Yeast putative transcription factors involved in salt tolerance

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Abstract Four putative yeast transcription factors (Hal6–9p) have been identified which upon overexpression in multicopy plasmids increase sodium and lithium tolerance. This effect is mediated, at least in part, by increased expression of the Ena1p Na^+/Li^+ extrusion pump. Hal6p and Hal7p are bZIP proteins and their gene disruptions affected neither salt tolerance nor *ENA1* expression. Hal8p and Hal9p are putative zinc fingers and their gene disruptions decreased both salt tolerance and *ENA1* expression. Therefore, Hal8p and Hal9p, but not Hal6p and Hal7p, qualify as transcriptional activators of *ENA1* under physiological conditions. Hal8p seems to mediate the calcineurin-dependent part of *ENA1* expression.

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Key words: Signal transduction; Salt stress; Leucine zipper; Zinc finger; *Saccharomyces cerevisiae*; Calcineurin

1. Introduction

Molecular mechanisms underlying cell adaptation to salt stress are being analyzed in the model organism *Saccharomyces cerevisiae*. Over the last years, genetic analysis has identified many yeast genes involved in halotolerance [1]. The *ENA/PMR2* locus, a gene tandem array encoding isoforms of a putative P-ATPase [2], is an important determinant of sodium and lithium tolerance [3]. Deletion of that locus makes cells hypersensitive to NaCl and LiCl. The first gene of that tandem, *ENA1/PMR2A* (in the following referred as to *ENA1*), is the most expressed and encodes a putative ATPase necessary for Na^+ and Li^+ efflux [4].

ENA1 expression is increased by osmotic and salt stress [4,5] and many components of the signal transduction pathways operating during these stresses have been identified as important determinants of halotolerance. The HOG pathway [6], activated by non-specific osmotic stress, mediates *ENA1* induction at low salt concentrations. Under severe sodium stress, the calcineurin mediated pathway further stimulates *ENA1* expression [5]. Unrelated to these pathways, yeast *HAL1* and *HAL3* genes, which confer halotolerance by overexpression, apparently contribute to reduction of intracellular sodium levels by increasing *ENA1* expression. In addition, protein kinase A and the PPZ protein phosphatases act as negative factors on *ENA1* expression [1].

Many transcription factors responsive to different stresses such as heat shock, oxidative damage, drugs, etc. have been characterized in *S. cerevisiae*, but none of them is involved in controlling ion homeostasis under salt stress [1]. To identify genes encoding transcription factors relevant to halotolerance, we have isolated yeast genes that, upon overexpression in multicopy plasmids, improve cell growth at high external sodium and lithium concentrations [7]. Four genes encoding as yet uncharacterized putative transcription factors have been found: two of them encode leucine zipper (bZIP) proteins and do not seem to be part of the physiological ion homeostasis machinery. The remaining two genes encode zinc binding proteins which are involved in regulating the expression of the Ena1p sodium pump.

2. Materials and methods

S. cerevisiae strains RS-16 [7] and W303-1A [8] have been described. The *ena1-4::HIS3* mutant in the W303-1A background was obtained from Dr. A. Rodriguez-Navarro (Madrid). Standard methods for yeast culture and manipulation were used. YPD and minimal medium were prepared as described [9]. Strains were derived by transformation using the lithium acetate procedure [10]. For halotolerance test, cells were grown for 48 h in liquid medium without salt, and 5 μl aliquots of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were dropped on salt restrictive plates. Growth was recorded after 5–7 days at 30°C. The calcineurin inhibitor FK506 was a generous gift of Dr. Ihor Bekersky (Fujisawa Pharmaceutical Company, Deerfield, IL, USA).

For the isolation and identification of the new *HAL* (halotolerance) genes, *S. cerevisiae* strain RS-16 was transformed with three independent genomic libraries in the *URA3* multicopy plasmid YEp24 [11]. Transformants were selected in minimal medium without uracil, pooled and plated either in the former medium containing 0.2 or 0.4 M LiCl, or in rich medium containing 1.5 M NaCl. Eighty-nine superresistant clones were selected, purified and confirmed after rescreening. Plasmid DNAs were isolated in bacteria and analyzed by restriction using *Eco*RI/*Hin*dIII and *Bam*HI/*Pst*I endonuclease pairs. To eliminate plasmids carrying already known halotolerance genes [1], digested DNAs were separated by electrophoresis, blotted to nylon membrane and hybridized with *HAL1*, *HAL2*, *HAL3*, and *ENA1* probes labeled with [α-^{32}P]dCTP. Twenty-five selected plasmids were partially sequenced using direct (D) 5′-ACTACGCGATCATGGCG-3′ and reverse (R) 5′-TGCCGGCCACGATGCGT-3′ primers (sequences adjacent to the cloning *Bam*HI site in YEp24) to obtain nucleotide sequences at the ends of the inserts. The complete sequence and physical map of each clone was inferred by comparing with the *Saccharomyces* genome sequences in the database (http://genome-www.stanford.edu./Saccharomyces/).

The four putative *HAL* genes were subcloned in YEp351 multicopy plasmid [12]. *HAL6* was subcloned as a *Hin*dIII-*Xba*I fragment. *HAL7*, *HAL8* and *HAL9* were amplified by standard polymerase chain reaction (PCR), and inserted at the *Bam*HI site in the polylinker. The primers used were:

HAL7(D) 5′-GCGGATCCAGCAGAACGTGACGTAT
HAL7(R) 5′-CGGGATCCTCGCTTGTTTCAGGGTT
HAL8(D) 5′-CGGGATCCTCTTGGCATCATCCACA
HAL8(R) 5′-CGGGATCCCCACGTAAAACGCATCC
HAL9(D) 5′-CGGGATCCAACTGGATACGTCTAGG
HAL9(R) 5′-CGGGATCCCTATTGCGTGCTATAGC

Gene disruptions were designed to delete the most relevant structural domains found in each open reading frame (ORF). For convenience of genetic markers, disruptions were made in *S. cerevisiae* strain

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W303-1A. Plasmid pJJ215 was used for *HAL6* and *HAL8* disruptions [13]. To construct *HAL7* and *HAL9* disruptions, a pJJ248 [13] derivative with the *TRP1* gene (*Bam*HI-*Bgl*II fragment) replaced by the *HIS3* gene (*Bam*HI fragment from pJJ215) was used. This is referred to below as pBDHIS.

HAL6 gene disruption: a 5′ flanking region of 1.1 kbp was PCR-amplified using as primers 5′-GCTCTAGATCCTTCTTGTTCCAAGC and 5′-GCTCTAGAAATCAGGTGTGCAGCTA, *Xba*I digested and subcloned at the *Xba*I site of pJJ215. The 3′ flanking region was obtained as a 0.6 kbp *Eco*RI-*Sca*I fragment and subcloned into the *Eco*RI and *Sma*I sites of the former plasmid. The resulting plasmid was digested with *Eco*RI and *Sph*I to promote complete disruption of the *HAL6* ORF upon yeast transformation.

HAL7 gene disruption: a 5′ flanking region of 1.0 kbp was amplified by PCR using as primers 5′-GCGGATCCAGCAGAACGTGACGTAT and P5′-CGGGATCCGCTGCTTCCCTGATTAT, *Bam*HI digested and subcloned at the *Bam*HI site of pBDHIS. The 3′ flanking region (0.35 kbp) was amplified by PCR using as primers 5′-CGGGATCCTCGCTTGTTTCAGGGTT and 5′-CCGCTCGAGGGAGGAAATAGTAGGG, digested with *Xho*I and *Nde*I nucleases, and subcloned into the *Sal*I and *Nde*I sites of the former plasmid. Digestion of the resulting plasmid with *Alw*44I and *Eco*RI released a 3 kbp fragment which was used for yeast transformation. Integration results in over 90% deletion of ORF YDR259c, including the bZIP domain.

HAL8 gene disruption was done by replacing the 1.2 kbp coding sequences between two *Bgl*II internal sites by the *Bam*HI fragment (including *HIS3*) from plasmid pJJ215. Cutting the resulting plasmid with *Nsi*I and subsequent ligation allowed elimination of a 1.0 kbp *Nsi*I fragment containing the C-terminal zinc finger region. Integration, achieved by transformation with a 2.5 kbp *Bam*HI fragment, results in 81% deletion of the *HAL8* coding sequence, including the zinc finger region.

HAL9 gene disruption: a 0.95 kbp *Kpn*I fragment of upstream sequences was subcloned into pJJ248. Then, a 0.98 kbp *Eco*RI fragment was transferred to pBDHIS. 3′ flanking sequences were then subcloned as a *Sph*I 1.26 kbp fragment. A 3.54 kbp *Asn*I-*Rca*I restriction fragment was used to promote a 1.9 kbp genomic deletion of the *HAL9* locus. All genomic deletions were confirmed by PCR and Southern analysis.

The *ENA1-lacZ* fusion plasmid pFR70i [14] was linearized with *Nco*I before transformation to direct integration into the *ura3* locus. For gene expression assays, NaCl was added to exponentially growing cells in rich medium (absorbance at 660 nm about 1), and cultures were incubated at 30°C for 3 h. β-Galactosidase activity was measured in permeabilized cells as described [7] and normalized to protein concentration. Values represent the average of at least three determinations, with three independent transformants.

The intracellular lithium content was measured by atomic absorption spectrometry as described [15].

3. Results

After screening over 200 000 yeast overexpressed clones, we identified 14 novel genetic determinants of salt tolerance. Four of them (Fig. 1A) contain putative ORFs showing high homology to known transcription factors. These four candidate genes, named *HAL6–9*, when subcloned in multicopy plasmid and transformed into yeast, were able to confer resistance to high sodium and lithium concentrations (Fig. 1B).

These new halotolerance genes are present in one copy per haploid genome: *HAL6* and *HAL9* genes belong to chromosome XV, whereas *HAL7* and *HAL8* are located on chromosomes IV and XIV, respectively (Fig. 1A). They were identified during the systematic *Saccharomyces* genome sequencing project as ORF YOR028c (Hal6p), ORF YDR259c (Hal7p), ORF YNL027w (Hal8p), and ORF YOL089c (Hal9p).

Examination of the predicted amino acid sequences of the novel halotolerance genes revealed the presence of DNA binding motifs in all of them. Hal6p (295 residues) and Hal7p (383 amino acids) sequences show, at positions 242–257 and 226–241 respectively, a structural motif known as bZIP (Fig. 2A), also present in a family of yeast transcription factors related to the mammalian c-Jun oncoprotein [16]. Among them, the *Saccharomyces* transcriptional activators Yap1p and Cad1p/Yap2p exhibit the highest homologies. These transcription factors have been described as mediators of stress responses to drugs, metals and oxidants [17,18].

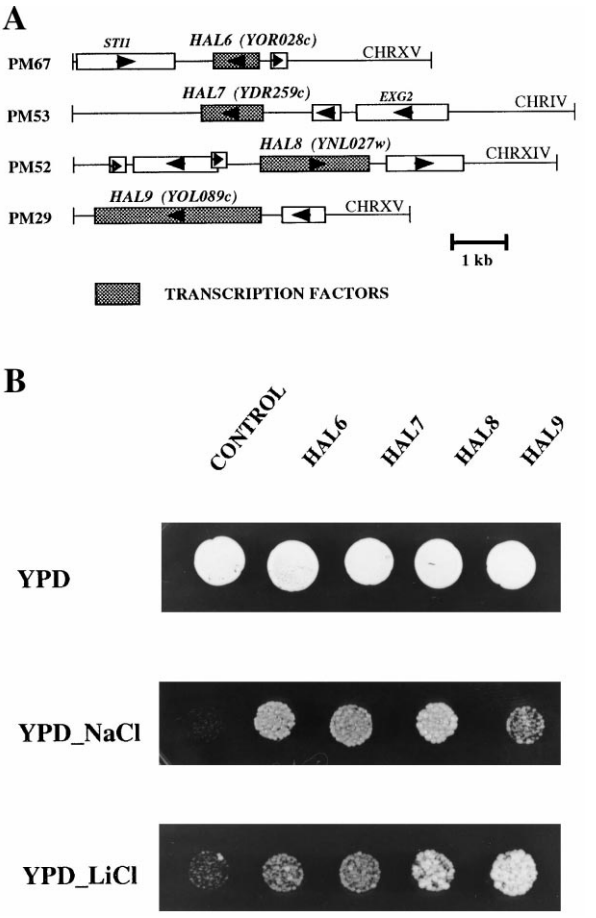


Fig. 1. A: Schematic representation of *S. cerevisiae* genomic fragments containing the halotolerance genes *HAL6–9*. B: Phenotypes conferred by multicopy plasmids containing the subcloned halotolerance genes. Transformed RS-16 cells (Ena^+) were spotted on plates with normal growth medium (YPD) or medium supplemented with 1.2 M NaCl or 0.25 M LiCl as indicated. Control refers to cells transformed with empty plasmid.

The other two open reading frames are predicted to be zinc-binding proteins. Hal8p (678 amino acids) shows two C2H2 zinc finger motifs [18,19] at the C-terminal region (residues 569–591 and 597–619) of the coding sequence, and an adjacent third unusual finger has also been predicted (amino acids 627–661) (Fig. 2B). In addition to this putative DNA binding domain, Hal8p has three regions (residues 115–140, 367–370, and 483–487) consisting of clusters of asparagine and glutamine residues. This kind of domain is involved in activation of gene expression by known transcription factors [16]. A recent *Saccharomyces* zinc finger protein classification, based on finger structure homologies, groups together the *HAL8* gene product with Swi5p and Ace2p, two cell cycle regulated transcription factors [19].

The remaining ORF YOL089c (Hal9p), a 1030 amino acids polypeptide, contains near the N-terminal end (amino acids

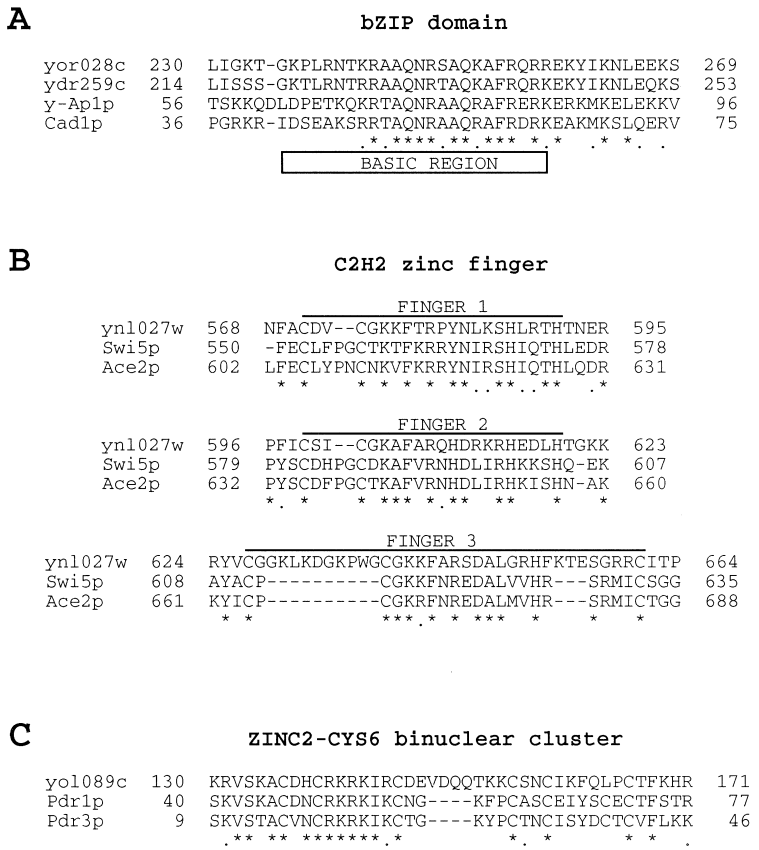


Fig. 2. Alignment of amino acid sequences of the DNA binding domains of Hal6p (ORF YDR259c), Hal7p (ORF YOR028c), Hal8p (ORF YNL027w) and Hal9p (ORF YOL089c) with those of representative yeast transcription factors containing bZIP, C2H2 zinc fingers and zinc2-cys6 binuclear cluster motifs [17]. Invariant residues are indicated by an asterisk, while conservative replacements are shown by a single dot.

135–166), a variation of the zinc finger motif known as the zinc2-cys6 binuclear cluster domain (Fig. 2C). This structural motif has been found in yeast transcription activators such as Pdr1p and Pdr3p, implicated in transcriptional control of genes responsive to drug stress [17,18].

In *S. cerevisiae*, the adaptation to salinity is primarily based on the Na^+/Li^+ extruding ATPase encoded by the *ENA1* gene [3]. Halotolerant cells often show high levels of *ENA1* expression, which alleviate internal toxic cation concentration [1]. We have measured the transcriptional activity of the *ENA1* promoter after transformation with the *HAL6–9* multicopy plasmids into a yeast strain having an integrated *ENA1-lacZ* reporter fusion. As shown in Fig. 3, when overexpressed, all of these halotolerance genes moderately (30–50%) increase *ENA1* expression under salt-induced conditions, with *HAL8*

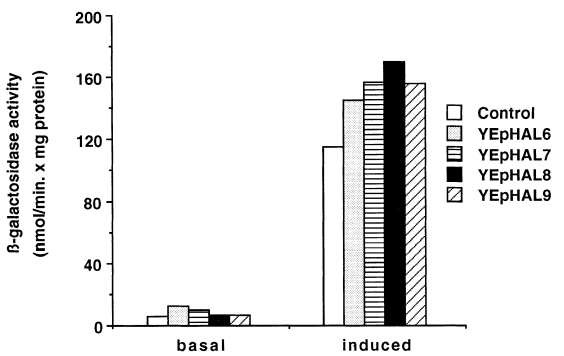


Fig. 3. Effect of overexpression of halotolerance genes *HAL6–9* on the transcriptional activity of the *ENA1* gene. Strain RS-16 with an *ENA1-lacZ* fusion integrated at the *ura3* locus was transformed with multicopy plasmids containing the halotolerance genes. β-Galactosidase activity was measured in exponential cells before (basal) and after 90 min incubation with 0.9 M NaCl (salt induced). Control refers to cells transformed with empty plasmid. Results are the average of four determinations. The standard deviations of basal and induced activities were less than 1 and 10 nmol/min/mg protein, respectively.

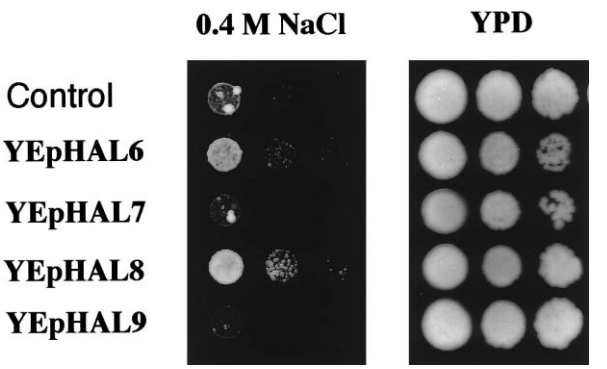


Fig. 4. Effect of overexpression of halotolerance genes *HAL6–9* on the salt tolerance of an *ena* mutant. Strain W303-1A with a deletion of the *ENA* locus was transformed with multicopy plasmids containing the halotolerance genes and plated on control media and in media supplemented with 0.4 M NaCl. Control refers to cells transformed with empty plasmid.

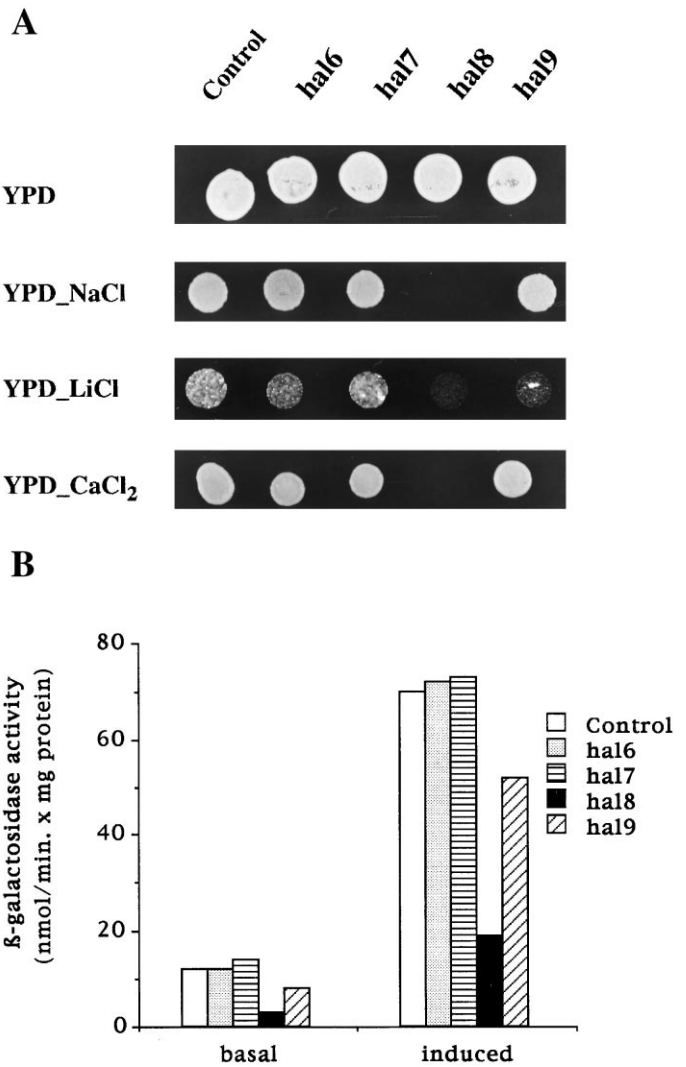


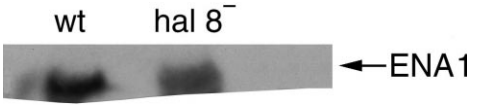
Fig. 5. A: Effect of disruption of *HAL6–9* genes on the tolerance to high sodium (1.3 M NaCl), lithium (0.25 M LiCl) and calcium (0.3 M $CaCl_2$) concentrations. W303-1A derivatives with the indicated null mutations were spotted on salt containing plates as described in Section 2 and photographed after 5 days. B: Effect of disruption of *HAL6–9* genes on the transcriptional activity of an *ENA1-lacZ* fusion under basal and salt induced conditions (see legend to Fig. 3). Incubation with 0.9 M NaCl was for 3 h. Control refers to cells transformed with empty plasmid. Results are the average of six determinations. The standard deviations of basal and induced activities were less than 1 and 5 nmol/min/mg protein, respectively.

exhibiting the strongest effect. These results suggest that the *ENA1* Na^+/Li^+ extrusion system is a component of the halotolerance mechanisms triggered by high dosage of these genes.

Mutants lacking the *ENA* gene cluster are helpful in revealing halotolerance mechanisms unrelated to this Na^+/Li^+ extrusion ATPase [15]. As shown in Fig. 4, when transformed in high copy plasmid into *ena* null mutants, *HAL6* and *HAL8* improve cell growth in high sodium medium while *HAL7* and *HAL9* are ineffective. Therefore, overexpression of *HAL6* and *HAL8* confers salt tolerance by increasing *ENA1* expression and also by mechanisms not involving this major Na^+/Li^+ efflux system. These could include other cation extrusion systems different from *ENA1*, such as *NHA1* [20] and *SNQ2* [21] or cation influx systems such as *TRK1* [22].

No data about the function of the *HAL6–9* genes have been found in the updated literature. In order to obtain some information on the biological function of these new halotolerance genes, mutants having the chromosomal gene replaced by a disrupted copy were obtained. All the *HAL6–9* genes appear not to be essential for cell growth in standard media, since growth of haploid null mutant cells at 30°C in rich and minimal media was indistinguishable from that of wild type strain (not shown). However, they show a non-uniform behavior in different salt selective media (Fig. 5A). While disruption of the bZip protein coding sequences (*HAL6* and *HAL7*) does not significatively affect growth in high salt media, disruption of the zinc binding protein genes (*HAL8* and *HAL9*) impairs growth in high salt media. When compared with wild type yeast, the *hal8* null mutant cannot grow in medium containing 1.3 M NaCl, and high Li^+ concentrations (0.25 M) impair growth of both *hal8* and *hal9* mutants. In addition, the *hal8* mutant is hypersensitive to calcium. These results suggest that the *HAL8* gene could be involved in the mechanisms controlling overall cation homeostasis, whereas *HAL9* is more specific to Li^+ toxicity. In addition, *HAL6* and *HAL7* genes appear to be dispensable for Li^+, Na^+, and Ca^{2+} homeostasis.

To analyze the effect of the Hal6–9p potential transcription factors on *ENA1* gene expression, we performed gene disruptions in a yeast strain having an *ENA1-lacZ* reporter fusion integrated into the chromosome. We measured β-galactosidase activity, indicative of the *ENA1* promoter transcriptional activity, in exponentially growing cells before and 3 h after addition of 0.9 M NaCl to the growth medium. Results from Fig. 5B show that disruption of the putative bZip transcription factors encoded by *HAL6* and *HAL7* does not affect *ENA1-lacZ* expression levels, either in basal or in salt induced conditions. On the other hand, deletions of putative zinc finger transcription factors *HAL8* and *HAL9* reduced *ENA1-lacZ* expression. Disruption of *HAL8* results in a extremely low β-galactosidase activity level (70% reduction) and this suggest that the *HAL8* gene product could play a physiological role as an *ENA1* transcriptional activator. The significant effect of *HAL8* disruption on *ENA1* expression level was corroborated by Northern analysis.



HAL9 gene deletion has a smaller but significant effect on *ENA1-lacZ* fusion expression, resulting in about 30% reduction of β-galactosidase activity, both in basal and in salt-induced conditions. Therefore the *HAL9* gene product could be a positive factor having a partial effect on *ENA1* gene expression.

The increase in *ENA1* gene expression caused by overexpression of *HAL8* and *HAL9* and the decreased expression resulting from disruption of these genes correlated with the intracellular accumulation of lithium. After 3 h incubation with 0.1 M LiCl, control cells accumulated intracellular lithium to 40 ± 3 mM, while cells overexpressing *HAL8* had 30 ± 2 mM and *hal8* null mutants 90 ± 8 mM. In the case of *HAL9*, overexpression reduced lithium accumulation to 35 ± 2 mM and the null mutant had 46 ± 3 mM. These results indicate that the changes in *ENA1* expression caused by gain and loss of function of *HAL8* and *HAL9* are important under in vivo conditions and determine the accumulation of toxic cations such as lithium. The greater effect of gain and loss of function of *HAL8* as compared to *HAL9* is observed in all phenotypes tested: salt tolerance, *ENA1* expression and lithium accumulation.

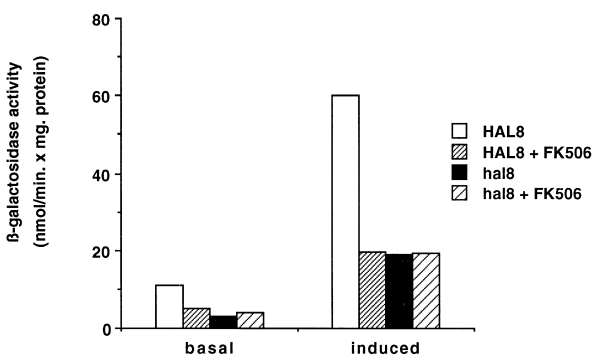


Fig. 6. Effect of *hal8* mutation and of calcineurin inhibitor FK506 on the transcriptional activity of *ENA1*. W303-1A cells contained the integrated *ENA1-lacZ* reporter gene (see legend to Fig. 3) and, when indicated, the *hal8* mutation. β-Galactosidase activity was determined in cultures growing in the absence and presence of FK506 (2 μg/ml) before (basal) and after treatment for 3 h with 0.9 M NaCl (induced). Results are the average of three determinations. The standard deviations of basal and induced activities were less than 1 and 5 nmol/min/mg protein, respectively.

Induction of *ENA1* expression in NaCl stressed cells is controlled by two signal transducing pathways, the osmotic (solute unspecific) HOG-MAP kinase cascade and the sodium specific calcineurin pathway. Both processes can be easily dissected in the presence of the calcineurin inhibitor FK506 [5]. We have investigated the induction of *ENA1* promoter activity by sodium stress in wild type and *hal8* mutant cells, in the absence and presence of this drug. Results in Fig. 6 show that FK506 sensitive expression, attributable to the calcineurin mediated pathway, is abolished in *hal8* defective cells. Therefore the *HAL8* gene could be considered a component of the sodium activated calcineurin transduction pathway.

4. Discussion

Overexpression of transcription factors could upregulate genes which under normal conditions may not be controlled by these proteins. Therefore, the effect of *hal6–9* gene disruptions on salt tolerance and *ENA1* expression is more indicative of their participation in the control of ion homeostasis under physiological conditions. By this criterion, neither Hal6p nor Hal7p is a normal component of ion homeostasis because their disruption produced neither salt sensitivity (Fig. 5A) nor decreased expression of *ENA1* (Fig. 5B). The effects of overexpression of these bZip proteins can therefore be regarded as artefactual, perhaps mediated by complex formation with other transcription factors. On the other hand, the two zinc fingers Hal8p and Hal9p are important determinants of ion homeostasis because null mutants exhibited decreased lithium tolerance (Fig. 5A) and *ENA1* expression (Fig. 5B). Hal8p is quantitatively more important than Hal9p and it qualifies as one of the important transcription factors mediating *ENA1* expression under physiological conditions.

The HOG-MAP kinase cascade and the calcium activated protein phosphatase calcineurin define the two signal transduction pathways mediating the salt induction of *ENA1* [5]. Hal8p seems to mediate the effect of calcineurin on the *ENA1* promoter because the *hal8* mutation abolish the calcineurin dependent (FK506 sensitive) part of *ENA1* expression and also confers calcium sensitivity, a typical phenotype of calcineurin mutants. Actually, when this work was in the process of being submitted for publication, we became aware (thanks to the *Saccharomyces* Genome Database, http://genome-www.stanford.edu./Saccharomyces/), that the ORF YNL027w (which we called Hal8p) has been identified by the laboratories of M.S. Cyert and K.W. Cunningham as the calcineurin dependent transcription factor of yeast and designated Crz1p/Tcn1p. Our results are in agreement with this information and indicate that in addition to *ENA1*, some other unidentified gene(s) contributing to salt tolerance is regulated by overexpression of Hal8p.

The transcription factor mediating the effect of the HOG pathway on *ENA1* expression has not been disclosed by our screening. We have preliminary evidence that this factor is a repressor counteracted by osmotic stress (M. Proft and R. Serrano, unpublished observations) and therefore overexpression of this factor could not result in tolerance.

5. Note added in proof

The work on Crz1p/Tcn1p mentioned above has recently been published [23,24]. Interestingly, the *ENA1* promoter contains two sequences (at positions −726 and −920) with strong homology to the calcineurin dependent response element of the *FKS2* promoter. This element has been shown to bind Crz1p/Tcn1p [23] and experiments are under way to determine the functionality of these putative elements in the *ENA1* promoter.

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