

Regulation of Cation Transport in *Saccharomyces cerevisiae* by the Salt Tolerance Gene *HAL3*

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Dynamic regulation of ion transport is essential for homeostasis as cells confront changes in their environment. The gene *HAL3* encodes a novel component of this regulatory circuit in the yeast *Saccharomyces cerevisiae*. Overexpression of *HAL3* improves growth of wild-type cells exposed to toxic concentrations of sodium and lithium and suppresses the salt sensitivity conferred by mutation of the calcium-dependent protein phosphatase calcineurin. Null mutants of *HAL3* display salt sensitivity. The sequence of *HAL3* gives little clue to its function. However, alterations in intracellular cation concentrations associated with changes in *HAL3* expression suggest that *HAL3* activity may directly increase cytoplasmic K^+ and decrease Na^+ and Li^+ . Cation efflux in *S. cerevisiae* is mediated by the P-type ATPase encoded by the *ENAI/PMR2A* gene, a putative plasma membrane Na^+ pump whose expression is salt induced. Acting in concert with calcineurin, *HAL3* is necessary for full activation of *ENAI* expression. This functional complementarity is also reflected in the participation of both proteins in recovery from α -factor-induced growth arrest. Recently, *HAL3* was isolated as a gene (named *SIS2*) which when overexpressed partially relieves loss of transcription of G_1 cyclins in mutants lacking the protein phosphatase Sit4p. Therefore, *HAL3* influences cell cycle control and ion homeostasis, acting in parallel to the protein phosphatases Sit4p and calcineurin.

The homeostasis of intracellular ion concentrations is a fundamental property of living cells. Many important physiological parameters such as cell volume, turgor, intracellular pH, ionic strength, and cation concentrations depend on the regulation of uptake and efflux systems for the major monovalent cations sodium and potassium (52, 61). Eukaryotes employ primary active transport, mediated by P-type ATPases, and secondary transport, mediated by channels and cotransporters, to maintain characteristic high intracellular concentrations of essential K^+ and low intracellular concentrations of toxic Na^+ . A secondary effect of the investment of ATP energy in ion pumping is that ion gradients are utilized for concentrative transport of many nutrients, plasma membrane excitability, and integrity of the membrane itself (60).

Most animal cells are bathed in an extracellular fluid regulated by action of the kidney to vary little from a characteristic osmotic pressure, pH, and ion concentrations. In these cells, the primary pump is the (Na^+ , K^+)-ATPase, which together with Na^+ and K^+ channels and with Na^+/H^+ antiporters and (Na^+ , K^+ , $2Cl^-$) symporters determine cell volume and intracellular cation concentrations. Cellular uptake of sugar and amino acids is mediated by cotransport with Na^+ (60). Even subtle changes in the extracellular milieu are transduced via the activity of regulated protein kinases and phosphatases to the different transporters (1, 19, 60).

In comparison with the predictable environment of most animal cells, the cells of plants, algae, and fungi must tolerate a wider range of osmotic pressures, pH, and ion concentrations

in their environments. The cell wall of such organisms is partially responsible for their remarkable tolerance to osmotic challenge. However, physiological studies of fungal microorganisms have shown that their capacity to proliferate over a wide range of ion concentrations reflects a strategy for transport at the plasma membrane different from that of animal cells. The plasma membranes of fungi and plants contain a highly active proton-exporting ATPase (57, 58). Secondary transport of nutrients and ions is largely coupled to the resulting proton gradient, allowing intracellular Na^+ and K^+ to be independently regulated. In the yeast *Saccharomyces cerevisiae*, *PMR1*, encoding the principal plasma membrane H^+ -ATPase, is an essential gene. *PMR1*-encoded ATPase comprises as much as 50% of plasma membrane protein, and its activity, relatively insensitive to Na^+ and K^+ , is regulated by pH and carbon source availability (58). Genes mediating transport of Na^+ and K^+ in nonanimal cells have only recently been characterized. In *S. cerevisiae*, a major system for potassium uptake is encoded by the *TRK1* and *TRK2* genes (18, 32, 48, 49), and a major sodium and lithium efflux system is encoded by the *ENAI/PMR2A* ATPase gene (20, 28, 53). The K^+ transporter genes *TRK1* and *TRK2* encode large homologous membrane proteins that lack sequence similarity to other classes of transport proteins, such as the ABC- and P-type ATPases. K^+ import through this system may be coupled to proton influx (50), as in the bacterial *trkG/trkH* system (3). Cells lacking *TRK1* are viable in normal media but are unable to concentrate K^+ from media with low K^+ content. Deletion of *TRK2* has consequences for K^+ uptake only in a *trk1* background, suggesting that *TRK1* is responsible for most of the high-affinity K^+ uptake of normal cells (32, 48). The Na^+ transporter gene *ENAI/PMR2A* is the first repeat in a tandem array of five open reading frames, the *PMR2* locus, encoding nearly identical proteins with high homology to P-type ATPases. Unlike the

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other *PMR2* repeats, *ENAI/PMR2A* is the only repeat that is highly expressed and is preceded by a significant region of 5' untranslated sequence required for inducible expression. Yeast cells tolerate deletions of the whole array (53), but such mutants, or cells lacking only the first repeat, are sensitive to high Na^+ and Li^+ (20, 28).

Both the *TRK1,2* K^+ uptake system and the *ENAI/PMR2A* Na^+ efflux system are regulated by extracellular ion concentrations. Concentrative uptake of K^+ is modulated by changes in the affinity of the Trk system triggered by intracellular K^+ levels (27, 49). Activity of the *ENAI/PMR2A* transporter is induced by osmotic stress and high pH at the level of transcription (20). Regulation of *TRK1,2* and *ENAI/PMR2A* is mediated, at least in part, by the calcium-dependent protein phosphatase calcineurin (40, 44), suggesting a potential connection between calcium signaling and salt tolerance. Calcineurin is a heterodimer that consists of a ≈ 60 -kDa catalytic A subunit (containing a carboxy-terminal autoinhibitory domain with a Ca^{2+} -calmodulin binding site) stably associated with a ≈ 20 -kDa regulatory B subunit (containing four EF-hand motifs characteristic of Ca^{2+} -binding proteins). Calcineurin is activated upon binding of Ca^{2+} to both calmodulin and the B subunit. Ca^{2+} -calmodulin binding to the A subunit displaces the autoinhibitory domain and activates the phosphatase (8). The immunosuppressants cyclosporin A and FK506 act by inhibiting calcineurin-mediated signal transduction in activated T cells. This inhibition involves a complex of the drugs with specific binding proteins, the immunophilins. Immunosuppressant-immunophilin complexes bind to calcineurin and thereby inactivate it (33). The blunted T-cell response in immunosuppressant-treated individuals is attributed to failure to transduce the calcium signal produced by stimulated T-cell receptors to activation of the transcription factor NF-AT. NF-AT, once dephosphorylated by calcineurin, binds to the promoter of the interleukin-2 growth factor gene to stimulate expression. Interleukin-2 production promotes expansion of clones of activated T cells.

The genes encoding the calcineurin subunits and binding proteins for cyclosporin A and FK506 have been isolated in *S. cerevisiae* (33). Null mutants lacking either the two genes encoding calcineurin catalytic subunits, *CNA1* and *CNA2*, or lacking the gene encoding the Cnb1 regulatory subunit each express no measurable calcineurin activity. Such mutants are viable under most conditions but demonstrate slow recovery from α -factor pheromone-mediated arrest (10, 17) and increased sensitivity to media with high Na^+ or Li^+ or alkaline pH (40, 44). Similarly, treatment of yeast cells with cyclosporin A or FK506 confers slow recovery from α -factor and salt sensitivity, but only in cells with intact immunosuppressant-binding proteins. The defect in salt tolerance associated with loss of calcineurin activity is attributable largely to failure to activate *ENAI/PMR2A* expression and secondarily to a failure to increase affinity of *TRK1* for K^+ upon salt stress (40). Nonetheless, calcineurin mutants retain some responsiveness to Na^+ challenge, and *ENAI/PMR2A* expression remains partially stimulatory by salt.

The regulatory circuit for ion transport may comprise several components responding to different stimuli and operating through multiple signal transduction pathways. In addition, ion homeostasis is probably linked to other basic regulatory circuits such as those operating during the cell growth and division cycle. To find genes relevant to ion homeostasis, we have isolated yeast genes that, by overexpression from plasmids, improve growth at high NaCl concentrations (*HAL*, or halotolerance, genes). Two genes have already been described. *HAL1* modulates the intracellular Na^+/K^+ ratio by unknown mech-

anisms (21), and *HAL2* (identical to *MET22*) corresponds to a salt-sensitive step in sulfate activation (23, 42). A novel halotolerance gene, *HAL3*, is described in the present work. *HAL3* overexpression confers superresistance to salt stress of wild-type cells and suppresses the salt sensitivity of calcineurin mutants. *HAL3* defines a novel regulatory pathway for cell growth and ion homeostasis.

MATERIALS AND METHODS

Yeast strains and culture conditions. Standard methods for yeast culture and manipulation were used (26) except as described. Minimal medium contained 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco), 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] adjusted to pH 6.0 with Tris, and either uracil (30 $\mu\text{g}/\text{ml}$) or leucine (100 $\mu\text{g}/\text{ml}$) as indicated. Synthetic complete medium lacking uracil was prepared as described previously (26). Rich medium contained 1% yeast extract (Difco), 2% Bacto Peptone (Difco), and either 2% glucose or 2% galactose. NaCl, KCl, LiCl, or sorbitol was added as indicated. FK506 was a kind gift of Ihor Bekersky (Fujisawa Pharmaceutical Company, Deerfield, Ill.). For most experiments, FK506 was dissolved at 5 mg/ml in dimethyl sulfoxide and added to media to a final concentration of 1 $\mu\text{g}/\text{ml}$. Solid media contained 2% bacteriological-grade agar. Salt and osmotic tolerance in solid and liquid media was determined as described previously (21, 23). Alternatively, wells of a 96-well microplate were inoculated with 100- μl aliquots of test media containing yeast cells at a nominal A_{600} of 0.1. After incubation at 30°C without agitation for the indicated times, cells were resuspended with a Flow Laboratories (Irvine, United Kingdom) Titertek microplate mixer, and the cell density of each well determined by light scattering at 570 nm, using a Dynatech (Alexandria, Va.) MR600 microplate reader.

The *S. cerevisiae* strains used for this work are described in Table 1. Strains were derived by standard genetic crosses or by transformation using the lithium acetate procedure (31). The double mutant *hal3::LEU2 cnb1::LEU2* (strain SKY682) was constructed by crossing the two single mutants and selecting Leu^+ spores in tetrads in which the *LEU2* gene segregated 2:2. The *pmr2::HIS3* allele, carrying a complete replacement of the *PMR2* locus, was obtained from Hans Rudolph.

Isolation and sequencing of the *HAL3* gene. The screen for superresistance to NaCl has been described previously (21). Briefly, strain RS-16 was transformed with a genomic library from strain A7A in plasmid YCp50 (51) and selected on agar plates containing minimal medium with leucine. Transformants were pooled and plated on the above-described medium containing 1.5 M NaCl. Colonies exhibiting improved growth were selected, and plasmids were isolated. One of them (clone 5-3) was identified as conferring the greatest halotolerance upon retransformation into RS-16. The plasmid was subjected to restriction analysis and subcloning. A 3.2-kb *EcoRI-HindIII* fragment conferring salt tolerance was subcloned in pBluescript (Stratagene), and each strand was sequenced by dideoxynucleotide chain termination with modified T7 DNA polymerase (Sequenase, United States Biochemicals), using an oligonucleotide primer walking strategy. Oligonucleotides were synthesized by Isogen (Amsterdam, The Netherlands).

The minimal 2.4-kb *BclI-HindIII* fragment containing *HAL3* (construction 4 in Fig. 1A) was subcloned in plasmids YEp351 (2 μm origin, *LEU2* marker) and YEp352 (2 μm origin, *URA3* marker) described by Hill et al. (29) to construct high-copy-number plasmids for overexpression of *HAL3*.

Disruption of *HAL3* and Southern analysis. Three different null alleles were constructed in two different strain backgrounds. To construct the disruption allele *hal3-1::LEU2*, the 3.2-kb *EcoRI-HindIII* fragment containing *HAL3* (Fig. 1A) was subcloned in pUC18 (45). The resulting plasmid was cut with *BamHI*, blunt ended with the Klenow fragment of DNA polymerase I, and ligated to *XhoI* linkers. A 2.2-kb *XhoI-SalI* fragment containing the *LEU2* gene was obtained from plasmid YEp13 (6) and inserted into the artificial *XhoI* site. The original *EcoRI-HindIII* fragment contained three *BglII* sites 0.55, 0.98, and 1.08 kb from the *EcoRI* site (not shown in Fig. 1A). As *EcoRI* is present within the *LEU2* sequence, the resulting plasmid was cut with *BglII* and *HindIII* to release the 4.3-kb fragment containing the interrupted *HAL3* gene (*hal3-1::LEU2*) and used for transformation of strains RS-16, RS-736, and W303-1A to generate genomic disruptions by homologous recombination.

Two disruption alleles, *hal3-2::HIS3* and *hal3-3::LEU2*, were constructed as follows from DNA flanking the *HAL3* open reading frame. Two fragments derived from the 3.2-kb *EcoRI-HindIII* fragment containing *HAL3*, a 0.50-kb *XbaI* fragment that extends to 12 bp 5' of the *HAL3* start codon (not shown in Fig. 1A), and the 0.51-kb *KpnI* fragment that contains the C-terminal 72 amino acids of *HAL3* were cloned into the polylinker of pBluescript II KS(+) (Stratagene). The *XbaI* fragment was isolated from DNA prepared from a *dam* *Escherichia coli* strain. The resulting construct represents the *HAL3* region extending from the *XbaI* site 0.51 kb 5' of the *HAL3* open reading frame to the *KpnI* site 0.30 kb 3' of the open reading frame, with an internal *XbaI-KpnI* fragment replaced with the pBluescript II polylinker. The *BamHI* fragment containing *HIS3* from plasmid pJH-H1 and the *BglII* fragment containing *LEU2* from plasmid YEp13 were cloned into the *BamHI* site between the *XbaI*

TABLE 1. Yeast strains used

| Strain | Genotype | Source or reference ^a |
|---------|--|----------------------------------|
| A7A | <i>MATa ade1,2 gal1 his7 lys2 tyr1 ura3</i> | R. Gaxiola |
| RS-736 | <i>MATa/MATα ade1,2/ade1,2 gal1/gal1 his7/his7 lys2/lys2 tyr1/tyr1 ura3/ura3</i> | R. Gaxiola |
| RS-16 | <i>MATa leu2-3,112 ura3-251,328,372</i> | 21 |
| RS-48 | RS-16 <i>hal3::LEU2</i> | |
| RS-44 | RS-16 [YEp351] | |
| RS-47 | RS-16 [YEp351- <i>HAL3</i>] | |
| RS-566 | RS-44 <i>ura3-251::ENA1/PMR2A-lacZ::URA3</i> | |
| RS-565 | RS-47 <i>ura3-251::ENA1/PMR2A-lacZ::URA3</i> | |
| RS-567 | RS-48 <i>ura3-251::ENA1/PMR2A-lacZ::URA3</i> | |
| RS-600 | A7A <i>ura3-251::HAL3-lacZ::URA3</i> | |
| DBY746 | <i>MATα leu2-3,112 ura3-52 his3-Δ1 trp1-289</i> | YGSC |
| RH16.6 | DBY746 <i>ena1,2::LEU2</i> | 27 |
| RS-825 | DBY746 [YEp352] | |
| RS-828 | DBY746 [YEp352- <i>HAL3</i>] | |
| RS-841 | RH16.6 [YEp352] | |
| RS-844 | RH16.6 [YEp352- <i>HAL3</i>] | |
| W303-1A | <i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i> | 62 |
| W303-1B | W303-1A <i>MATα</i> | |
| SKY624 | W303-1A <i>cmd1-3 cnb1::LEU2</i> | |
| SKY684 | W303-1A <i>hal3-1::LEU2</i> | |
| SKY683 | W303-1A <i>cnb1::LEU2</i> | |
| SKY697 | W303-1A <i>pmr2::HIS3</i> | 53 |
| SKY682 | W303-1A <i>hal3-1::LEU2 cnb1::LEU2</i> | |
| SKY699 | W303-1A <i>hal3-1::LEU2 pmr2::HIS3</i> | |
| SKY700 | W303-1A <i>cnb1::LEU2 pmr2::HIS3</i> | |
| SKY696 | W303-1A <i>hal3-1::LEU2 cnb1::LEU2 pmr2::HIS3</i> | |
| SKY802 | W303-1A <i>hal3-2::HIS3</i> | |
| SKY806 | W303-1A <i>hal3-3::LEU2</i> | |
| SKY723 | W303-1A <i>PMR2::ENA1-lacZ::LEU2</i> | |
| SKY724 | SKY684 <i>PMR2::ENA1/PMR2A-lacZ::LEU2</i> | |
| SKY725 | SKY683 <i>PMR2::ENA1/PMR2A-lacZ::LEU2</i> | |
| SKY726 | SKY682 <i>PMR2::ENA1/PMR2A-lacZ::LEU2</i> | |

^a Unless otherwise indicated, from this study. YGSC, Yeast Genetic Stock Center, Berkeley, Calif.

and *KpnI* fragments to make the *hal3-2::HIS3* and *hal3-3::LEU2* deletion constructs, respectively. To generate a genomic deletion by homologous recombination, strain W303A-1A was transformed with the *PvuII* digest of the pBluescript-*hal3-2::HIS3* and pBluescript-*hal3-3::LEU2* plasmids, and cells were selected for histidine or leucine prototrophy, respectively.

Deletions were confirmed by PCR and Southern analysis. For PCR, two primers, HAL3L (GTGCTCTGGATCAGCATCCC, 170 bp 5' of the start codon) and HAL3R (CTCTATGTACGCTAGTATGGC, 160 bp 3' of the stop codon), were used under standard conditions to determine the size of the *HAL3* region. For Southern analysis, chromosomal DNA was prepared (64), digested with *HindIII*, separated by electrophoresis, blotted to nylon membrane, and hybridized (7) with the 2.1-kb *KpnI-KpnI* fragment of *HAL3* (Fig. 1A) labeled by the random-priming method (15).

Measurement of intracellular ion concentrations. After incubation in medium containing either 1 M NaCl or 0.1 M LiCl, cells were harvested by centrifugation for 5 min at 2,000 × *g* and 4°C, resuspended with a cold solution containing 20 mM MgCl₂ and iso-osmotic sorbitol (1.5 or 0.2 M, respectively), centrifuged as described above, resuspended with the same solution, filtered through a glass fiber filter (Whatman GF/C), washed in the filter with the same solution, and extracted by incubation with 20 mM MgCl₂ for 15 min at 95°C. After centrifugation, aliquots of the supernatant were analyzed with an atomic absorption spectrometer (Varian) in flame emission mode.

***lacZ* fusions and determination of β-galactosidase activity.** The *HAL3-lacZ* fusion was made by subcloning the 2-kb *EcoRI-BamHI* fragment containing the promoter region and about half of the *HAL3* reading frame (Fig. 1A) into integrative plasmid YIp353 (43). The resulting plasmid was linearized with *KpnI* before yeast transformation to direct integration at the *HAL3* locus. An *ENA1/PMR2A-lacZ* fusion, containing the *ENA1/PMR2A* promoter and first 10 codons fused in frame to the *E. coli lacZ* gene in the integrative *URA3* plasmid Yip356R, was obtained from Alonso Rodriguez-Navarro (40). This plasmid was linearized with *NcoI* before transformation to direct integration at the *ura3-251* locus. A similar *lacZ* fusions in which the *ENA1/PMR2A* promoter and first 10 codons present in plasmid B2001 (53) are cloned into YIp366R (43) was a kind gift of Kyle Cunningham. The YIp366R *ENA1/PMR2A-lacZ::LEU2* plasmid was linearized at the *BglII* site within the *ENA1/PMR2A* promoter to direct integration to the *PMR2* locus to produce a tandem duplication of the *ENA1/PMR2A* promoter surrounding an integrated YIp366R plasmid.

Overnight cultures of strains carrying a *lacZ* fusion reporter construct were diluted into fresh media containing various concentrations of salt as noted and grown into log phase. Alternatively, salt was added to log-phase cultures, which were incubated for the indicated times. β-Galactosidase activity was measured in permeabilized cells as described previously (21). Alternatively, cells were collected by centrifugation, resuspended in reaction buffer containing 0.1% Triton X-100 and 1 mM β-mercaptoethanol, and frozen at −80°C. Cells thawed at room temperature were subjected to β-galactosidase assay. Units of activity were normalized to protein concentration or cell density.

Expression in *E. coli* and antibody methods. The complete reading frame of *HAL3* was amplified from plasmid construction 3 (Fig. 1A; see above) by standard PCR methodology. The upstream primer was 5'-GGGCTAGAGATGACTGCCGTCGCCTCT, which introduces an *XbaI* site (underlined) before the start ATG codon. The downstream primer was 5'-GGGAAGCTTGATGCTTATCTATTAT, which introduces a *HindIII* site (underlined). The 1.7-kb amplified fragment was digested with *XbaI* and *HindIII* and subcloned into expression plasmid pGEX-KG (24). The glutathione *S*-transferase-Hal3p fusion protein was expressed in *E. coli*, affinity purified with glutathione-Sepharose 4B (Pharmacia), and cleaved with thrombin to isolate Hal3p, which was used to inject rabbits for antibody production. Antibodies specific to Hal3p were precipitated by ammonium sulfate and affinity purified by binding to Hal3p blotted on nitrocellulose (34). Purified antibody was used at 1/50 dilution for both Western blot (immunoblot) analysis (4) and immunofluorescence (46). The specificity of immunodecoration was demonstrated by preincubation of the diluted anti-Hal3p antibody with purified Hal3p (100 μg/ml) for 30 min at room temperature.

Preparation and fractionation of homogenates. Protein extracts were prepared from exponentially growing cells by shaking with glass beads as described previously (56). The homogenization medium contained 20% sucrose, 50 mM Tris-HCl (pH 8), 0.1 M KCl, 5 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 20 μg of chymostatin per ml. After removal of debris by centrifugation for 10 min at 2,000 rpm (Beckman JA-20 rotor), the homogenate was fractionated into soluble and particulate fractions by centrifugation for 30 min at 30,000 rpm (Beckman 80 Ti rotor; 80,000 × *g*). The pellet was resuspended in homogenization medium, and protein was quantified by the Bio-Rad Bradford reagent with bovine gamma globulin as the standard.

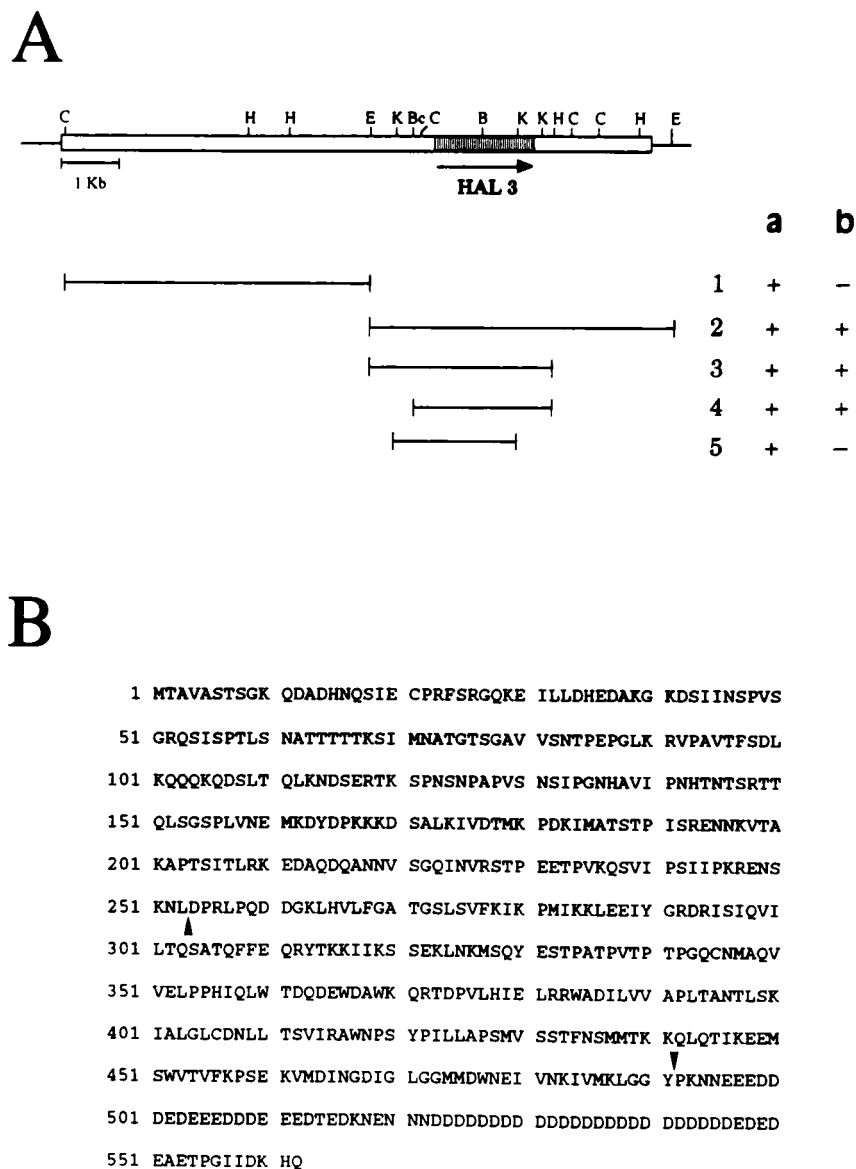


FIG. 1. Isolation and encoded protein sequence of the *HAL3* gene. (A) Restriction map of an insert of 10 kb (bar) present in clone 5-3 from the genomic library in YCp50. The open reading frame of *HAL3* (filled bar) and direction of transcription (arrow) are indicated. B, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I. The following restriction fragments of the insert (indicated below the map) were subcloned in YCp50 and transformed into strain RS-16: 5.5-kb *Cla*I-*Eco*RI (construction 1), 5.5-kb *Eco*RI-*Eco*RI (construction 2), 3.2-kb *Eco*RI-*Hind*III (construction 3), 2.4-kb *Bcl*I-*Hind*III (construction 4), and 2.1-kb *Kpn*I-*Kpn*I (construction 5). Transformants were tested for growth in normal minimal medium with leucine (a) and in this medium supplemented with 1.4 M NaCl (b). The results were qualitatively scored as + or -. (B) Predicted amino acid sequence of Hal3p. The upward- and downward-pointing arrowheads indicate the points of truncation in one gene disruption (*Bam*HI site) and in the carboxyl-terminal deletion (*Kpn*I site), respectively.

RESULTS

A screen for superresistance to NaCl reveals a new mediator of salt tolerance. We have previously described two halotolerance genes, *HAL1* (21) and *HAL2* (23), isolated in a screen for genomic clones that when present in an episomal plasmid confer marked ability for strain RS-16 wild-type cells to tolerate otherwise toxic concentrations of NaCl. Here, we report characterization of a third gene identified in this screen, which we name *HAL3*.

The original genomic clone in the centromeric vector YCp50 (51), the map of the insert, and the localization of the salt tolerance gene are depicted in Fig. 1A. Restriction fragments of this insert were subcloned in YCp50, transformed into strain

RS-16, and tested for salt tolerance. A *Bcl*I-*Hind*III fragment of 2.4 kb was the minimal length of DNA conferring enhanced salt tolerance (construction 4).

***HAL3* overexpression bypasses the salt sensitivity of cells lacking calcineurin.** The activity of the protein phosphatase calcineurin has been shown to be required for tolerance to high Na⁺ and Li⁺ concentrations (40, 44). We have confirmed and extended these observations. We found that similar ranges of cation sensitivities are displayed by yeast strains carrying alleles of the yeast calmodulin gene, including *cmd1-3* (22), previously shown to be defective in Ca²⁺ binding (unpublished results). Such calmodulin mutants are likely defective in Ca²⁺-calmodulin-dependent activation of calcineurin. To better under-

stand the pathways connecting Ca^{2+} signaling to Na^+ tolerance, the highly salt-sensitive strain SKY624 (*cmd1-3 cnb1::LEU2*) was transformed with a yeast cDNA library in the pRS-316-*GALI*-cDNA vector (36). Transformants were selected, pooled, and plated on rich agar media containing 2% galactose and 1.0 M NaCl. Salt-resistant clones were selected, and plasmids were isolated. Several plasmids conferred galactose-dependent salt tolerance when retransformed into the salt-sensitive strain and were studied further. Sequence analysis revealed both novel and previously described genes (unpublished results), including several plasmids carrying complete cDNAs encoding the *HAL3* gene. Transformation of the *cmd1-3 cnb1::LEU2* strain or any other strain lacking calcineurin activity and/or functional calmodulin with plasmid YEp-*HAL3* conferred salt tolerance on these otherwise salt-sensitive strains.

The *HAL3* gene encodes a novel expressed protein which confers salt tolerance. Sequencing of a 3.2-kb region including the *HAL3* gene revealed a single open reading frame of 562 amino acids. The predicted product, Hal3p, contains no sequence motifs characteristic of proteins with biochemically defined functions. Hybridization to yeast chromosomes separated by pulsed-field electrophoresis indicated that *HAL3* maps to chromosome XI. The recently published complete DNA sequence of this chromosome (13) confirms our sequence and putative assignment of a reading frame, identifying *HAL3* as the open reading frame YKR072c, at 576 to 578 kb from the left telomer. Comparison of the predicted Hal3p sequence with sequences in the databases (35) revealed a striking homology to only one other sequenced protein, open reading frame YKL088w, also present on yeast chromosome XI (13). Hal3p and YKL088w are of similar length and share protein sequence homology throughout their reading frames.

The predicted protein (Hal3p) contains an acidic domain at its carboxyl terminus (Fig. 1B). This conspicuous domain consists of 58 amino acids, of which 35 are aspartates and 16 are glutamates. We infer that this domain is essential for *HAL3* function because deletion at a *Kpn*I site immediately 5' to the acidic domain caused loss of salt tolerance activity (Fig. 1A; compare constructions 5 and 4).

Hal3p is constitutively expressed. A construct containing an in-frame fusion of the 5' portion of the coding region of *HAL3* to the *E. coli lacZ* reporter gene expressed β -galactosidase activity when transformed into yeast cells. Both β -galactosidase activity and Northern (RNA) analysis demonstrated that *HAL3* expression is constitutive and is not induced by salt stress. Nonetheless, the salt-resistant activity of cells overexpressing *HAL3* is highly dose dependent. Overexpression of *HAL3* from its own promoter on multicopy (2 μ m, YEp) plasmids results in significantly greater salt tolerance than the equivalent construct in a centromeric plasmid.

Hal3p was expressed in *E. coli* as a fusion protein to glutathione *S*-transferase, purified by affinity chromatography, cleaved from the glutathione *S*-transferase domain, and injected into rabbits for polyclonal antibody production. Affinity-purified antibodies recognized a protein band with an apparent molecular mass 70 kDa (Fig. 2A, lanes 1 to 3). All immunoreactivity of this band was lost after preincubation of the antibodies with purified antigen (Fig. 2A, lanes 4 to 6). The apparent molecular mass is slightly greater than the value of 62.4 kDa predicted from the amino acid sequence. The discrepancy was not due to posttranslational modification restricted to yeast cells, as the *E. coli*-expressed protein shared the aberrant apparent molecular mass. Rather, the decreased mobility on polyacrylamide gel electrophoresis may reflect the extremely acidic character of the carboxyl terminus, which

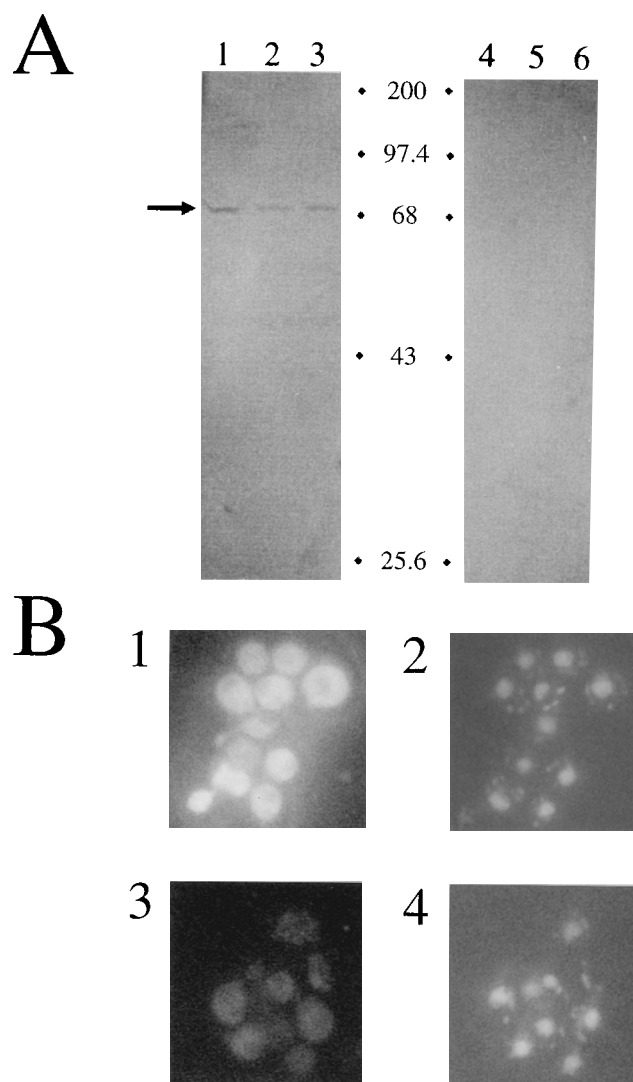


FIG. 2. Immunological analysis of Hal3p localization. (A) Western blot. Each lane contained 30 μ g of protein from either homogenates (lanes 1 and 4), soluble fractions (lanes 2 and 5), or particulate fractions (lanes 3 and 6) from wild-type yeast strain RS-16. The distributions of homogenate protein between soluble and particulate fractions were 85 and 15%, respectively. Blots were immunodecorated with affinity-purified antibodies against Hal3p (lanes 1 to 3). In lanes 4 to 6, the antibodies were preincubated with purified Hal3p to demonstrate the specificity of the reaction. The positions of molecular weight standards (in kilodaltons) are indicated at the center. The arrow at the left indicates the position of Hal3p. The relative distribution of Hal3p between fractions was similar in the case of strain RS-47, which overexpresses Hal3p (data not shown). (B) Immunofluorescence analysis. Cells of strain RS-47, containing the *HAL3* gene in multicopy plasmid YEp-*HAL3*, were fixed and immunodecorated with affinity-purified antibodies against Hal3p (panels 1 and 2). In panels 3 and 4, the antibodies were preincubated with purified Hal3p to demonstrate the specificity of the reaction. After staining with a second antibody coupled to fluorescein and with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI), samples were visualized with either fluorescein (panels 1 and 3) or DAPI (panels 2 and 4) filters.

could result in altered sodium dodecyl sulfate binding and aberrant charge-to-length ratio.

Hal3p is a soluble component of the yeast cytoplasm. Using the 70-kDa immunoreactive band as an assay for Hal3p content, we found that Hal3p was present with similar enrichments in the soluble and particulate fractions of a homogenate of wild-type cells (Fig. 2A, lanes 2 and 3). As the soluble fraction contained 85% of total yeast protein, most of Hal3p is soluble.

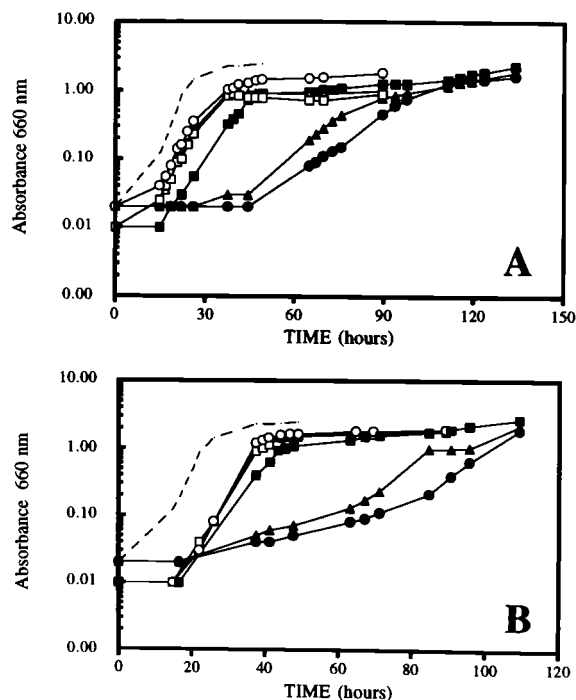


FIG. 3. Effects of *HAL3* on growth inhibition by different salts and sorbitol. In each panel, the discontinuous line reflects growth in normal minimal medium with uracil, which was identical in all strains. Squares, the Hal3p-overexpressing strain (containing YEp351-*HAL3*); triangles, the wild-type strain (containing YEp351); circles, the strain with the *hal3-1::LEU2* gene disruption. (A) Open symbols, medium supplemented with 1 M NaCl. (B) Open symbols, medium supplemented with 1.5 M sorbitol; closed symbols, medium supplemented with 0.1 M LiCl. Essentially identical results were obtained with three different transformants from every plasmid.

This relative distribution was unaffected in cells overexpressing Hal3p and in cells stressed with NaCl. The nature of the particulate fraction containing about 15% of Hal3p has not been identified. After isopycnic sucrose gradient centrifugation, it equilibrates at a sucrose concentration of 38 to 43% (wt/wt), where most yeast organelles such as the endoplasmic reticulum, Golgi bodies, vacuolar vesicles, and mitochondria are found (55). Nuclei and plasma membranes equilibrate at higher sucrose concentrations. Particulate Hal3p could be solubilized by neither detergent (Triton X-100) nor high ionic strength.

The predominant cytoplasmic localization of Hal3p was confirmed by indirect immunofluorescence (Fig. 2B). Uniform staining of the cells with apparent exclusion of immunoreactivity from the nuclei was observed. This localization was not affected by growing the cells under salt stress conditions.

Salt-sensitive growth of mutants lacking functional Hal3p.

As the last 71 amino acids of Hal3p are essential for its salt tolerance function (Fig. 1A), a construct, *hal3-1::LEU2*, in which the carboxy-terminal 310 amino acids and the 3' untranslated sequence were replaced by the *LEU2* gene, was used to disrupt the chromosomal *HAL3* locus in diploid wild-type cells. When the diploid transformant was sporulated and individual meiotic products were examined, in each case four viable haploid progeny with 2:0 segregation of leucine prototrophy were produced. As these experiments indicate that *HAL3* is not an essential gene, gene disruption was also performed in haploid strains RS-16 and W303-1A. Southern analysis confirmed that *HAL3* had been replaced with the *hal3-1::LEU2*

construct. The interruption of the gene was also reflected by the disappearance of the Hal3p-specific 70-kDa immunoreactive band in Western analysis (data not shown). *hal3-1::LEU2* cells demonstrated no significant phenotypes when grown under normal medium conditions. However, when the cells were challenged with medium containing 1 M NaCl or 0.15 M LiCl, a significant growth disadvantage in comparison with wild-type cells was observed (Fig. 3). When challenged with 1 M KCl or 1 M sorbitol, the mutant and wild-type cells grew equally well. Microscopic observation of *hal3-1::LEU2* cells undergoing salt stress showed an accumulation of large round unbudded cells, while the wild-type control strain showed a mixture of cells of various sizes, many of which contained buds.

To confirm that the *hal3-1::LEU2* mutation reported the true phenotype of a complete loss of function in *HAL3*, two more deletion mutations were constructed and introduced into the W303-1A background. The *hal3-2::HIS3* and *hal3-3::LEU2* mutations replaced the N-terminal 490 amino acids of the *HAL3* open reading frame with the *HIS3* and *LEU2* genes, respectively. Both alleles conferred LiCl and NaCl sensitivities identical to those conferred by *hal3-1::LEU2* mutation. When *hal3* mutants were crossed to the wild type and then subjected to tetrad analysis, poor growth on 150 mM LiCl or 1.0 M NaCl plates was found to be a recessive phenotype that segregated precisely with the leucine or histidine prototrophy, demonstrating linkage between the deletion and salt sensitivity. These results indicate that *HAL3* encodes an important determinant of adaptation to salt stress which is limiting for growth in high-salt media.

Ion relationships in *HAL3* mutants. The phenotypes of yeast cells overexpressing *HAL3* (YEp351-*HAL3*) and of the null mutant (*hal3-1::LEU2*) are specific for sodium and lithium (Fig. 3). In medium with added KCl or sorbitol, alterations of *HAL3* had no detectable effect on growth. Only in medium with added sodium or lithium could improved growth by overexpression and decreased growth in the null mutant be observed. Therefore, *HAL3* is a determinant of ion homeostasis and not of osmotic adjustment. The salt tolerance conferred by overexpression of *HAL3* was also observed in medium supplemented with methionine, pointing to a mechanism unrelated to the sodium-sensitive methionine biosynthetic enzyme encoded by *HAL2* (23, 42).

The intracellular levels of sodium and potassium were dependent on the levels of *HAL3* expression (Fig. 4). Overexpression of this gene increased K^+ and decreased Na^+ , with a null mutation having opposite effects. A kinetic study of ion

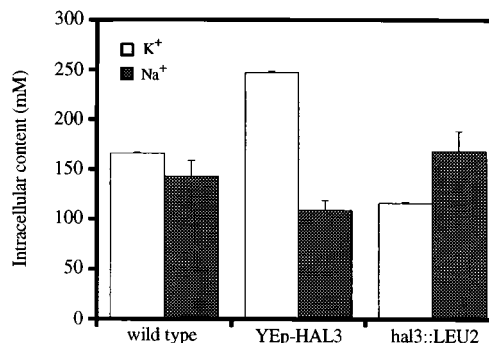


FIG. 4. Effects of *HAL3* on the intracellular ion concentrations of growing cells. Cells were grown in minimal medium with uracil supplemented with 1 M NaCl. Values are the means of three experiments, and bars represent the standard deviations. Essentially identical results were obtained with three different transformants from every plasmid.

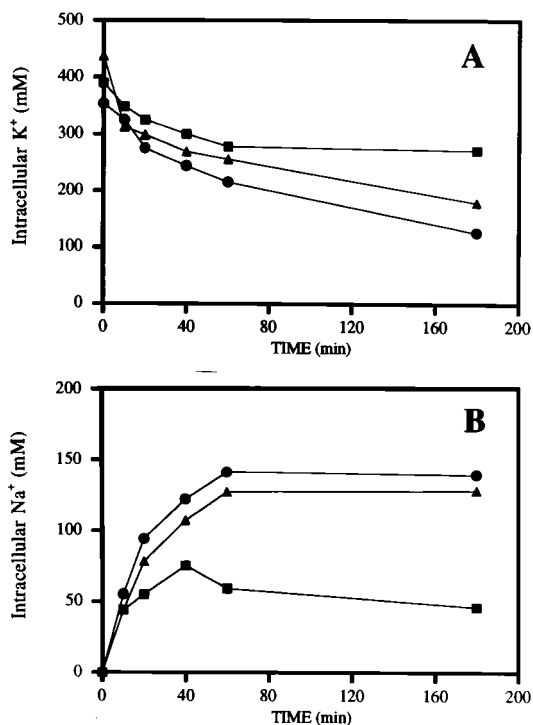


FIG. 5. Effects of *HAL3* on the time course of intracellular Na⁺ and K⁺ during salt stress. Exponentially growing cells in minimal medium with uracil were stressed by supplementation at time zero with NaCl to a final concentration of 0.85 M. Aliquots were taken at the indicated times for determination of the intracellular potassium (A) and sodium (B) concentrations. Squares, the Hal3p-overexpressing strain (containing YEp351-*HAL3*); triangles, the control strain (containing the YEp351); circles, the strain with the *hal3::LEU2* gene disruption. Essentially identical results were obtained with three different transformants from every plasmid.

movements during salt shock is shown in Fig. 5. The mechanism of K⁺ efflux indicated in Fig. 5A has not been characterized. It may reflect a normal efflux system compensated for during normal growth by K⁺ influx, with this influx being blocked during salt shock. Alternatively, it may correspond to a novel efflux pathway activated by salt shock (25). A null mutation in *HAL3* clearly accelerated K⁺ loss from the cells at early times. On the other hand, overexpression of *HAL3* did not affect the initial loss of K⁺ but inhibited loss at longer times. In this case, an effect on the recapture of K⁺ by activating the influx system could be involved.

HAL3 did not affect the initial rate of sodium influx, but it determined the final level of the ion. Cytoplasmic Na⁺ is reduced by *HAL3* overexpression and increased in the null mutant (Fig. 5B). This finding suggests an effect of *HAL3* on the previously described sodium efflux system which opposes influx after a lag period (20, 27, 28). This delayed response has been attributed to activation of the major sodium and lithium efflux system encoded by the *ENA1/PMR2A* gene. This mechanism was confirmed by analysis of a yeast strain carrying a disrupted *ENA1/PMR2A* gene, in which overexpression of *HAL3* had no effect on the final level of lithium uptake (Fig. 6). Similar results were obtained with sodium uptake.

Parallel effects of Hal3p and calcineurin on salt tolerance and *ENA1/PMR2A* expression. We examined the interaction between Hal3p and the other known modulator of ion homeostasis in *S. cerevisiae* calcineurin (40, 44) (Fig. 7A). Salt tolerance of wild-type yeast cells was compromised by mutation either in *HAL3* or in the *CNB1* gene encoding the regu-

latory subunit of calcineurin. A further decrease in salt tolerance was observed in the *hal3-1::LEU2 cnb1::LEU2* double mutant. NaCl sensitivity similar to that of *hal3-1::LEU2 cnb1::LEU2* double mutant was observed in *hal3-1::LEU2* mutants exposed to FK506 (1 μg/ml) to inhibit calcineurin activity and in a *cnb1::URA3 cnb2::HIS3 hal3::LEU2* triple mutant. These data indicate that calcineurin and *HAL3* make independent contributions to NaCl tolerance. The sensitivity of the *hal3-1::LEU2 cnb1::LEU2* double mutant was comparable to that conferred by a complete deletion of the *PMR2* locus (Fig. 7A). The *pmr2::HIS3* mutation (53) replaces all five *PMR2* repeats, corresponding to *ENA1/PMR2A* through *ENA5/PMR2E*, with the *HIS3* gene.

The salt-sensitive growth phenotypes of the wild-type and *hal3-1::LEU2* and *cnb1::LEU2* mutants correlated with the level of expression of the *ENA1/PMR2A* gene (Fig. 8). As previously described (40), calcineurin is required for maximal *ENA1/PMR2A* expression, both in the absence and in the presence of salt stress. As for calcineurin, a functional *HAL3* gene is required for normal *ENA1/PMR2A* expression in uninduced and induced conditions. Each of these two determinants of *ENA1/PMR2A* expression, calcineurin and *Hal3p*, have additive and independent effects: *CNB1* improves *ENA1/PMR2A* expression in both *HAL3* and *hal3-1::LEU2* strains, and *HAL3* improves *ENA1/PMR2A* expression in both *CNB1* and *cnb1::LEU2* strains. Calcineurin and *hal3-1* mutants are specially defective in the induction of *ENA1/PMR2A* expression at high-salt concentrations (above 0.4 M; Fig. 8). At lower salt concentrations, the level of induction is less significantly affected by either mutation.

Another correlation between *HAL3* expression, salt tolerance, and *ENA1/PMR2A* expression is that overexpression of Hal3p from high-copy-number *HAL3* plasmids promotes increased expression of *ENA1/PMR2A* in otherwise wild-type cells. With an integrated *ENA1/PMR2A-lacZ* reporter gene, the magnitude of the effect in normal media is about threefold (from 15 to 50 U of β-galactosidase specific activity). In medium with 0.8 M NaCl, the effect of overexpressing *HAL3* is 1.5-fold (from 550 to 800 U of β-galactosidase specific activity). Increased *ENA1/PMR2A* expression resulting in enhanced Na⁺/Li⁺ transport capacity may confer the superresistance to

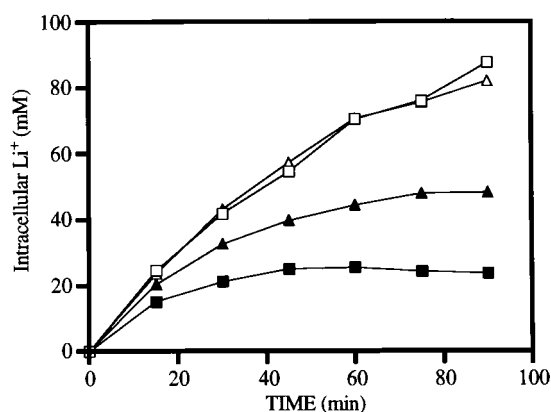


FIG. 6. Effects of *HAL3* and *ENA1* on lithium uptake. Exponentially growing cells in minimal medium with histidine and tryptophan were supplemented at time zero with 0.1 M LiCl. Aliquots were taken at the indicated times for determination of intracellular lithium concentrations. Open symbols, *ena1.2::LEU2* null mutant; closed symbols, wild type (containing YEp351). Triangles, cells with YEp352; squares, cells with YEp352-*HAL3*. Essentially identical results were obtained with three different transformants from every plasmid.

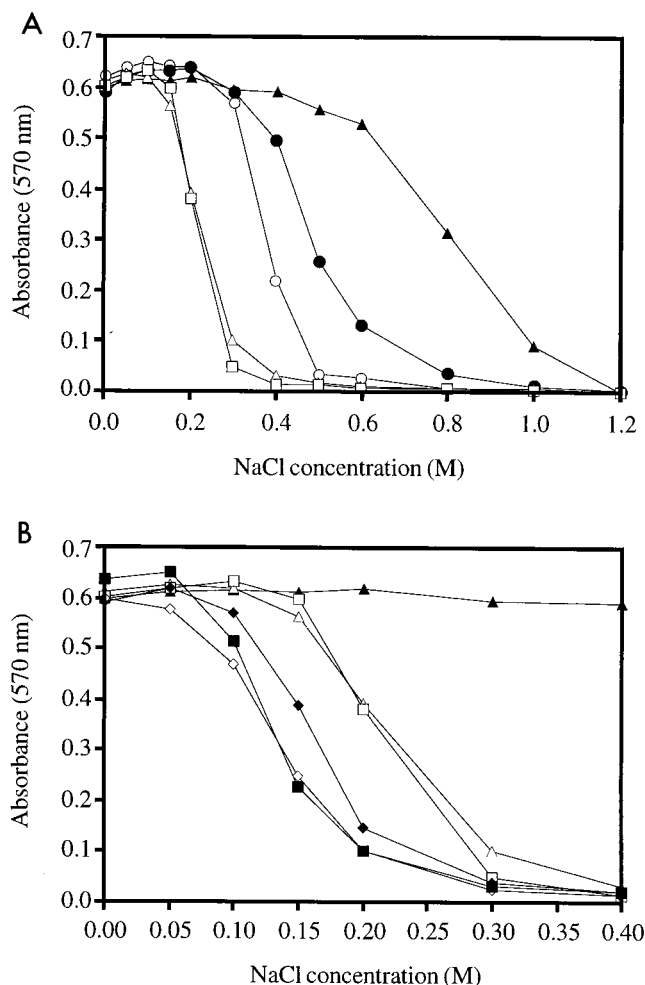


FIG. 7. Salt tolerance of yeast cells with *cnb1*, *hal3*, and *ena1,2* mutations. Saturated cultures of the different strains were diluted in fresh media with the indicated concentrations of salt, and growth was recorded after 18 h. The experiment was repeated twice with similar results, and essentially identical results were obtained with three different transformants from every plasmid. (A) Strains used were W303-1A (*CNB1 HAL3 ENA1*; closed triangles), SKY683 (*cnb1::LEU2*; open circles), SKY684 (*hal3::LEU2*; closed circles), SKY697 (*pmr2::HIS3*; open triangles), and SKY682 (*cnb1::LEU2 hal3::LEU2*; open squares). (B) Strains used were W303-1A (*CNB1 HAL3 ENA1*; closed triangles), SKY697 (*ena1::HIS3*; open triangles), SKY682 (*cnb1::LEU2 hal3::LEU2*; open squares), SKY699 (*hal3::LEU2 pmr2::HIS3*; closed diamonds), SKY700 (*cnb1::LEU2 pmr2::HIS3*; open diamonds), and SKY704 (*cnb1::LEU2 hal3::LEU2 pmr2::HIS3*; closed squares).

salt found in cells expressing plasmid-borne copies of the *HAL3* gene.

The presence of a YEp24 high-copy-number plasmid carrying *ENA1/PMR2A* (53) significantly improved the growth of *hal3-1::LEU2*, *cnb1::LEU2*, and *cnb1::LEU2 hal3-1::LEU2* mutant strains exposed to solid medium containing 1 M NaCl or 0.15 M LiCl over that of the same strains carrying the vector alone (data not shown). Suppression of the salt sensitivity of calcineurin and *hal3* mutants by overexpression of *ENA1/PMR2A* confirms that failure of such mutants to induce *ENA1/PMR2A* expression is a plausible mechanism for their salt sensitivity.

Evidence for targets of *HAL3* and calcineurin mediating salt tolerance other than *PMR2*. Crossing a *cnb1::LEU2 hal3-1::LEU2* strain to a strain carrying a complete deletion of the *PMR2* locus, *pmr2::HIS3*, yielded a series of strains with all

combinations of *hal3-1::LEU2*, *cnb1::LEU2*, and *pmr2::HIS3*. These strains were tested for NaCl sensitivity (Fig. 7B). Combination of either *hal3-1::LEU2* or *cnb1::LEU2* with *pmr2::HIS3* induced slightly decreased tolerance for NaCl over that of the *pmr2::HIS3* mutant alone. One interpretation of these data is that Hal3p and calcineurin each affect activities of targets beyond *ENA1/PMR2A* that are also involved in adaptation to NaCl challenge.

Effect of *HAL3* on potassium transport. As demonstrated in Fig. 9A, overexpression of *HAL3* on a high-copy-number plasmid confers improved salt tolerance even to an *ena1,2::LEU2* mutant lacking the inducible Na⁺ efflux system. This phenotype correlates with an increase in intracellular K⁺ without affecting the intracellular Na⁺ (Fig. 9B). Therefore, in concert with its effect on *ENA1/PMR2A* expression, Hal3p may modulate a potassium transport system, and this effect likely contributes to salt tolerance. This observation indicates that the altered K⁺ efflux in *hal3-1::LEU2* mutants and in cells overexpressing *HAL3* depicted in Fig. 5A is likely independent of *ENA1/PMR2A* activity and may be a significant component of the altered salt tolerance in these mutants.

Hal3p is involved in adaptation to peptide mating pheromone. In addition to its role in ion homeostasis, calcineurin is required for recovery from α -factor pheromone-induced growth arrest (10). Therefore, we investigated if *HAL3* also participates in this phenomenon. Overexpression with YEp-*HAL3* plasmids improved adaptation to the pheromone, as measured by filling in of the halo of arrested *MATa* cells surrounding a source of α -factor. Enhanced recovery was observed both in normal medium and in the presence of the calcineurin inhibitor FK506 (Fig. 10). Disruption of *HAL3*, however, had no significant effect on adaptation on any media.

DISCUSSION

Tolerance for environmental stresses is an important determinant of survival in organisms that lack the capacity to escape. Fungi and plants are often confronted with large transient changes in ion concentrations in their environments, challenging homeostatic mechanisms to maintain intracellular

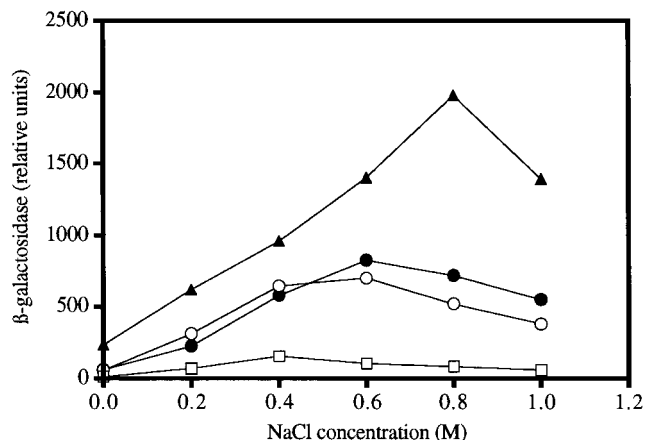


FIG. 8. Expression of an integrated *ENA1-lacZ* fusion in yeast cells with *cnb1* and *hal3* mutations. Strain used were SKY723 (*CNB1 HAL3*; triangles), SKY725 (*cnb1::LEU2*; open circles), SKY724 (*hal3::LEU2*; closed circles), and SKY726 (*cnb1::LEU2 hal3::LEU2*; squares). Cells were grown in rich medium to exponential phase, and β -galactosidase activity was measured after 90 min of incubation with the indicated concentrations of NaCl. Values are the means of three experiments with standard deviations of less than 10%. Essentially identical results were obtained with three different transformants from every plasmid.

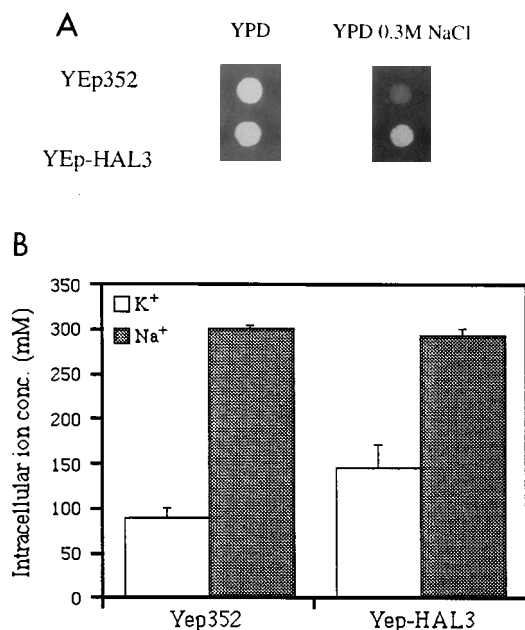


FIG. 9. Effects of *HAL3* in yeast cells without the *ENA1* efflux system. The *ena1,2::LEU2* mutant was transformed with either YEp352 or YEp352-*HAL3* (YEp-*HAL3*). (A) Growth in rich medium and in medium supplemented with 0.3 M NaCl. (B) Intracellular Na⁺ and K⁺ after incubation of cells growing exponentially in rich medium with 1 M NaCl during 3 h. Values are the means of three experiments, and bars represent the standard deviations. Essentially identical results were obtained with three different transformants from every plasmid.

ions within the relatively small concentration range consistent with life. We have studied genes regulating the response to NaCl salt stress in a model organism, the budding yeast *S. cerevisiae*. By screening both for superresistance to NaCl and for bypass of an NaCl-sensitive mutant, we have isolated a gene, *HAL3*, that when overexpressed confers resistance to salt stress and that when disrupted causes salt sensitivity. We conclude that this novel salt tolerance gene encodes a critical component of the machinery that modulates sodium and potassium transport in *S. cerevisiae*.

HAL3 does not fall easily into a previously described class of effectors of transport or homeostasis. The predicted sequence of Hal3p shares no significant sequence homology with previously described transporters, signaling molecules, or transcription factors. Southern analysis under nonstringent conditions suggests that at least two other yeast genes may have significant nucleic acid homology to *HAL3*. One of these may correspond to the predicted yeast gene YKL088w on chromosome XI (13), an open reading frame that has both domain structure and sequence homology to *HAL3*. *HAL3* and YKL088w likely establish a new gene family of regulatory molecules.

As previously found for calcineurin (40, 44), Hal3p has positive effects in salt adaptation, increasing both potassium uptake and sodium efflux during salt stress. We find that both components contribute to salt tolerance. As for calcineurin (40), we find that activation of sodium efflux by Hal3p is based on increased expression of the *ENA1/PMR2A* gene, a sodium efflux transporter whose expression is increased significantly with salt stress (20). Mutants lacking either functional Hal3p or functional calcineurin demonstrate decreased induction of *ENA1/PMR2A* expression. Double mutants have a greatly diminished *ENA1/PMR2A* transcriptional response. In accordance with these effects, overexpression of *ENA1/PMR2A* in

multicopy plasmids increases the salt tolerance of cells lacking calcineurin and/or Hal3p. Clearly, lack of *ENA1/PMR2A* expression is a critical defect producing salt sensitivity in *hal3* and calcineurin mutants.

Independent of its role in stimulating Na⁺ efflux, Hal3p may have some role in Na⁺ tolerance via its effects on activation of K⁺ uptake during salt stress. Physiological studies have shown calcineurin-dependent modulation of the relative affinities of the *TRK1,2* cation import system for K⁺ and Na⁺ (27, 40), and it is plausible that this modulation is also dependent on Hal3p.

In addition to their common effects on ion homeostasis, calcineurin and Hal3p are each involved in the recovery from α -factor-induced-growth arrest. Cell cycle arrest of *MAT α* cells by the α -factor pheromone secreted by a *MAT α* mating partner requires a mitogen-activated protein kinase cascade and results in inactivation of cyclin-dependent kinase and reduced expression of G₁ cyclins, leading to G₁ arrest in preparation for conjugation (59). Arrested cells that fail to conjugate, as when stimulated with synthetic pheromone, eventually return to vegetative growth in a process termed recovery. Calcineurin mutants are defective in recovery and lose viability if kept in the continuous presence of α -factor (10). Although the targets of calcineurin in pheromone adaptation remain unidentified, calcineurin-dependent dephosphorylation of some target of the pheromone kinase cascade could be essential for renewed synthesis of G₁ cyclins and growth recovery. Unlike the case with calcineurin, disruption of *HAL3* does not inhibit recovery. However, *HAL3* overexpression in high-copy-number plasmids speeds recovery and suppresses the adaptation defect in calcineurin mutants.

The conventional explanation for the genetic results indicating that either Hal3p or calcineurin can operate in the absence of the other is that these two proteins define independent and parallel pathways involved in homeostatic responses to salt stress and adaptation to mating pheromone. Several activities making up components of the calcineurin pathway can be reconstructed from these and other experiments. Calcineurin is a Ca²⁺-calmodulin-dependent protein phosphatase (8). Its activity *in vivo* is almost completely limited to intervals where the cytoplasmic calcium concentration increases significantly above its basal level. Large Ca²⁺ influxes and/or significant increases in cytoplasmic Ca²⁺ have been measured during the yeast α -mating pheromone response (30) and upon salt stress of maize protoplasts (38) and yeast cells (unpublished observations). Therefore, one arm of this dual response to salt stress

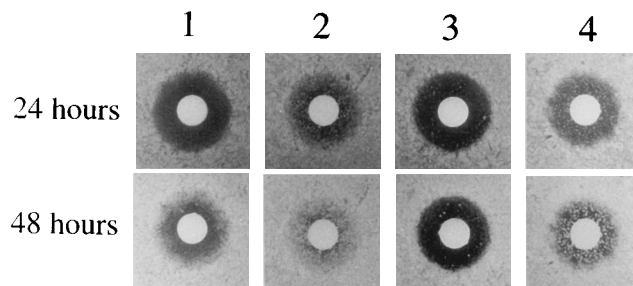


FIG. 10. Pheromone response and recovery of *HAL3* mutants and calcineurin-inhibited cells. Immediately after solidification of the top agar-containing cells, sterile cellulose discs (0.6 cm; Difco) with 14 μ g of synthetic α -factor (Sigma) were placed on the nascent lawn. The plates were incubated at 30°C and photographed after 24 and 48 h, as indicated. Plates: 1 and 3, control cells (transformed with YEp351); 2 and 4, Hal3p-overexpressing cells (transformed with YEp351-*HAL3*); 1 and 2, YPD plates; 3 and 4, plates supplemented with the calcineurin inhibitor FK506 (2 μ g/ml). Essentially identical results were obtained with three different transformants from every plasmid.

consists of a pathway leading to calcium influx and calcium activation of calmodulin and thereby of calcineurin. Were the paradigm of calcineurin function in the response of stimulated T cells to hold in adaptation responses in *S. cerevisiae*, the principal function of calcineurin would be to dephosphorylate and thereby activate a transcription factor. In salt adaptation, the as yet unidentified transcription factor could then activate expression of the *ENA1/PMR2A* gene to promote Na^+ efflux.

We can only speculate about the signaling mechanism of the second pathway defined by Hal3p. The predicted sequence of the *HAL3* open reading frame offers little clue to its function. A conspicuous feature is an essential acidic domain at the Hal3p carboxyl terminus. Polyacidic regions are present in many other proteins such as the nuclear proteins nucleoplamin, nucleolin, high-mobility-group proteins, and UBF nuclear transcription factors (14), as well as in proteins with other localizations such as calsequestrin, a calcium-binding protein of the endoplasmic reticulum (65), and Kex1 protein, a carboxypeptidase of the yeast Golgi apparatus (12). While preparing this report, we became aware that Di Como et al. (11) isolated *HAL3* as *SIS2*, a multicopy suppressor of *sit4* mutants. *SIT4* encodes a protein phosphatase of type 2A (2) whose function is required for the normal expression of G_1 cyclin genes at the start of the cell cycle (16). Overexpression of *SIS2* resulted in increased expression of the *CLN1*, *CLN2*, and *CLB5* cyclin genes, but disruption of *HAL3/SIS2* does not confer the *sit4* phenotype of impaired cyclin expression. Di Como et al. (11) find a predominant nuclear localization for Hal3p in cell extracts and therefore propose that it may be stably associated with chromatin. Indeed, that many proteins with polyacidic domains are involved in gene expression at the level of control of transcription or chromatin structure suggests that Hal3p could function in the control of expression of cyclin genes and *ENA1/PMR2A* by directly binding to their promoters. However, using both fractionation and immunolocalization, we find that Hal3p is predominantly a soluble cytoplasmic protein. The cytosolic localization of the bulk of Hal3p in yeast cells would argue for a function outside the nucleus. We are concerned that the acidic domain of Hal3p may make it prone to nonspecific interactions with other cellular components, perhaps causing it to associate with the nuclear fraction at the nonphysiological ionic strength used during homogenization by Di Como et al. (11). Further work is needed to clarify this point.

That Hal3p participates as a parallel element to two distinct phosphatase signaling pathways is highly significant. Hal3p may be involved in the function of yet a third phosphatase pathway. We have considered what cytoplasmic functions might be ascribed to Hal3p. One class of relatively poorly characterized proteins, the 14-3-3 proteins (41), has been found to participate in signaling multiple kinase pathways, including the protein kinase C and Raf pathways, via direct interaction with the kinases. An attractive mechanism that explains how Hal3p appears to act in parallel to two distinct protein phosphatases (calcineurin and Sit4p) is that it may have a role analogous to that of 14-3-3 proteins, but acting in multiple regulated protein phosphatase pathways. Hal3p could act as a scaffold mediating the interaction of signaling phosphatases, including calcineurin, Sit4p, and others, with specific substrates. Different genetic and biochemical approaches are under way to explore this novel regulatory machinery.

Nonetheless, we have not eliminated an alternate explanation for our results. The apparently independent activities of Hal3p and calcineurin do not rule out the possibility that Hal3p is one of two or more partially redundant targets for calcineurin in Na^+ adaptation. In the absence of activated

calcineurin, Hal3p and its redundant partner would be persistently phosphorylated and therefore largely inactivated. In a calcineurin mutant, a large excess of newly synthesized and therefore unphosphorylated Hal3p as produced by overexpression from multicopy plasmids could overwhelm the capacity of the relevant protein kinase to inactivate it. If phosphorylation became limiting, the increased level of unphosphorylated Hal3p resulting from this situation could then promote ectopic *ENA1/PMR2A* expression. On the other hand, in order to explain the differences in salt tolerance and *ENA1/PMR2A* expression between the *cnb1* mutant and the *hal3 cnb1* double mutant, Hal3p should have some activity in the absence of calcineurin. Experiments are under way to reveal genetic evidence for a redundant activity with Hal3p. Also, the state of phosphorylation of Hal3p as influenced by calcineurin is under investigation.

Some residual induction of *ENA1/PMR2A* expression by salt was detected even in the *hal3 cnb1* double mutant. We have evidence (unpublished observations) for the participation of the osmoregulated *PBS2-HOG1* mitogen-activated protein kinase pathway (5, 54) and of the nitrogen catabolite repression pathway mediated by the *URE2* and *GLN3* gene products (63) in regulating *ENA1/PMR2A* in a calcineurin- and *HAL3*-independent manner.

An important issue not resolved by our work is the specificity of Hal3p action. Hal3p is not a necessary component of all responses to salt stress. Neither *HAL3* nor calcineurin is involved in the expression of other yeast genes induced by salt stress such as *HAL1* (21), *CTT1* (39), and *DDR48* and *PAI3* (unpublished observations). In turn, our data do not rule out a role for Hal3p in expression of genes completely unrelated to calcineurin function. It is reasonable that in addition to *ENA1/PMR2A* and G_1 cyclins, expression of other genes may be modulated by this novel regulatory pathway.

We can try to rationalize the coordinate effects of calcineurin and Hal3p on K^+ uptake, Na^+ efflux, and G_1 cyclin synthesis on the basis of cellular strategies for growth control and stress tolerance. A connection between ion homeostasis and the cell cycle is an obvious postulate. Proper intracellular ion concentrations are part of the nutrient requirement for Start at the beginning of the yeast cell cycle (47). A common regulatory mechanism for ion transport and G_1 cyclin synthesis may provide the required coordination. Neither calcineurin nor Hal3p is required for growth under normal laboratory conditions, and their physiological role is apparent only under inhibitory conditions such as high sodium concentrations or presence of α -factor. The activating effects of calcineurin and Hal3p on K^+ uptake, Na^+ efflux, and G_1 cyclin synthesis are important only under stress conditions, where these otherwise highly regulated functions may be compromised.

A final point is that, given the similarity between plants and fungi in terms of basic ion transport mechanisms (55, 57), *S. cerevisiae* serves as model system for the understanding of ion homeostasis in plants. Calcineurin seems to be involved in the regulation of ion transport in plant cells (37). Hal3p may also play a role. Southern analysis at reduced stringency indicates the presence in plant genomes of a gene family related to yeast *HAL3*.

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