence (CRE_{HAL1}) at position −222 to −215 with the palindromic core sequence TTACGTAA is essential for stress-induced expression of *HAL1*. Down-regulation of *HAL1* under normal growth conditions requires specific binding of Sko1p to CRE_{HAL1} and the corepressor gene *SSN6*. Release from this repression depends on the function of the high-osmolarity glycerol pathway. The Gcn4p transcriptional activator binds in vitro to the same CRE_{HAL1} and is necessary for up-regulated *HAL1* expression in vivo, indicating a dual control mechanism by a repressor-activator pair occupying the same promoter target sequence. *gcn4* mutants display a strong sensitivity to elevated K^+ or Na^+ concentrations in the growth medium. In addition to reduced *HAL1* expression, this sensitivity is explained by the fact that amino acid uptake is drastically impaired by high Na^+ and K^+ concentrations in wild-type yeast cells. The reduced amino acid biosynthesis of *gcn4* mutants would result in amino acid deprivation. Together with the induction of *HAL1* by amino acid starvation, these results suggest that salt stress and amino acid availability are physiologically interconnected.

The transcriptional response of cells to stress conditions has two general requirements, a low expression of defense genes during periods of favorable conditions and a fast increase of their expression during adverse conditions. A low abundance of stress gene transcripts can be achieved by repression of transcription or by the absence or inactivation of positive factors. The immediate up-regulation of defense genes upon stress, on the other hand, will require the inactivation of repressors and/or the operation of gene activators. Repression and activation mechanisms have been implicated in the hyperosmotic and salt stress response of yeast (15, 43).

The yeast *Saccharomyces cerevisiae* responds to hyperosmotic challenge by inducing more than 180 different genes (32, 37). Among the signal transduction pathways contributing to this adaptive response, the high-osmolarity glycerol (HOG) pathway (2) plays a dominant role. This osmosensing mitogen-activated protein (MAP) kinase pathway very rapidly activates the Hog1p MAP kinase by phosphorylation, leading to its nuclear import and subsequently to stress gene induction (6, 22, 31, 35). Additionally, the Ras-cyclic AMP (cAMP)-protein kinase A pathway, which generally responds to stresses and availability of nutrients (for a review, see reference 48), plays an important role in osmotic adaptation (30).

Some mechanisms of transcriptional modulation by these signaling cascades are beginning to be defined. Depending on the promoter architecture of the osmotic stress-regulated genes, various gene activators like Msn2p, Msn4p, Msn1p, and Hot1p (24, 36, 37, 41) or Crz1p/Hal8p (25, 26, 45) contribute to various extents to the transcriptional induction.

Other stress defense genes, such as *ENA1*, show a negative regulation. The Sko1p repressor has been found to participate in the transcriptional response by binding a cAMP response element (CRE)-like promoter sequence. Sko1p is controlled by the HOG pathway (33) and belongs to the bZIP family of transcription factors that recognize their DNA target sequence via a conserved basic region (16) and dimerize by using the adjacent leucine zipper domain (21). Apparently, *S. cerevisiae* has a set of different transcriptional activators and repressors that impose specific expression patterns on certain stress defense genes.

The Gcn4p transcriptional activator, another member of the yeast bZIP family, was originally identified as up-regulating amino acid biosynthetic genes upon amino acid starvation (reference 12 and references therein). Gcn4p dimers bind to AP-1 sites located in the upstream control regions of a multitude of amino acid biosynthetic genes (1, 16) and activate their transcription.

The *HAL1* gene plays an important role in maintaining cellular Na^+/K^+ ion homeostasis and confers salt tolerance when overexpressed in yeast cells (9, 38). *HAL1* is induced by osmotic stress (9) via a derepression mechanism involving the general corepressor Ssn6p/Tup1p (23). Here we demonstrate that *HAL1* transcriptional regulation depends on a single CRE promoter element that confers repression under normal growth conditions by binding the Sko1p bZIP repressor and is activated upon hyperosmotic challenge by Gcn4p. Our findings point to a general role of Gcn4p in hyperosmotic stress adaptation and identify *HAL1* as a natural target for the competitive operation of a bZIP repressor-activator pair.

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TABLE 1. Yeast strains used in this work

Strain	Genotype	Source or reference
W303-1A	*MAT***a** *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*	51
MAP6	W303-1A *ssn6::loxp-KAN-loxp*	33
MAP19	W303-1A *sko1::loxp-KAN-loxp*	33
MAP32	W303-1A *hog1-Δ1::TRP1*	33
MAP33	W303-1A *hog1-Δ1::TRP1 sko1::loxp-KAN-loxp*	This work
APA73	W303-1A *gcn4::loxp-KAN-loxp*	This work
APA75	W303-1A *sko1::loxp gcn4::loxp-KAN-loxp*	This work

MATERIALS AND METHODS

Strains and growth conditions. All the strains of *S. cerevisiae* used in this work are listed in Table 1. Gene disruptions were made as described previously (10) and confirmed by genomic PCR. YPD contained 2% glucose, 2% peptone, and 1% yeast extract. Synthetic medium (SD) contained 2% glucose, 0.7% yeast nitrogen base (Difco) without amino acids, 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] adjusted to pH 6 with Tris, and the amino acids and purine and pyrimidine bases required by the strains. The growth of yeast strains under different osmotic and salt stress conditions was assayed by spotting dilutions of saturated cultures onto YPD plates with the indicated concentration of osmotic agents or salts.

Plasmids. The *HAL1-lacZ* fusion plasmid pRS-909 (*URA3* 2μm) was reported previously (9) and contains 1,071 bp of the *HAL1* promoter fused to *lacZ*. The CRE_{HAL1}-*lacZ* plasmid pPY9 and the 2×CRE_{HAL1}-*lacZ* plasmid pPY17 reporter fusions were constructed by inserting one or two double-stranded oligonucleotides TCGACGGGAAAAATTACGTAAAGCATCG, respectively (giving *Sal*I-compatible ends, representing nucleotides −209 to −231 of the *HAL1* promoter), into the *CYC1-lacZ* fusion pMP206 (33) (*URA3* 2 μm), which contains 250 bp of the *CYC1* upstream control region without upstream activator sites fused to the *lacZ* gene. The point-mutated CRE^*_{HAL1}-*lacZ* reporter (pPY10) was obtained in the same way by inserting TCGACGGGAAAAATT ATTTAAAGCATCG (the 2-base exchange in the CRE-core sequence is underlined). All plasmids were confirmed by sequencing. Site-directed mutagenesis of the *HAL1-lacZ* fusion plasmid pRS-909 was performed as described previously (14). An internal primer pair was used to change the TTACGTAA CRE sequence to TTATTTAA to obtain the *HAL1*-lacZ* fusion plasmid pPY18, which was confirmed by sequencing. The *ENA1-lacZ* fusion plasmid was pFR70, a kind gift of Alonso Rodríguez-Navarro (7).

Northern blot analysis. Total RNA was isolated (3) from YPD-grown yeast cells that were either untreated or subjected to the indicated salt stress conditions. Approximately 30 μg of RNA per lane was separated in formaldehyde gels and blotted onto nylon membranes (Hybond-N; Amersham). Radioactively labeled probes were hybridized in PSE buffer (300 mM sodium phosphate [pH 7.2], 7% sodium dodecyl sulfate, 1 mM EDTA). The probes used were a 0.8-kb PCR fragment of almost the entire *HAL1* gene amplified from pRS903 (9), a 3.3-kb PCR fragment spanning the whole *ENA1* gene amplified from plasmid ML80 (a kind gift of Martin Leube), and PCR fragments representing nucleotides 77 to 706 of *TBP1* and 1 to 1035 of *ATR1* amplified from chromosomal yeast DNA. Signal quantification was carried out using a Fujifilm BAS-1500 phosphorimager.

Expression and purification of epitope-tagged proteins. Almost the entire *GCN4* open reading frame (lacking the sequence encoding first five N-terminal amino acids) was cloned by *Eco*RI-*Pst*I into the pET-28b His tag vector (Novagen). His-tagged Gcn4 protein was produced in *Escherichia coli* BL21, bound to His-bind resin (Novagen), and eluted with 300 mM imidazole-containing buffer. Construction of *GST-SKO1* and purification of glutathione *S*-transferase (GST)-tagged Sko1p were described previously (33).

Gel retardation. The GST-Sko1 fusion protein was tested for CRE_{HAL1} interaction as described previously (33). Binding conditions for His-tagged Gcn4 protein and general conditions of electrophoresis were as described previously (23). Yeast protein extracts were prepared as in reference (23). Oligonucleotides representing CRE_{HAL1} and the point-mutated CRE^*_{HAL1} (the same as that used to construct the corresponding CRE_{HAL1}-*lacZ* plasmids) were labeled by filling the *Sal*I protruding ends with Klenow polymerase.

β-Galactosidase assay. Transformed yeast strains were grown until saturation in SD medium without uracil and then diluted into YPD. Exponentially growing cells were then directly measured or subjected to salt stress by adding 0.4 M NaCl (final concentration) for 20 min or transferred to minimal medium for 1 h. β-Galactosidase activity was determined as described previously (9). All results presented in this work are mean values for at least three independent clones measured in duplicate.

Leucine uptake assay. Exponentially grown cells for transport studies (either untreated or treated for 2 h with 1 M NaCl or 200 mM LiCl) were diluted at 10 mg/ml in 50 mM succinate–Tris buffer (pH 5.5) (either without or with 1 M NaCl or 200 mM LiCl). After 20 min, leucine uptake assays were started by adding L-[U-^{14}C]leucine (American Radiolabelled Chemicals, St. Louis, Mo.) to a final concentration of 10 μM (specific radioactivity, 20 Ci/mol). Measurements were performed as described elsewhere (49).

RESULTS

Sko1p is a repressor of *HAL1* expression. The regulation of *HAL1* gene expression has been previously reported to depend on a repression mechanism occurring on an upstream promoter region, URS_{HAL1} (−231 to −156) (23). A database search (MatInspector 34) revealed a CRE-like sequence at position −222 to −215. To date, only one transcription factor from *S. cerevisiae*, Sko1p, has been described that represses transcription from CRE (28, 33, 50). Therefore, we investigated the role of Sko1p in the transcriptional control of *HAL1*. As shown in Fig. 1A, *sko1* mutants show derepressed *HAL1* mRNA levels under nonstress conditions as compared to the wild-type strain, showing that Sko1p is the repressor acting on the *HAL1* promoter. While *HAL1* transcription in wild-type cells is induced during osmotic stress (0.4 M NaCl), we observed a constant high level of *HAL1* mRNA in *sko1* mutant cells. A very similar result was obtained using a *HAL1*-promoter-*lacZ* fusion (Fig. 1B). Additionally, we tested a *hog1* mutant for repression and derepression of *HAL1-lacZ* since genetic data place Sko1p downstream of Hog1p (33). Accordingly, upon brief hyperosmotic shock, we did not observe any derepression of *HAL1* (Fig. 1B). The use of a *sko1 hog1* double mutant, which showed very similar high *HAL1-lacZ* expression to *sko1* single mutants, indicated that the Hog1 MAP kinase acts through Sko1p on *HAL1* expression (Fig. 1B).

CRE_{HAL1} mediates osmotic stress-dependent repression by binding Sko1p. We next asked whether CRE_{HAL1} is sufficient to mediate the observed stress-regulation. We therefore inserted CRE_{HAL1} (−231 to −209) into a *CYC1-lacZ*-based test system. A single insertion of the 23-base CRE was sufficient to mediate repression under normal conditions and derepression under hyperosmotic stress conditions (Fig. 2). A tandem insertion of CRE_{HAL1} led to a more pronounced repression of the fusion gene (Fig. 2). A 2-bp exchange in the CRE_{HAL1} core sequence completely abolished its function, and *sko1* mutants failed to repress the fusion gene through CRE_{HAL1}. The same behavior was observed for *ssn6* mutants, confirming the previously reported dependence of *HAL1* on the Ssn6p-Tup1p core-

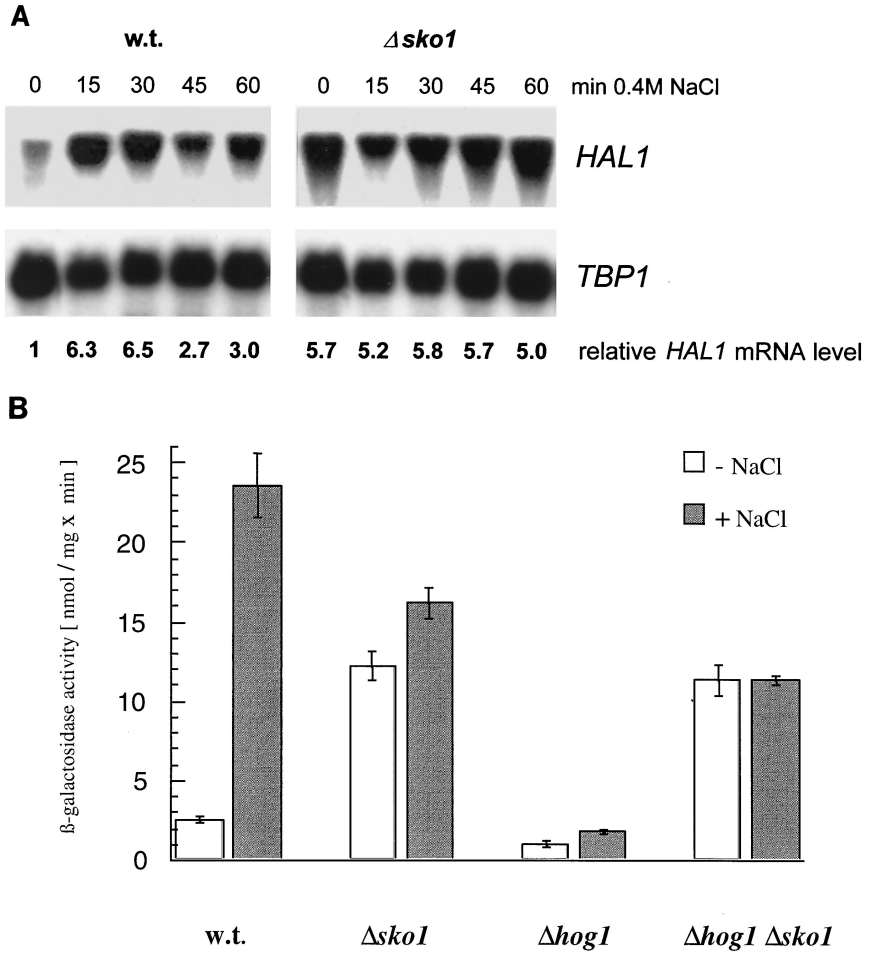

FIG. 1. *HAL1* expression is repressed by Sko1p. (A) Northern analysis of total RNA from the wild type (w.t.) (W303-1A) and *sko1* mutant (MAP19). Cells were subjected to hyperosmotic stress (0.4 M NaCl) for the indicated times. The *TBP1* gene was used as loading control. The relative *HAL1* mRNA level corrected for the *TBP1* control is given below the gels. (B) Repression-derepression of *HAL1-lacZ* depends on Sko1p and Hog1p. *HAL1* expression was monitored using a *HAL1-lacZ* reporter (pRS-909) transformed in wild-type (W303-1A) and *sko1* (MAP19), *hog1* (MAP32), and *hog1 sko1* (MAP33) mutant strains. Transformants were grown in YPD (−NaCl) or treated for 30 min with 0.4 M NaCl (+NaCl).

pressor complex (23). The dependence on the HOG pathway was shown by measuring CRE-driven *lacZ* expression in a *hog1* mutant strain that was highly repressed under both nonstress and stress conditions (Fig. 2).

Using recombinant GST-Sko1p fusion protein, we tested its binding to CRE_{HAL1} (Fig. 3B). Gel retardation assays demonstrated a specific GST-Sko1p-CRE complex that was not observed when the point-mutated and inactive CRE* probe was used. Furthermore, using total-protein extracts from wild-type yeast cells, a protein-CRE complex was found that was not detected in extracts from *sko1* mutant cells (Fig. 3A). Taken together, our results demonstrate that repression of *HAL1* occurs through the Sko1p bZIP repressor bound to its CRE_{HAL1} recognition site.

Gcn4p is an activator of *HAL1* expression. To prove the importance of the CRE_{HAL1} site for stress-dependent regulation, we constructed a point-mutated *HAL1-lacZ* reporter (designated *HAL1*-lacZ*) by changing the TTACGTAA core sequence to TTATTTAA. *HAL1*-lacZ* showed low β-galactosidase activities independently of osmotic stress conditions (Fig. 4). Therefore, we concluded that CRE_{HAL1} did not behave like a pure repressor element but displayed gene activation properties upon osmotic stress. Since mutation of CRE_{HAL1} completely abolished the responsiveness to stress, *HAL1* cannot be regulated simply by the loss of Sko1p-mediated repression, implying the operation of an activator under hyperosmotic stress conditions. Moreover, given the requirement of an intact CRE site within the *HAL1* promoter, this activator should recognize the same CRE_{HAL1} site. Previously it has been shown that a single CRE sequence can repress and activate transcription in artificial promoter fusions (42). The bZIP activator Gcn4p flexibly recognizes both the so-called AP-1 site (consensus, TGACTCA) and the CRE site (consensus, TGACGTCA) (42). Therefore, we investigated the possible role of Gcn4p as an antagonist to Sko1p at the *HAL1* promoter. By using the recombinant His-tagged Gcn4 protein, we examined whether Gcn4p (like Sko1p) can bind in vitro to the CRE_{HAL1} element. As shown in Fig. 3C, we detected a specific binding of Gcn4p to CRE_{HAL1} that was absent when a point-mutated CRE* probe was used. Moreover, the binding of Gcn4p and

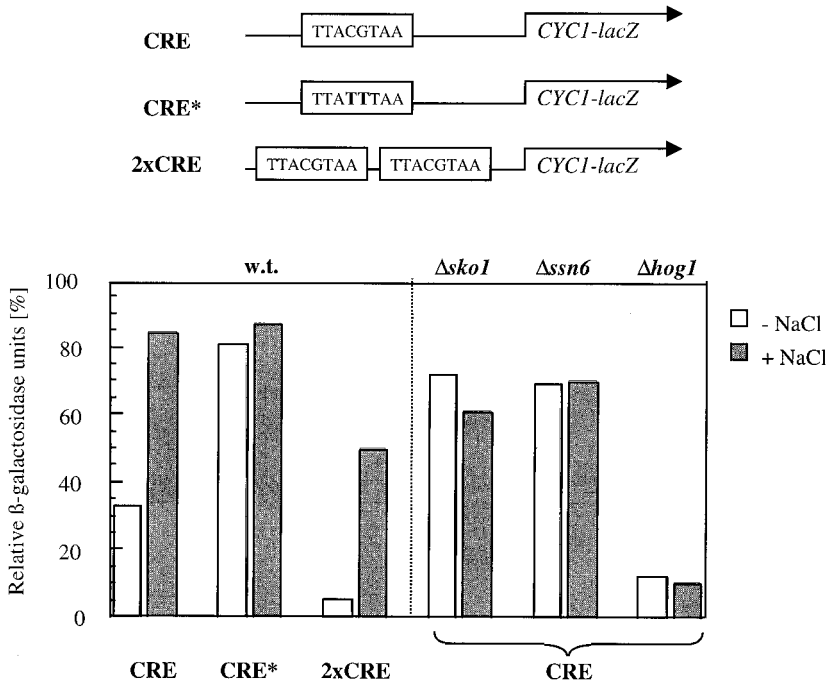

FIG. 2. CRE_{HAL1} confers repression dependent on Sko1p and Ssn6p. A CRE_{HAL1}-*lacZ* reporter was assayed in wild-type (w.t.) (W303-1A) and *sko1* (MAP19), *hog1* (MAP32), and *ssn6* (MAP6) mutant strains. Growth conditions were the same as in the experiment in Fig. 1B. CRE* refers to the insertion of a point-mutated CRE_{HAL1}, and 2×CRE indicates the tandem insertion of two CRE_{HAL1} sites. The degree of expression is given as relative β-galactosidase values compared to the constitutive empty *CYC1-lacZ* vector, which has a value of about 1,000 nmol min^{-1} mg^{-1} in all the strains used. Absolute values are accurate to ±10%.

Sko1p to the CRE_{HAL1} sequence occurs in a competitive manner, as shown in Fig. 3D.

Analysis of *HAL1* mRNA levels revealed that *gcn4* mutants failed to induce *HAL1* transcription upon NaCl shock (Fig. 5A). Moreover, the *gcn4 sko1* double mutant showed the same defect, indicating that derepressed *HAL1* levels in the absence of Sko1p are due to Gcn4p-mediated activation. The slightly elevated *HAL1* transcription in a *gcn4 sko1* double mutant might indicate the existence of a minor Gcn4p-independent activation of *HAL1*. The dependence on Gcn4p was also shown by using a *HAL1-lacZ* reporter, as illustrated in Fig. 5C. In turn, constitutive overexpression of *GCN4* increased *HAL1* transcript levels significantly (data not shown).

We also tested the very recently reported CRE-binding activators Aca1p and Aca2p (8) for their effect on *HAL1* expression. However, we did not find a diminished transcriptional activation when comparing *aca1 aca2* double mutants with the wild type by Northern blot analysis or by the *HAL1-lacZ* reporter assay (data not shown).

Sko1p participates in regulating the transcription of the *ENA1* gene from a CRE site that does not display a palindromic structure (TGACGTTT) (33) and therefore possibly does not fulfill the optimal binding requirements of Gcn4p (42). Accordingly, *ENA1* transcript levels (Fig. 5B), as well as *ENA1-lacZ* expression (Fig. 5D), were unaffected by mutation of *GCN4*. These results also demonstrate that the loss of *HAL1* transcriptional induction in *gcn4* mutants does not result from a general sensitivity of *gcn4* cells to salt stress (see also the following section).

We next tested whether the conditions that activate Gcn4p also increase *HAL1* expression. We therefore examined *HAL1-lacZ* expression before and after the switch from rich medium to minimal medium containing only the amino acids needed to satisfy the auxotrophies (Fig. 5C). By the use of a *GCN4-lacZ* fusion, we confirmed that β-galactosidase production was stimulated by this treatment. Under these conditions, *HAL1* was also induced (Fig. 5C). Moreover, it was similarly dependent on Gcn4p and Sko1p, as was found for salt induction. Gcn4p has been previously shown to activate the transcription of *ATR1* (encoding a multidrug resistance transporter) in response to amino acid starvation in cooperation with Yap1p (4, 17) from an AP-1-binding site, TTAGTAA, suggesting a general role of Gcn4p in stress resistance. Therefore we also examined *ATR1* expression levels under salt stress conditions. As shown in Fig. 5B, the *ATR1* transcript, like *HAL1*, was induced severalfold by NaCl and this induction was absent in a *gcn4* mutant.

***gcn4* mutants are sensitive to salt and osmotic stress.** Our results indicate that the Gcn4p activator plays an important role in the osmotic induction of putative defense genes like *HAL1* and *ATR1*. Therefore we tested the resistance of *gcn4* mutant cells to osmotic and salt stress. We found that the lack of *GCN4* function decreased the tolerance of yeast cells to salt (NaCl and KCl) and osmotic (sorbitol) stresses (Fig. 6). However, stress caused by the highly toxic Li^+ ions did not result in a greater inhibition than in wild-type cells, but, as described previously (33), it was improved by deletion of *SKO1*. It is clear, however, that the salt and osmotic sensitivities of *gcn4* mutants cannot be explained by reduced *HAL1* expression because the observed phenotype is much stronger than in the

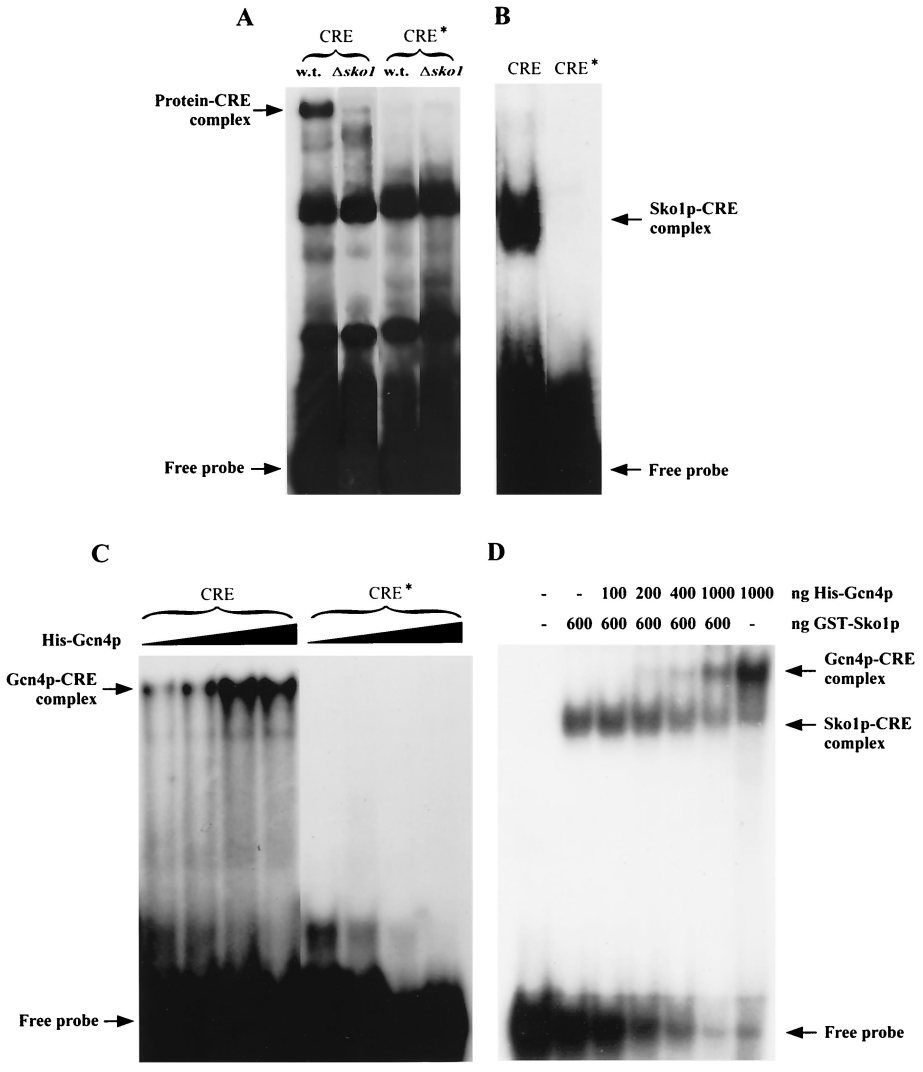

FIG. 3. Sko1p and Gcn4p bind specifically to CRE_{HAL1}. (A) Gel retardation assay using whole-cell extracts from YPD-grown wild-type (W303-1A) or *sko1* (MAP19) mutant cells. Wild-type CRE_{HAL1} or the point-mutated CRE^*_{HAL1} sequence were used as a probe. (B) Gel retardation assay using bacterially expressed and purified Sko1-GST protein. Both lanes contain 600 ng of fusion protein incubated with the wild-type CRE_{HAL1} probe or the point-mutated CRE^*_{HAL1} probe. (C) Gel retardation assay using bacterially expressed and purified His-tagged Gcn4 protein. Increasing amounts of fusion protein (200, 400, 600, and 800 ng) were incubated with wild-type CRE_{HAL1} probe or the point-mutated CRE^*_{HAL1} probe. (D) Competition of Sko1p and Gcn4p for CRE_{HAL1} binding. Bacterially expressed and purified GST-Sko1 and His-tag-Gcn4 proteins were used in a gel retardation assay. Numbers indicate nanograms of fusion protein in the binding reaction using wild-type CRE_{HAL1} probe.

case of *hal1* mutants, which do not exhibit increased sensitivity to either sorbitol or KCl (9). Therefore, *GCN4* function must be important for the expression of additional genes, which become rate limiting for growth under osmotic stress and especially under salt stress. These unknown genes may also be regulated by Sko1p, because deletion of this repressor improves the salt and sorbitol tolerance of *gcn4* mutants (Fig. 6).

Amino acid uptake is inhibited by Na^+. Given that Gcn4p is the major transcriptional activator of a multitude of structural genes involved in amino acid biosynthesis (12), we examined the physiological connection between salt stress conditions and intracellular amino acid abundance. One reason for the sensitivity of *gcn4* mutants could be that high Na^+ or K^+ concentrations impair amino acid uptake and therefore reduce intracellular amino acid pools to a critical level. Therefore we quantified leucine uptake in the absence or presence of salt stress. Figure 7 shows the results of a typical leucine uptake assay comparing cells grown in YPD or treated with 1 M NaCl or 0.2 M LiCl. Both treatments result in a similar growth inhibition of wild-type cells. In these assays, the uptake rate was inhibited by more than 90% by Na^+ (and K^+ [data not shown]), while Li^+ treatment did not significantly affect the transport rate. Identical results were obtained using salt-adapted cells (>20 h of NaCl treatment [data not shown]), showing that the observed inhibition is independent of intracellular adaptation processes. We also measured inhibition of leucine uptake over a range of Na^+ and K^+ concentrations and found that as little as 0.4 M NaCl or KCl inhibited the uptake

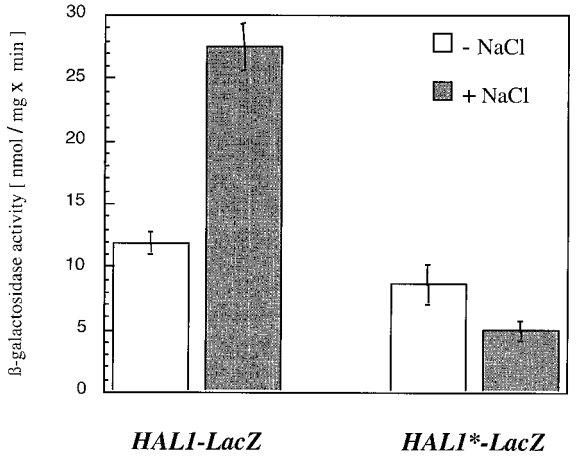

FIG. 4. CRE_{HAL1} function is crucial for salt stress induction. Wild-type yeast cells (W303-1A) were transformed with *HAL1-lacZ* (pRS909, wild-type sequence) or *HAL1*-lacZ* (pPY18, *HAL1* promoter point mutated in the CRE_{HAL1} core). β-Galactosidase activity was determined under the growth conditions described in the legend to Fig. 1B.

efficiency significantly (about 40%), in agreement with the observed growth inhibition of *gcn4* mutants by these ion concentrations (data not shown).

DISCUSSION

Here we present evidence for an interplay between the transcriptional activator and repressor bound at a natural promoter CRE site as a mechanism of hyperosmotic stress response in yeast cells (a schematic overview is given in Fig. 8). The experimental data supporting this model are as follows: (i) the single *HAL1* CRE-like sequence TTACGTAA is essential for osmotic stress-induced expression of *HAL1* and confers osmotic stress regulation to a heterologous test promoter; (ii) Sko1p repressor binds specifically to CRE_{HAL1} in vitro and is necessary for repression of *HAL1* transcription in vivo; and (iii) Gcn4p activator binds specifically to CRE_{HAL1} in vitro and is necessary for activation of *HAL1* transcription in vivo.

Our findings and the originally reported in vitro binding studies (28, 50) indicate that the target sequence for Sko1p can be depicted as T(G/T)ACGT(C/A)A. Suckow and Hollenberg (47) also identified a CRE sequence matching the CRE_{HAL1} core as a possible in vivo target of Sko1p by using a systematic approach based on the use of artificial *CYC1-lacZ* reporters. Sko1p plays a dominant role in the regulation of *HAL1* since a *sko1* mutant strain shows highly elevated *HAL1* mRNA levels that are no longer increased during salt stress (Fig. 1). Therefore, up-regulation of *HAL1* and *ENA1* (and probably other Sko1p target genes) contributes to the salt resistance phenotype of *sko1* mutant cells (33).

However, high *HAL1* expression in the absence of Sko1p is not simply due to the lack of repression but is also due to the operation of an activator. Here we demonstrate that the Gcn4p activator is essential for the stress-induced expression of *HAL1* since neither *gcn4* nor *gcn4 sko1* mutants could increase *HAL1* transcription over the basal level upon salt stress (Fig. 5A and C). In turn, overexpression of *GCN4* increases *HAL1* transcript levels (data not shown). Furthermore, we demonstrate here that Gcn4p (like Sko1p) binds specifically to the CRE_{HAL1} site in vitro (Fig. 3C and D), indicating that Gcn4p activates *HAL1* expression from CRE_{HAL1} while Sko1p represses *HAL1* from the same site.

Gcn4p, which up-regulates the transcription of at least 40 genes under amino acid starvation conditions (for a review, see reference 12), is the best-characterized bZIP factor of yeast. Although initial evidence restricted Gcn4p binding specificity to AP-1 sites (1, 11), it has been found that Gcn4p can bind flexibly to AP-1 and CRE sites (42, 46). Therefore, our results are in perfect agreement with those of in vitro binding assays that qualify CRE sequences as targets for Gcn4p. However, when tested in artificial promoter hybrids, CRE sites failed to activate transcription significantly in a Gcn4p-dependent manner (8, 47). One reason for this discrepancy could be that the in vivo reporters used previously did not fulfill the steric requirements of correct Gcn4p binding. This hypothesis is reinforced by X-ray structural data obtained with Gcn4-bZIP peptides bound to either AP-1 or CRE sequences (5, 19), which revealed that binding of Gcn4p to CRE (but not to AP-1) involves a bending of the target DNA. It was therefore concluded that the flexibility of natural CRE sites might be an important determinant for Gcn4p accessibility (19, 47). Here we show that removal of CRE from its *HAL1* promoter context inactivates *HAL1* induction, as disruption of *GCN4* does. It is very likely that CRE_{HAL1} is a functional Gcn4p-binding site in vivo and therefore meets its bending requirements. Also, it is possible that activation of *HAL1* by Gcn4p requires an additional protein(s) that binds to other promoter sequences outside CRE_{HAL1}. From these results, it is clear that the relevance of a given CRE site for one or another bZIP factor can be estimated only when the natural promoter environment is experimentally maintained.

HAL1 is the first natural target gene of the antagonistic Sko1p-Gcn4p pair. The identification of more CRE sites that function in stress-regulated promoters should reveal more examples of a mechanism that implies the occupancy of CRE by a negative bZIP factor preventing transcription, its stress-induced inactivation, and the operation of a bZIP activator(s) (Fig. 8). This model was originally derived from the overlapping sequence specificities of Sko1p, Gcn4p, and other bZIP activators, as well as from the regulation of artificial CRE-*lacZ* reporters (50). With *HAL1*, we have not found evidence for the participation of additional bZIP transcription factors, at least not under the conditions tested. However, CRE sites can be regulated very differently depending on their surrounding promoter sequences (42), and therefore the relative importance of each bZIP factor might be different in each specific promoter.

Differential gene regulation by competitive occupancy of either an activator or a repressor of one or two neighboring promoter elements has been reported in yeast for some genes. For example, Mig1p repressor and Mal63p activator compete for binding at two adjacent sites in the *MAL62* promoter (52). Also, expression of some genes during sporulation depends on the competitive interplay of Sum1p repressor and Ndt80p activator at middle sporulation elements (54). Moreover, Mig1p-binding sites also bind activators, and the elimination of Mig1p sites in the *SUC2* promoter led to its inactivation, pointing to

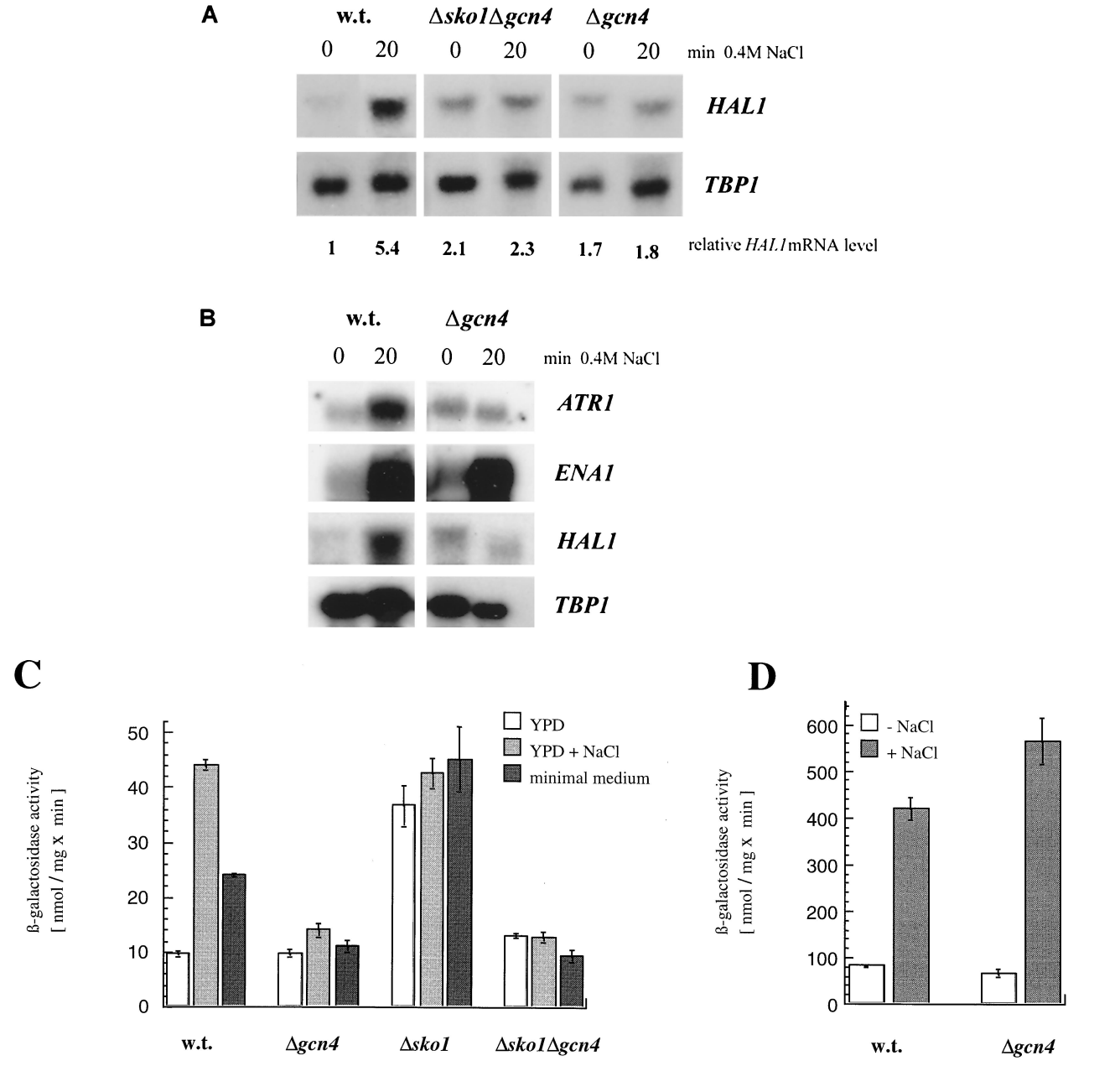

FIG. 5. Gcn4p is involved in salt stress-induced gene expression. (A) Effect of *gcn4* and *sko1* on *HAL1* mRNA levels. A Northern blot analysis of total RNA from wild-type (w.t.) (W303-1A), *gcn4* (APA73), and *sko1gcn4* (APA75) cells grown in YPD without salt or treated for 20 min with 0.4 M NaCl was performed. Relative *HAL1* mRNA levels corrected for the *TBP1* loading control are given below. (B) Effect of *gcn4* on *ATR1* and *ENA1* expression. A Northern blot analysis of total RNA from wild-type (w.t.) (W303-1A) and *gcn4* (APA73) cells grown as indicated in panel A was performed. (C) Effect of *gcn4* and *sko1* mutations on *HAL1-lacZ* reporter expression. *HAL1-lacZ* expression was assayed in wild-type (w.t.) (W303-1A), *gcn4* (APA73), *sko1* (MAP19), and *sko1gcn4* (APA75) strains in the absence (YPD) or presence (YPD + NaCl) of 0.4 M NaCl or after a shift to minimal medium for 1 h. (D) *ENA1-lacZ* expression was assayed in wild-type (w.t.) (W303-1A) and *gcn4* (APA73) strains in the absence or presence of 0.4 M NaCl.

the existence of currently unidentified transcriptional activators competing with Mig1p on glucose starvation (53).

A functional HOG pathway is absolutely required for the immediate derepression of *HAL1*, as well as CRE_{HAL1}-*lacZ*, upon a sudden increase in osmolarity (Fig. 1B and 2). Very recently we showed that Sko1p repressor activity is indeed regulated by direct phosphorylations of the Hog1 MAP kinase (M. Proft, A. Pascual-Ahuir, E. de Nadal, J. Ariño, R. Serrano, and F. Posas, unpublished data). Additionally, Gcn4p could be a target of HOG-mediated activation. However, this is unlikely to play an important role, since in the absence of Sko1p under normal conditions that do not activate HOG, *HAL1* and CRE_{HAL1}-*lacZ* are largely derepressed. Therefore we can speculate that osmotic induction of *HAL1* implies mainly the

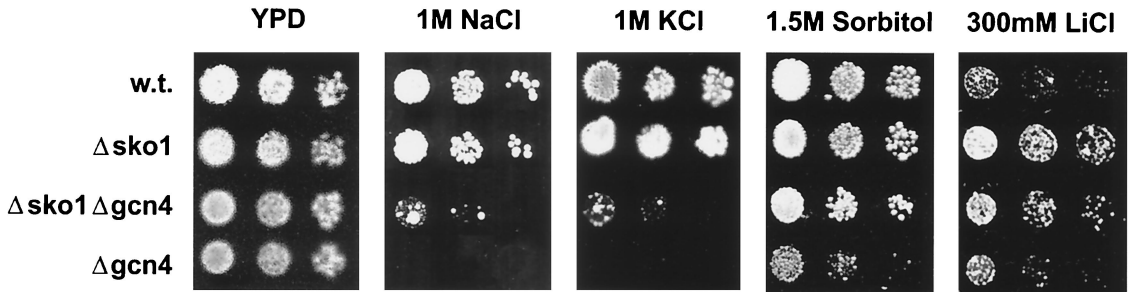

FIG. 6. *gcn4* mutants are sensitive to high Na^+ and K^+ concentrations. The growth of mutant strains *sko1* (MAP19), *gcn4* (APA73), and *sko1gcn4* (APA75) under salt stress or in high-osmolarity media is compared with that of wild-type (w.t.) (W303-1A) cells.

inactivation of the Sko1p repressor by Hog1 that is specifically activated under such conditions. Gcn4p then accounts for high *HAL1* transcription. Under amino acid starvation conditions, the HOG pathway will not be activated, but Gcn4p levels will increase by the well-known translational activation mechanism (13) and thereby will activate *HAL1* expression, probably by competing with Sko1p.

In mammalian cells, various external stimuli lead to the phosphorylation of the bZIP CRE-binding protein (CREB), which subsequently triggers changes in gene expression. CREB is one of the best-characterized stimulus-induced transcription factors, and several kinases (like protein kinase A, Ca^{2+}-calmodulin-dependent kinases, MAPKAP kinase 2, which acts downstream of the mammalian Hog1 homolog p38, and RSK1-3) modulate CREB activity by direct phosphorylation (for a recent review, see reference 44). Interestingly, an important regulatory mechanism has been established in the mammalian system that implies that competition between the CREB activator and the induced cAMP early repressor is necessary for the correct timing of the transcriptional response to cAMP (for reviews, see references 20 and 40). However, in this case the bZIP repressor ICER is expressed from an alternative intronic promoter (27).

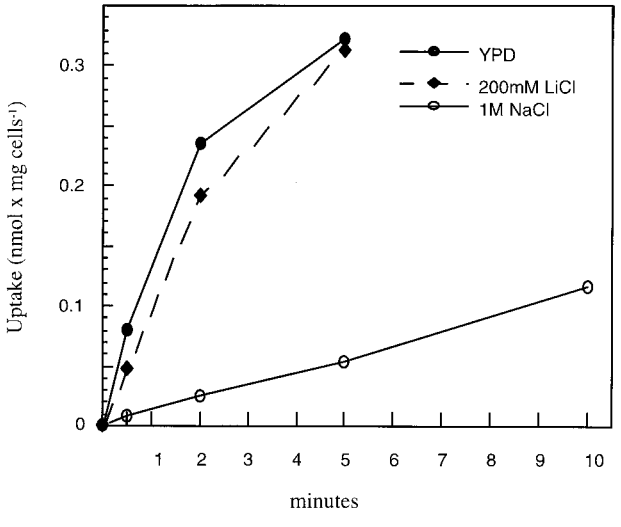

FIG. 7. Leucine uptake is inhibited by NaCl. Yeast wild-type cells (W303-1A) grown in YPD, YPD plus 1 M NaCl, or YPD plus 200 mM LiCl were assayed for uptake of 10 μM leucine.

Gcn4p is the dominant transcriptional activator in the general amino acid control of *S. cerevisiae*. A multitude of genes encoding enzymes of distinct amino acid synthesis pathways are induced in a Gcn4p-dependent manner upon amino acid starvation (for a review, see reference 12). Here we report a role of Gcn4p in the salt stress induction of *HAL1* and *ATR1* (Fig. 5). Both genes are induced by amino acid starvation and salt stress (4, 17; also see above), pointing to a common transcriptional response elicited by both stresses, and this may also be true for other stress-regulated genes. However, the *ATR1* case is different from the regulation we report here for *HAL1*. *ATR1* is up-regulated by the two activators Gcn4p and Yap1p, which have a common AP-1 site in the *ATR1* promoter (4), while *HAL1* differential expression is achieved by competitive binding of Sko1p repressor and Gcn4p activator at a CRE site. Gcn4p function seems to be crucial for the adaptation and survival under severe salt stress (1 M NaCl and KCl) (Fig. 6). This phenotype is the opposite of that reported for the loss of Sko1p function, which leads to hyperresistance to high Na^+ and Li^+ concentrations (33), again reflecting the antagonistic roles of both transcription factors in salt stress adaptation.

Having found that a key regulator of general amino acid control also plays a regulatory role in the expression of genes that are important during salt challenge, we asked whether salt stress is interconnected with amino acid starvation. We hypoth-

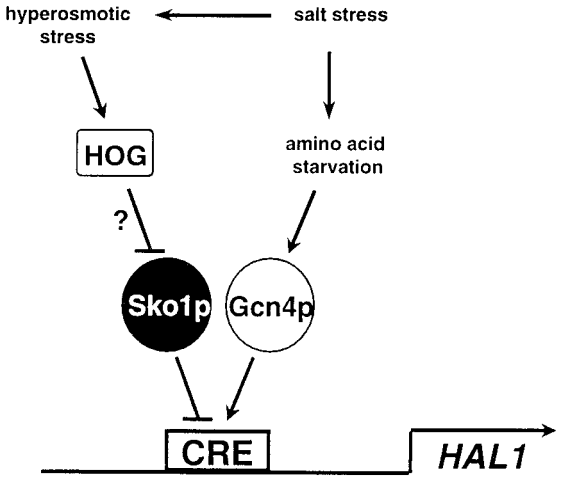

FIG. 8. Schematic overview of transcriptional regulation of *HAL1*.

esized that growth in the presence of elevated salt concentrations could provoke a starvation situation for some or all amino acids. Experimentally we provide strong evidence for this hypothesis, at least at the level of NaCl-dependent inhibition of amino acid uptake (Fig. 7). Our results are in agreement with those obtained previously with *S. cerevisiae* and *Candida membranefaciens*, where NaCl inhibited the uptake of several amino acids (18, 29). Therefore, one important consequence of salinity stress is the general inhibition of amino acid import. Active uptake of amino acids depends on the electrochemical proton gradient generated by the plasma membrane H^+-ATPase (49). High concentrations of Na^+ and K^+ would depolarize the yeast plasma membrane through uptake by the low-affinity monovalent cation system of yeast (39). This, in turn, would cause an internal amino acid depletion, which we have actually measured (A. Pascual-Ahuir, J. Calvete, and R. Serrano, unpublished data). This situation is normally counteracted by activation of *GCN4* transcription and translation and the subsequent up-regulation of amino acid biosynthetic genes.

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