

Regulation of *ENA1* Na⁺-ATPase Gene Expression by the Ppz1 Protein Phosphatase Is Mediated by the Calcineurin Pathway

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Saccharomyces cerevisiae strains lacking the Ppz1 protein phosphatase are salt tolerant and display increased expression of the *ENA1* Na⁺-ATPase gene, a major determinant for sodium extrusion, while cells devoid of the similar Ppz2 protein do not show these phenotypes. However, a *ppz1 ppz2* mutant displays higher levels of *ENA1* expression than the *ppz1* strain. We show here that the increased activity of the *ENA1* promoter in a *ppz1 ppz2* mutant maps to two regions: one region located at -751 to -667 , containing a calcineurin-dependent response element (CDRE), and one downstream region (-573 to -490) whose activity responds to intracellular alkalization. In contrast, the increased *ENA1* expression in a *ppz1* mutant is mediated solely by an intact calcineurin/Crz1 signaling pathway, on the basis that (i) this effect maps to a single region that contains the CDRE and (ii) it is blocked by the calcineurin inhibitor FK506, as well as by deletion of the *CNB1* or *CRZ1* gene. The calcineurin dependence of the increased *ENA1* expression of a *ppz1* mutant would suggest that Ppz1 could negatively regulate calcineurin activity. In agreement with this notion, a *ppz1* strain is calcium sensitive, and this mutation does not result in a decrease in the calcium hypertolerance of a *cnb1* mutant. It has been shown that *ENA1* can be induced by alkalization of the medium and that a *ppz1 ppz2* strain has a higher intracellular pH. However, we present several lines of evidence that show that the gene expression profile of a *ppz1* mutant does not involve an alkalization effect. In conclusion, we have identified a novel role for calcineurin, but not alkalization, in the control of *ENA1* expression in *ppz1* mutants.

The *Saccharomyces cerevisiae* Ppz phosphatases are encoded by the *PPZ1* and *PPZ2* genes (21, 37, 38), and they are characterized by a catalytic carboxyl-terminal half related to type 1 phosphatases and an amino-terminal extension. The catalytic moieties of the two proteins are very similar (92%). In contrast, the sequences of the amino-terminal halves are much less related. These phosphatases are involved in a variety of cell processes, including regulation of salt tolerance (39), maintenance of cell wall integrity (26, 37), and regulation of the cell cycle at the G₁/S transition (5, 6). In all cases, the function of Ppz1 appears to be more important than that of Ppz2. For instance, a *ppz1* deletion mutant is tolerant to high concentrations of sodium or lithium cations, while a *ppz2* mutant is not. However, the *ppz1 ppz2* double mutant is more tolerant than the *ppz1* single mutant (39). Genetic and biochemical evidence has identified the halotolerant determinant Hal3, also known as Sis2 (12, 13), as a negative regulatory subunit of Ppz1 (11). Therefore, overexpression of Hal3 results in increased salt tolerance. Hal3 binds to the carboxy-terminal catalytic moiety of Ppz1, thus modulating the diverse physiological functions of the phosphatase. No evidence of Hal3 interaction with Ppz2 has been reported so far.

In *S. cerevisiae*, the *ENA1* gene plays an important role in salt tolerance. This gene encodes a P-type Na⁺-ATPase that represents the most important element for the efflux of Na⁺

and Li⁺. Consequently, an *ena1* mutant is highly sensitive even to low concentrations of Na⁺ or Li⁺ (14). The *ENA1* gene is barely expressed under standard growth conditions, but it is strongly induced by exposure to high salt concentrations and to an alkaline pH. This transcriptional response of *ENA1* is based on a complex regulation of its promoter (28). Expression of *ENA1* is repressed by the presence of glucose in the medium (2), through a mechanism that involves the general repressor complex Mig1-Ssn6-Tup1 (40). Saline induction is mediated by two pathways: the Hog1 mitogen-activated protein kinase pathway and the calcineurin pathway. The Hog1 pathway responds to increased osmolarity and acts through the Sko1 transcriptional inhibitor (40), which binds to a cyclic AMP response element present in the *ENA1* promoter.

The Ca²⁺-calmodulin-activated protein phosphatase calcineurin is structurally and functionally conserved from yeast to humans. The enzyme is a heterodimer composed of a catalytic A subunit and a regulatory B subunit. In *S. cerevisiae*, the catalytic subunit is encoded by two genes (*CNA1* and *CNA2*), while a single gene (*CNB1*) codes for the regulatory subunit (9, 10, 24, 27, 48). Calcineurin regulates *ENA1* transcription in response to sodium and lithium toxicity by dephosphorylating and activating the transcriptional activator Crz1/Tcn1/Hal8 (29, 31, 44), which binds specific DNA sequences known as calcineurin-dependent response elements (CDRE). Two such elements are present in the *ENA1* promoter, at positions -813 to -821 and -727 to -719 ; the downstream element is more relevant for the transcriptional response under stress conditions (30). The function of calcineurin can be regulated by a conserved family of proteins termed calcipressins, which are represented in *S. cerevisiae* by a single gene known as *RCN1*.

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Interestingly, although it seems clear that Rcn1 binds and inhibits calcineurin, it is not evident whether this protein acts as a regulator of calcineurin or as a downstream effector of the phosphatase (17, 23). Recently, a role for the TOR (target of rapamycin) pathway in the regulation of *ENAI* expression through the Gln3 and Gat1 transcription factors has been documented (7).

The transcriptional response of *ENAI* to an alkaline pH is also rather complex and not yet fully understood. Although the effect of a high pH on *ENAI* induction was described about 10 years ago (14, 33), only recently has a report (43) addressed the molecular basis of this effect and identified two responsive regions (ARR1 and ARR2) in the *ENAI* promoter. ARR1 maps to the downstream CDRE and is responsible for the calcineurin-dependent component of the response. ARR2 spans nucleotides -573 to -490 and integrates Rim101-dependent and -independent inputs. A very recent report has suggested that Rim101 may inhibit the function of the Nrg1 transcription factor, which in turn would negatively regulate the *ENAI* promoter (25).

The early observation that a *ppz1 ppz2* mutant showed increased expression of the *ENAI* gene (39) provided a reasonable explanation for the observed salt-tolerant phenotype. However, it has been shown very recently that a *ppz1 ppz2* mutant strain displays an augmented intracellular pH and, under NaCl stress conditions, a higher-than-normal intracellular potassium content. This is likely the result of an inhibitory role of the Ppz phosphatases on the Trk1/Trk2 potassium transporters, and it may explain most of the phenotypes observed in cells lacking both phosphatase genes (49). Despite the evident link between lack of Ppz phosphatases and Trk function, a number of important issues remain to be resolved. For instance, there was evidence that deletion of both *PPZ* phosphatases in a *trk1 trk2* background still produced a detectable increase in salt tolerance (49), suggesting that Trk-independent mechanisms may exist. In addition, since the saline tolerances of *ppz1* and *ppz2* mutants are not equivalent, it was important to evaluate the relative contributions of the two gene products to this phenotype.

The fact that the *ENAI* ATPase gene is a major determinant for salt tolerance prompted us to investigate how the absence of Ppz1, Ppz2, or both phosphatases affects *ENAI* expression. In this report we present a detailed dissection of the relative contributions of the Ppz phosphatases to the regulation of *ENAI*, providing evidence that Ppz1 negatively controls *ENAI* expression through mechanisms that involve calcineurin but not intracellular alkalinization.

MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast cells were grown at 28°C in YPD medium (containing, per liter, 10 g of yeast extract, 20 g of peptone, and 20 g of dextrose) or, when indicated, in synthetic minimal (SD) or complete minimal (CM) medium (1). The relevant genotypes of the strains described in this work are given in Table 1. Sensitivity to LiCl and NaCl was monitored essentially as in reference 39.

Gene disruptions and plasmids. Disruption of the *PPZ1* gene with the *URA3* marker was carried out as in reference 38. Deletion of this gene with the *TRP1* marker was performed as follows. Plasmid pYC2Z1 (6) was digested with *Bam*HI/*Hind*III, and the 1.6-kbp insert was cloned into the same sites of plasmid pGEM-3Z (Promega). This construct was then digested with *Sal*I/*Stu*I, and the DNA fragment released was replaced with a 0.85-kbp *TRP1* marker, previously released from plasmid YDp-W (4) by digestion with *Sal*I/*Sma*I. The disruption

TABLE 1. *S. cerevisiae* strains used in this work

Strain	Relevant genotype	Source or reference
DBY746	<i>MATα ura3-52 leu2-3, 112 his3-Δ1 trp1-Δ239</i>	D. Botstein
EDN2	DBY746 <i>ppz1::TRP1</i>	This work
EDN85	DBY746 <i>ppz1::TRP1 ppz2::KAN</i>	This work
MAR55	DBY746 <i>ppz2::TRP1</i>	This work
MAR15	DBY746 <i>cnb1::KAN</i>	43
EDN92	DBY746 <i>crz1::KAN</i>	43
MAR18	DBY746 <i>crz1::KAN ppz1::URA3</i>	This work
MAR19	DBY746 <i>cnb1::KAN ppz1::URA3</i>	This work
EDN308	DBY746 <i>crz1::KAN ppz1::TRP1</i>	This work
MAR40	DBY746 <i>cnb1::KAN ppz1::TRP1</i>	This work
ESV212	DBY746 <i>trk1::LEU2 trk2::HIS3</i>	This work
MAR37	DBY746 <i>ppz1::TRP1 trk1::LEU2 trk2::HIS3</i>	This work
RSC25	DBY746 <i>rcn1::KAN</i>	This work
MAR39	DBY746 <i>ppz1::TRP1 rcn1::KAN</i>	This work
MAR43	DBY746 <i>mck1::KAN</i>	This work
MAR46	DBY746 <i>ppz1::TRP1 mck1::KAN</i>	This work
JA100	<i>MATα ura3-52 leu2-3, 112 his4 trp1-1 can-1^r</i>	11
EDN75	JA100 <i>ppz1::KAN</i>	11
JA-103	JA100 <i>ppz2::TRP1</i>	6
EDN76	JA100 <i>ppz1::KAN ppz2::TRP1</i>	This work

cassette was then digested with *Sac*I, and the 1.3-kbp fragment was used for yeast transformation. This strategy removes most of the Ppz1 protein (from residue 1 to residue 547). Deletion of *PPZ2* with a *TRP1* marker was performed as described by Clotet et al. (6). To disrupt the *PPZ2* gene with the KanMX marker, a 2.2-kbp *Bam*HI/*Bam*HI fragment of the gene was cloned into pUC18 and then digested with *Hpa*I and *Bgl*II to remove approximately 1.5 kbp corresponding to a small fragment of the 5' untranslated region plus the first 460 codons. This fragment was replaced by the KanMX marker, obtained by digestion of plasmid pFA6-kanMX4 (45) with *Bgl*II and *Eco*RV. The final construct was then cleaved by *Bam*HI, and the insert was used to transform yeast cells. Disruptions of *CNB1* and *CRZ1* with the *KAN* marker have been described previously (43). The *TRK1* and *TRK2* genes were disrupted with the *LEU2* and *HIS3* markers, respectively, by using deletion cassettes described previously (20). Strains carrying deletions of the *RCN1* and *MCK1* genes were generated as follows. Deletion cassettes were amplified by PCR from genomic DNA prepared from *rcn1::KanMX* and *mck1::KanMX* deletion mutants in the BY4741 background (46). The oligonucleotides used spanned nucleotides -842 to +746 (*RCN1*) and -467 to +1782 (*MCK1*). Positions are relative to the initiating ATG codon. The amplification fragments were purified and used to transform the desired strains.

For high-copy expression of *HAL3*, the gene was recovered from plasmid YEep351-HAL3 (13) by digestion with *Eco*RI/*Hind*III and cloned into these sites of plasmid YEplac181 (16).

β -Galactosidase reporter constructs and assays. Plasmid pKC201, containing the entire promoter of *ENAI*, has been described previously (2, 8). The pMP and pMR series, containing defined fragments of the *ENAI* promoter, have also been described previously (40, 43). pMRK211 was constructed similarly to pMRK212 and pMRK213 (43). Plasmid pLA, a gift from I. Fernandez de Larrinoa (Universidad del Pais Vasco, San Sebastián, Spain), contains the -732-to-711 sequence of the *ENAI* promoter, corresponding to the high-affinity Crz1-binding element (30), and derives from plasmid pLG Δ 312s (19). Plasmid pAMS366 contains four copies in tandem of the wild-type CDRE present in the *FKS2* promoter, while pAMS364 contains a mutated version that cannot bind Crz1 (44). The construction of pPHO84-LacZ, pPHO89-LacZ, and pPHO12-LacZ has been described previously (43). Plasmids pFRE1-LacZ, pTRR1-LacZ, and pFIT2-LacZ were constructed by PCR amplification of the -792-to-+73, -758-to-+82, and -695-to-+82 regions of the respective genes, which were cloned into the *Eco*RI/*Hind*III sites of plasmid YEep357 (34). Plasmid pSIT1-LacZ was constructed by cloning the -786-to-+52 region of *SIT1* into the *Eco*RI/*Sac*I site of YEep357. In all cases, cloning resulted in translational fusions with the β -galactosidase gene.

The levels of expression driven by the various promoters tested (as well as by specific CDRE) in different mutant backgrounds were determined by using β -galactosidase reporter constructs as follows. Yeast cells were transformed with the desired plasmids, and positive clones were grown in selective medium (lacking uracil) to an A_{660} of 1.5 to 2.0. These cultures were used to inoculate YPD medium (2.5 ml), and growth was resumed until an A_{660} of 0.6 to 0.8 was reached

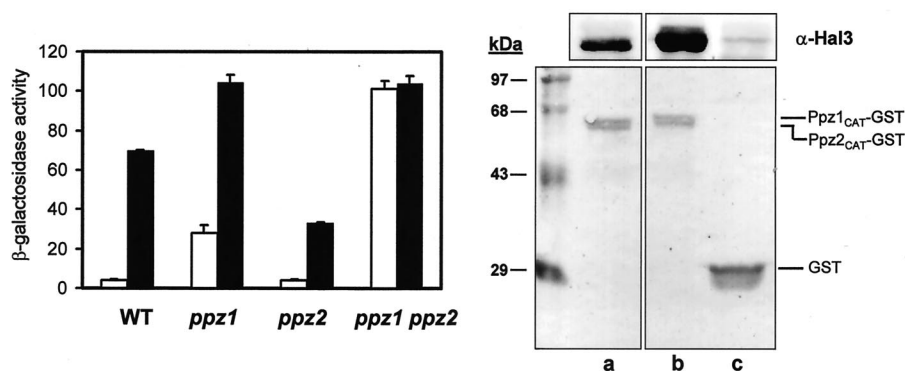


FIG. 1. Effects of overexpression of Hal3 on *ENAI* expression in different Ppz backgrounds. (Left) Strains JA100 (wild type [WT]), EDN75 (*ppz1*), JA103 (*ppz2*), and EDN76 (*ppz1 ppz2*) bearing plasmid pKC201 (2), which encodes the β -galactosidase reporter gene fused to the *ENAI* promoter, were transformed with the high-copy-number plasmid YEplac181 carrying no insert (open bars) or the same plasmid carrying the entire *HAL3* gene. Cells were grown as described in Materials and Methods, and β -galactosidase activity was measured. Data are means \pm standard errors of the means from three to nine independent clones. (Right) The catalytic domains of both Ppz1 and Ppz2 were purified from *E. coli* as GST fusion proteins. Approximately 3 μ g of purified protein was then used as an affinity resin to copurify Hal3 from crude yeast extracts. The material retained by the resin after extensive washing was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with antibodies specific for Hal3. The bottom panel shows the Ponceau S staining of the membrane, as a control for protein loading. The upper panel shows the result of Western blot analysis with Hal3-specific antibodies. Lane a, Hal3 purified by using the catalytic domain of Ppz2; lane b, Hal3 purified by using the catalytic domain of Ppz1). No Hal3 was detected by using GST alone as an affinity resin (lane c).

(approximately 5 h). When sensitivity to FK506 was tested, YPD medium was made 1.5 μ g/ml FK506 from a 10-mg/ml stock solution of the drug (in 90% ethanol–10% Tween P-20 [vol/vol]). Control cells were exposed only to the vehicle. Cells were recovered by centrifugation, and β -galactosidase assays were performed as described previously (41). The sensitivity to ambient pH of the response of the ARR1 and ARR2 elements of the *ENAI* promoter was determined essentially as described previously (43).

In vitro binding experiments. Interaction experiments were performed essentially as described previously (11). The construction of the glutathione *S*-transferase (GST) fusion protein containing the catalytic moiety (amino acids 345 to 692) of Ppz1 (Ppz1CAT-GST) has been documented previously (6). The catalytic domain (amino acids 379 to 710) of Ppz2 (Ppz2CAT) was cloned by PCR from genomic DNA using primers 5' ATATCTAGATAGGACTAATACTATGGTCGA and 3' ATAATAGTCGACTCAGCGATTGGCTAATTTAC. The resulting PCR product was then digested with *Xba*I and *Sal*I and was subcloned into the pGEX-KG vector (18). The yeast strain (LY127) used as a source of Hal3 has been described previously (49).

Intracellular cation measurements. Intracellular sodium and potassium concentrations were determined essentially as described previously (3). Briefly, YPD medium containing 0.3 M sodium chloride was inoculated with the different strains, and growth was resumed until an optical density at 660 nm (OD_{660}) of 0.9 to 1.0 was reached. Cells were recovered by filtration and washed with a 0.6 M sorbitol solution containing 10 mM magnesium chloride, and extracts were prepared by boiling for 30 min. Extracts were centrifuged for 1 min at 10,000 \times g, and the concentrations of the cations were measured in the supernatants by flame spectroscopy.

RESULTS

The Hal3-dependent regulation of *ENAI* expression is mediated by both Ppz1 and Ppz2 phosphatases. Hal3 was identified some time ago as a negative regulatory subunit of Ppz1. To characterize the relative influences of Ppz1 and Ppz2 phosphatases on *ENAI* expression, we first compared the effects of high-copy expression of Hal3 on the expression of the ATPase gene in wild-type cells and cells deficient in Ppz1, Ppz2, or both phosphatases. As shown in Fig. 1, overexpression of Hal3 in wild-type cells resulted in increased *ENAI* expression. When Hal3 was overexpressed in *ppz1* cells, the activity of the *ENAI* promoter reached virtually the same level as that observed in a

ppz1 ppz2 strain carrying an empty plasmid. Interestingly, expression of *ENAI* did not increase further when Hal3 was overexpressed in the *ppz1 ppz2* double mutant. A *ppz2* mutant did not show altered *ENAI* expression, and in this background, overexpression of Hal3 produced a level of activity of the *ENAI* promoter almost identical to that observed in a *ppz1* strain bearing an empty plasmid. These results indicated that Hal3 influences both Ppz1 and Ppz2 functions. To confirm the possibility of Ppz2 being a target for Hal3, the catalytic domains of both Ppz1 and Ppz2 were purified from *Escherichia coli* as GST fusion proteins and used as an affinity system to purify Hal3 from crude yeast extracts. As shown in Fig. 1, Hal3 was retained in similar amounts by Ppz1 and Ppz2, proving that Hal3 is able to interact in vitro with both phosphatases.

The increased *ENAI* expression of a *ppz1* mutant functionally maps to a CDRE and depends on an intact calcineurin-signaling pathway. The *ENAI* promoter is rather complex and is able to respond to different stimuli. We have analyzed the levels of expression in wild-type, *ppz1*, and *ppz1 ppz2* cells by using a number of reporter constructs that allow functional mapping of this promoter. As shown in Fig. 2, the increased *ENAI* expression characteristic of a *ppz1* mutant could be detected in all constructs including the -751 -to- -667 region, which contains the downstream CDRE defined for this promoter, but not in those lacking this element. In addition, incubation of the cells with the calcineurin inhibitor FK506 reduced the activity of the promoter in the *ppz1* mutant to the levels observed in wild-type cells. Interestingly, when the same experiments were performed with a *ppz1 ppz2* mutant, expression levels from the full promoter increased twofold and were reduced only partially after addition of FK506. As observed, the FK506-inhibitable expression fully maps to the -751 -to- -667 region. In contrast, the non-FK506-inhibitable component maps to a downstream region, delimited by nucleotides

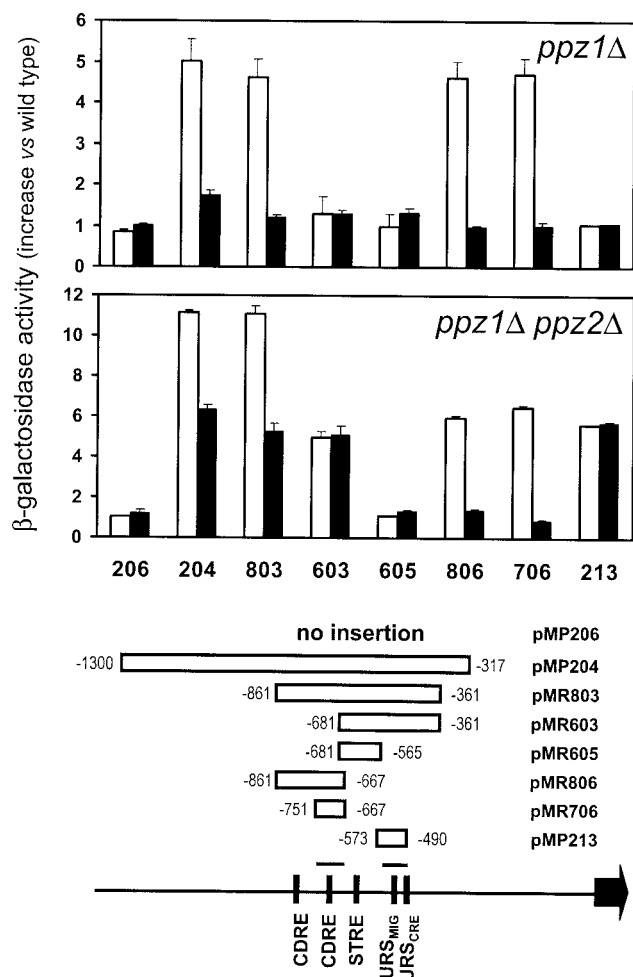


FIG. 2. Mapping the activity of the *ENA1* promoter in *ppz1* and *ppz1 ppz2* mutants. Strains JA100 (wild type), EDN75 (*ppz1*), and EDN76 (*ppz1 ppz2*) were transformed with the indicated constructs. (Bottom) Relevant regulatory elements described in the main text are schematically depicted. Rectangles represent the fragments of the *ENA1* promoter contained in the constructs, and numbers indicate their relative nucleotide positions (from the initiating Met codon). (Top) Data represent the ratio of β -galactosidase activity between each mutant and the isogenic wild-type strain in the absence (open bars) or presence (solid bars) of 1.5 μ g of FK506/ml. Each data point corresponds to the mean \pm standard error of the mean from three to nine independent clones.

–573 and –490. The level of expression driven from this region was not increased at all in a *ppz2* mutant.

The results described above suggested that the increased *ENA1* expression of a *ppz1* mutant was fully dependent on the existence of a functional calcineurin pathway. Consequently, we show (Fig. 3, left) that this increase in expression was completely abolished in a strain lacking the *CNB1* gene, which encodes the regulatory subunit of calcineurin. It is accepted that most, if not all, of the transcriptional response triggered by activation of calcineurin appears to be mediated by the transcriptional factor Crz1. As shown, lack of Ppz1 did not result in increased *ENA1* expression in a *crz1* mutant, providing additional support to the idea that the Ppz1-dependent effect on *ENA1* promoter activity requires an intact calcineurin/Crz1-

signaling pathway. Furthermore, expression driven exclusively by a synthetic sequence reproducing the CDRE found at positions –732 to –711 of the *ENA1* promoter was also increased in a *ppz1* strain (Fig. 3, right), and this increase was fully dependent on the presence of Crz1 (data not shown). Finally, we found that expression from the pAMS366 construct, which contains four tandem copies of the CDRE found in the calcium-responsive gene *FKS2*, was also increased in a *ppz1* mutant. This increase, however, was not detected when a mutated version of the *FKS2* CDRE (pAMS364), unable to bind Crz1 (44), was used (Fig. 3, right). The levels of expression driven from these chimeric promoters were only slightly increased by further deletion of the *PPZ2* phosphatase gene in a *ppz1* background.

Cation tolerance and *ENA1* gene expression analysis of strains lacking Ppz phosphatases and components of the calcineurin pathway. These results suggested that a *ppz1* strain may have an activated calcineurin pathway, and they were compatible with the notion of Ppz1 acting as a negative regulator of calcineurin. Accordingly, we observed that *ppz1* and *ppz1 ppz2* mutants were more sensitive to addition of exogenous CaCl_2 than the wild-type strain (Fig. 4A). Because deletion of *CNB1* in an otherwise wild-type background has been shown to confer calcium tolerance (47), we tested whether lack of *ppz1* affected this tolerance. We observed that a strain lacking Ppz1 and Cnb1 displayed a tolerance identical to that of a *cnb1* single mutant, demonstrating that Ppz1 is not required for this tolerance and lending further support to the idea that Ppz1 may act upstream of calcineurin. We next examined the salt tolerance phenotypes of strains lacking the Ppz1 phosphatase and components of the calcineurin pathway. As shown in Fig. 4B, we observed a decrease in the salt tolerance of the *ppz1* mutant upon further removal of either *CNB1* or its downstream transcription factor, *CRZ1*. Mutation of the *CNB1* gene in a *ppz1* background has more pronounced effects in terms of salt tolerance, as expected, since calcineurin is known to have cellular effects independent of Crz1.

Because Rcn1 has been reported as a protein that is able to interact with calcineurin and regulate its function, we were interested in evaluating whether the lack of Rcn1 could affect the level of expression of *ENA1* in a *ppz1* mutant. To this end, the *RCN1* gene was disrupted in wild-type and *ppz1* backgrounds, and the resulting strains were transformed with the *ENA1*-LacZ reporter pKC201. As shown in Fig. 5, in the absence of Rcn1, expression from the *ENA1* promoter was almost unaffected. We also tested the effect of disrupting *MCK1*, a gene encoding one of the four putative yeast homologs of glycogen synthase kinase-3, which has recently been postulated to be an upstream activator of calcineurin (K. Cunningham, personal communication). Interestingly, mutation of *MCK1* fully abolished the increased *ENA1* expression of the *ppz1* mutant (Fig. 5). The *mck1* mutant displayed a tolerance to high calcium concentrations virtually identical to that of a *cnb1* strain, and this tolerance was not decreased by deletion of *PPZ1* (data not shown).

The increased *ENA1* expression of a *ppz1* mutant is likely not due to intracellular alkalization. Recent evidence (49) has shown that the absence of both Ppz1 and Ppz2 results in an increased intracellular pH and that this increase is quantitatively similar to the transient rise in the intracellular pH ob-

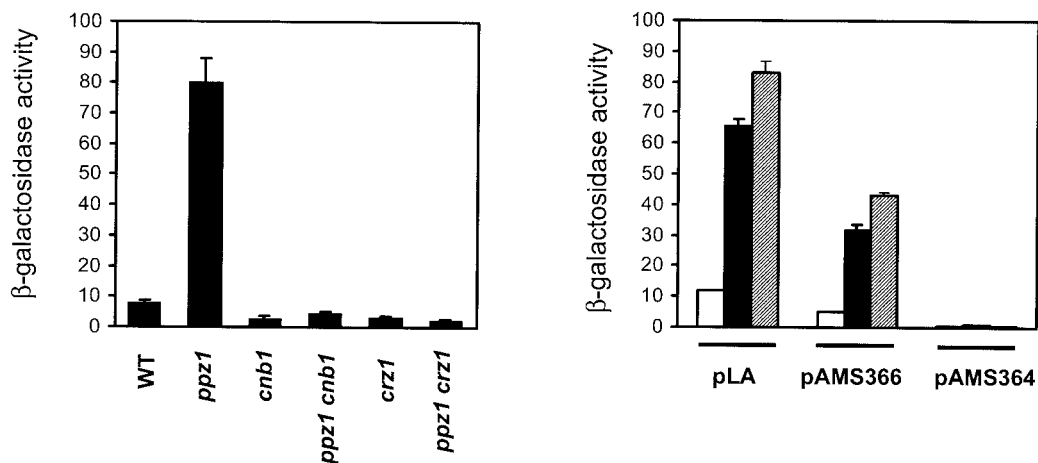


FIG. 3. Regulation of *ENA1* expression by Ppz1 requires the integrity of the calcineurin-Crz1 pathway. (Left) Strain DBY746 (wild type [WT]) and the indicated isogenic derivatives were transformed with plasmid pKC201, and β -galactosidase activity was measured. Data are means \pm standard errors of the means from 6 to 12 independent clones. (Right) Strains DBY746 (wild type) (open bars), EDN2 (*ppz1*) (filled bars), and EDN85 (*ppz1 ppz2*) (hatched bars) were transformed with plasmid pLA (containing the -732-to-711 CDRE found in the *ENA1* promoter as the only regulatory element), pAMS366 (bearing the wild-type CDRE found in the *FKS2* promoter), or pAMS364 (bearing a mutated, nonfunctional version of the CDRE in pAMS366). Data are means \pm standard errors of the means of β -galactosidase activities measured from three to nine independent clones.

served after shifting of a standard yeast culture to pH 5.8 to 8.5. Because it is known that expression from the *ENA1* promoter is substantially enhanced by exposure of cells to a high pH, it can be postulated that the increase in *ENA1* expression found in the *ppz1 ppz2* double mutant is due to intracellular alkalinization. In this context, it was reasonable to ask whether the higher *ENA1* expression characteristic of a *ppz1* mutant, which we have now observed to be calcineurin dependent, could also be influenced by intracellular pH.

To test this hypothesis, we first compared the expression of four different genes known to be induced by exposure of cells

to an alkaline environment (*ENA1*, *PHO84*, *PHO89*, and *PHO12*) in wild-type, *ppz1*, *ppz2*, and *ppz1 ppz2* strains, both in the presence and in the absence of the calcineurin inhibitor FK506. Interestingly, as documented in Fig. 6, while *ENA1* promoter activity (plasmid pKC201) was increased in *ppz1* and *ppz1 ppz2* cells, expression of *PHO84* and *PHO12* was increased only in the *ppz1 ppz2* double mutant and was not affected by the presence of the drug FK506. A remarkable observation was that the expression of *PHO89* was also increased in a single *ppz1* mutant (but not in a *ppz2* strain), and this increase was fully abolished by exposure of the cells to

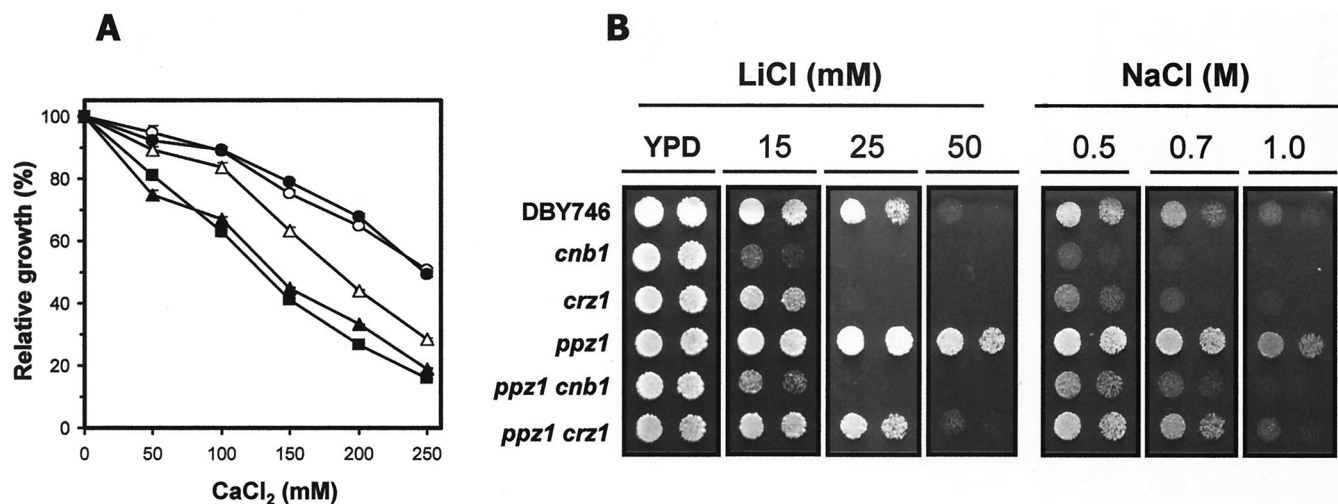


FIG. 4. Effects on tolerance to calcium, lithium, and sodium ions of mutation of *CNB1* and *CRZ1* in a *ppz1* background. (A) Strains DBY746 (wild type) (Δ), EDN2 (*ppz1*) (\blacktriangle), EDN85 (*ppz1 ppz2*) (\blacksquare), MAR15 (*cnb1*) (\circ), and MAR19 (*ppz1 cnb1*) (\bullet) were tested for growth in liquid cultures at the indicated concentrations of CaCl_2 . Data are expressed as the percentage of growth relative to that in cultures without added salt. Each data point is the mean \pm standard error of the mean from four independent cultures. (B) Cultures (OD_{660} , 0.05 and 0.01) of the indicated strains were spotted onto YPD plates containing the indicated concentrations of LiCl or NaCl. Growth was monitored after incubation at 28°C for 60 h.

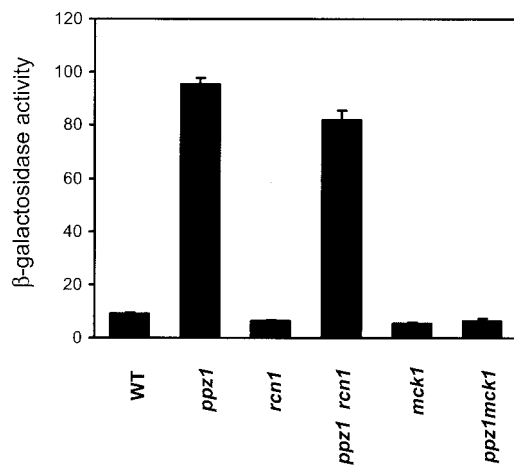


FIG. 5. Lack of Ppz1 does not result in increased *ENAI* expression in an Mck1 kinase-deficient background. The indicated strains were transformed with plasmid pKC201, and β -galactosidase activity was measured. Data are means \pm standard errors of the means from six independent clones. WT, wild type.

FK506. The level of *PHO89* expression in the *ppz1 ppz2* double mutant was higher than that observed in the *ppz1* single mutant, and in this case, a substantial part of the response was blocked by the calcineurin inhibitor. We extended the study to four additional genes whose expression has been found to

increase under alkaline stress: *FRE1*, *SIT1*, *TRR1*, and *FIT2*. The appropriate reporter constructs were transformed into wild-type, *ppz1*, *ppz2*, and *ppz1 ppz2* cells in two different genetic backgrounds, and the level of expression of each construct was monitored. As shown in Table 2, none of these promoters showed increased activity in *ppz1* or *ppz2* cells, while all of them were more active in the double mutant. It is worth noting that all four genes respond to an alkaline pH essentially in a calcineurin-independent manner (L. Viladevall and J. Ariño, unpublished data). We considered then the possibility that the lack of *ppz1* could result in a relatively light increase in pH and that the response of the calcineurin-responsive region could be particularly sensitive to small increases in pH. To test this, we determined the responses of reporter constructs containing the -742 -to- -490 (pMRK211), -742 -to- -577 (pMRK212), or -573 -to- -490 (pMRK213) region of the *ENAI* promoter when cells were grown at different ambient pHs (Fig. 7). As can be observed, the profile of the response was essentially the same when expression was driven from the region containing the CDRE or from the downstream region to which the FK506-insensitive increase in *ENAI* expression of a *ppz1 ppz2* strain was mapped (note that the region of the *ENAI* promoter in plasmid pMRK213 is the same as that in plasmid pMP213). Therefore, our results do not support the hypothesis that the region of the *ENAI* promoter containing the CDRE is more sensitive to an increase in pH than the downstream responsive region.

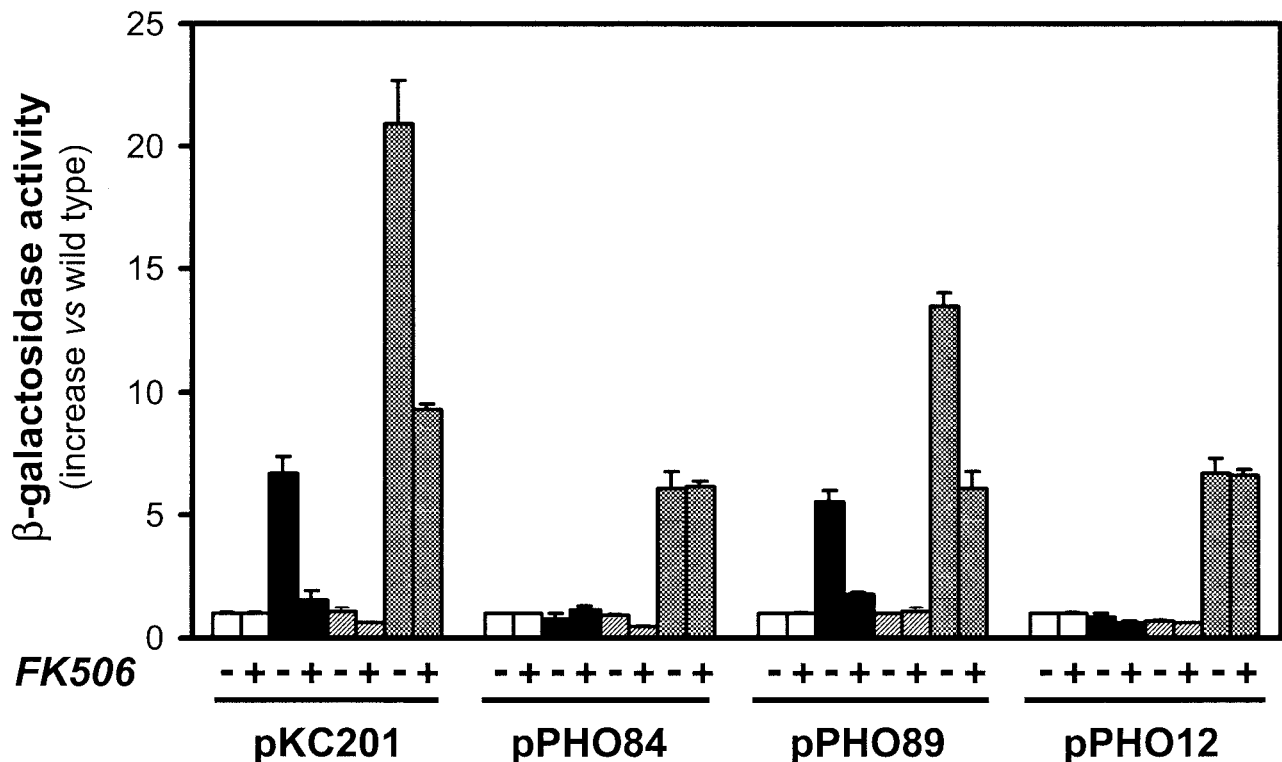


FIG. 6. Effects of calcineurin inhibition on the expression levels of different alkali-inducible genes in wild-type and Ppz-deficient backgrounds. Strains JA100 (wild type) (open bars), EDN75 (*ppz1*) (filled bars), JA103 (*ppz2*) (hatched bars), and EDN76 (*ppz1 ppz2*) (stippled bars) were transformed with plasmid pKC201, pPHO84, pPHO89, or pPHO12. Cultures were incubated in the presence (1.5 μ g/ml) or absence of FK506 as described in Materials and Methods, and β -galactosidase activity was measured. Data are means \pm standard errors of the means from 3 to 12 independent clones.

TABLE 2. Effects of the absence of Ppz1 and Ppz2 functions on the activities of the *FRE1*, *FIT2*, *SIT1*, and *TRR1* promoters

Reporter and background	β-Galactosidase activity (fold induction) ^a in the following mutant:			
	Wild type	<i>ppz1</i>	<i>ppz2</i>	<i>ppz1 ppz2</i>
pFRE1				
JA100	4.4 ± 0.3 (1.0)	4.1 ± 0.8 (0.9 ± 0.2)	3.0 ± 0.4 (0.7 ± 0.1)	14.8 ± 1.6 (3.4 ± 0.4)
DBY746	1.7 ± 0.1 (1.0)	1.3 ± 0.2 (0.8 ± 0.1)	1.3 ± 0.2 (0.7 ± 0.1)	27.9 ± 8.7 (16.6 ± 2.2)
pFIT2				
JA100	4.2 ± 0.5 (1.0)	3.2 ± 0.2 (0.8 ± 0.0)	2.1 ± 0.2 (0.8 ± 0.1)	41.3 ± 3.0 (9.9 ± 0.7)
DBY746	0.6 ± 0.1 (1.0)	1.1 ± 0.3 (1.9 ± 0.5)	1.0 ± 0.1 (1.8 ± 0.2)	47.8 ± 5.1 (83.1 ± 8.8)
pSIT1				
JA100	166.6 ± 9.9 (1.0)	128.3 ± 11.8 (0.8 ± 0.1)	140.0 ± 6.0 (0.8 ± 0.0)	350.4 ± 25.4 (2.1 ± 0.2)
DBY746	174.4 ± 6.9 (1.0)	185.4 ± 7.4 (1.1 ± 0.0)	179.0 ± 5.3 (1.0 ± 0.0)	627.1 ± 35.7 (3.6 ± 0.2)
pTRR1				
JA100	111.1 ± 9.9 (1.0)	132.7 ± 9.1 (1.2 ± 0.1)	118.8 ± 2.6 (1.1 ± 0.0)	424.2 ± 39.6 (3.8 ± 0.4)
DBY746	164.5 ± 6.0 (1.0)	148.6 ± 9.2 (0.9 ± 0.1)	151.7 ± 3.9 (0.9 ± 0.0)	336.0 ± 21.1 (2.0 ± 0.1)

^a β-Galactosidase activity is expressed in Miller units. Fold induction is given relative to activity in the isogenic wild-type strain.

Marked increases in intracellular concentrations of potassium can lead to alkalinization of the cytoplasm. Because the Trk1/Trk2 transporters represent by far the most important K⁺ uptake system, we considered it important to evaluate whether the increase in *ENAI* expression derived from the absence of Ppz1 was dependent on intact Trk1/Trk2-mediated transport.

As shown in Fig. 8A, cells carrying deletions of both the *TRK1* and *TRK2* genes retain most of the increased *ENAI* expression characteristic of a *ppz1* mutant, and under these circumstances, the expression is still completely blocked by incubation of the cells with FK506. When the sensitivities of these strains to sodium and lithium cations were tested, we observed (Fig. 8B)

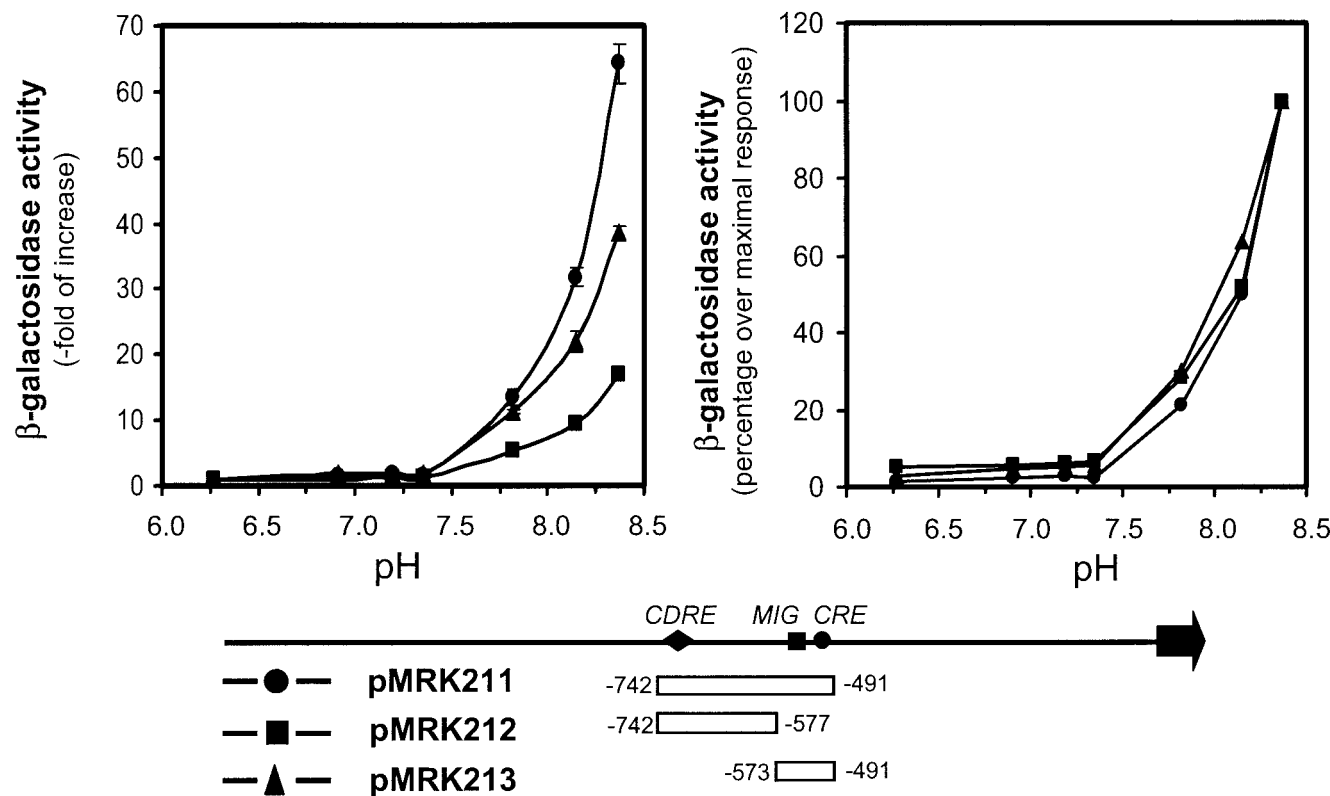


FIG. 7. Sensitivities of Ppz-regulatable regions of the *ENAI* promoter to different levels of alkalinization. The wild-type strain DBY746 was transformed with the indicated constructs bearing the regions of the *ENAI* promoter depicted schematically at the bottom. Cells were exposed to the indicated pH for 60 min and then processed for β-galactosidase activity measurement. The responses of each construct are expressed as the ratio of expression between each pH tested and the lowest pH used in the experiment (6.3) (left graph) and as the percentage of the maximal response (right graph). Data are means ± standard errors of the means from three independent clones.

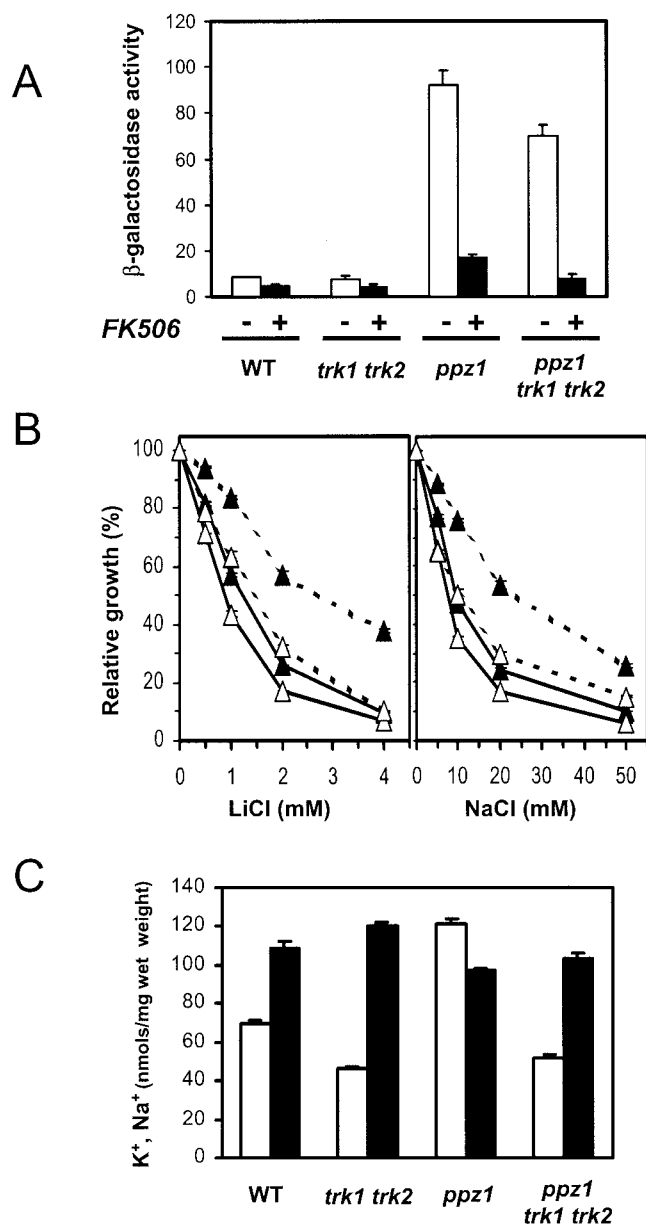


FIG. 8. Effects of the absence of Ppz1 on the expression of *ENAI* and on salt tolerance in a Trk-deficient background. (A) The wild-type (WT) strain DBY746 and its derivatives EDN2 (*ppz1*), ESV212 (*trk1 trk2*), and MAR37 (*ppz1 trk1 trk2*) were transformed with plasmid pKC201 and cultured in the presence or absence of FK506. β -Galactosidase activity was measured, and data are presented as means \pm standard errors of the means from six independent clones. (B) Strains ESV212 (solid lines) and MAR37 (dashed lines) were tested for growth in liquid cultures at the indicated concentrations of LiCl or NaCl in the absence (solid symbols) or presence (open symbols) of 1.5 μ g of FK506/ml. Data, expressed as percentages of the growth of cultures without added salt, are means \pm standard errors of the means from three independent cultures. (C) The indicated strains were grown in the presence of 0.3 M NaCl, extracts were prepared as described in Materials and Methods, and the concentrations of potassium (open bars) and sodium (solid bars) were measured. Data, expressed as nanomoles per milligram of cells (fresh weight), are means \pm standard errors of the means from two independent experiments, performed in duplicate.

that, as was observed with the *ppz1 ppz2* mutant (49), the absence of Ppz1 increased tolerance to these cations in the hypersensitive *trk1 trk2* background. Interestingly, this increase was abolished in the presence of the calcineurin inhibitor FK506. Addition of the drug slightly increased the sensitivity of the *trk1 trk2* mutant. Finally, the intracellular concentrations of sodium and potassium ions were determined in wild-type, *trk1 trk2*, *ppz1*, and *trk1 trk2 ppz1* cells grown in the presence of 0.3 M NaCl (Fig. 8C). As previously reported for the *ppz1 ppz2* mutant, disruption of *PPZ1* clearly increased the intracellular potassium ion concentration and slightly reduced the content of sodium, while lack of the Trk system decreased the potassium and increased the sodium ion concentration. Deletion of *PPZ1* in the *trk*-deficient background showed a slight decrease in the intracellular Na⁺ concentration relative to that in the *trk1 trk2* mutant. Equivalent results were obtained when cells were grown in the presence of a higher NaCl concentration (0.5 M). This decrease in Na⁺ accumulation in the *ppz1 trk1 trk2* strain is consistent with the continued induction of *ENAI* in this strain and likely explains the relative tolerance to NaCl and LiCl.

DISCUSSION

It has been known for some time that the Ppz1 and Ppz2 phosphatases are not functionally equivalent. For instance, the absence of Ppz1 results in clear-cut phenotypes, while cells lacking Ppz2 are indistinguishable from wild-type cells. Although the recent finding that the Ppz phosphatases are negative effectors of potassium influx, probably by regulating the Trk1/Trk2 potassium transporters (49), has provided a very fruitful framework for understanding the diverse phenotypes associated with the absence or the overexpression of these proteins, we considered it necessary to initiate a detailed evaluation of the function and regulation of these proteins independently, in order to better understand their individual physiological roles. Because it was known that Hal3 was able to interact with and inhibit Ppz1, we first raised the question of whether Hal3 could be considered fully specific for Ppz1. We show here that Hal3 can bind Ppz2 *in vitro* with roughly the same efficiency as that for Ppz1 binding. Furthermore, high-copy expression of Hal3 in the *ppz1* mutant results in a level of *ENAI* expression virtually identical to that observed in the *ppz1 ppz2* double mutant, suggesting that high levels of Hal3 could fully inhibit Ppz2 function. The observation that high-copy expression of Hal3 fails to increase expression of the *ENAI* promoter in a *ppz1 ppz2* mutant is in agreement with the notion that the regulation of the expression of the ATPase gene by Hal3 is fully mediated by both the Ppz1 and Ppz2 phosphatases. Consistent with these results, overexpression of Hal3 also fails to increase salt tolerance in a *ppz1 ppz2* double mutant (data not shown). In conclusion, Hal3 appears to regulate both the Ppz1 and Ppz2 phosphatases. A practical consequence of these observations would be that high-copy expression of Hal3 could not be considered a suitable tool for specific inhibition of Ppz1.

Since the absence of Ppz1 and Ppz2 affects *ENAI* expression, we considered that a detailed functional analysis of the *ENAI* promoter in *ppz1* and *ppz1 ppz2* mutants might provide useful information on the mechanism of action of these phos-

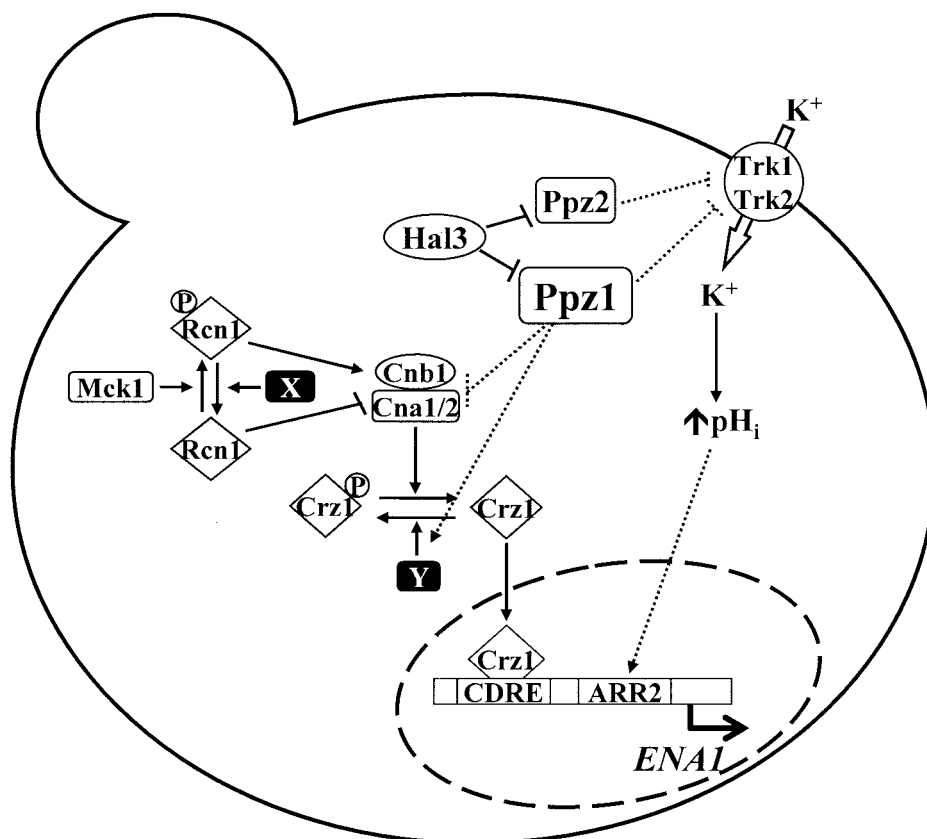


FIG. 9. Schematic depiction of the relationship of Hal3, Ppz1, and Ppz2 with the calcineurin-dependent pathway and the transcriptional regulation of the *ENA1* gene. “X” represents the protein phosphatase(s) that dephosphorylates Rcn1, and “Y” represents the protein kinase that phosphorylates Crz1. Discontinuous lines indicate functional relationships whose mechanisms have yet to be clarified.

phatases. Here we present evidence that a region containing the downstream CDRE of *ENA1* (30, 43) is necessary and sufficient to mimic the increased expression of the full promoter in a *ppz1* mutant. It should be noted that this region also contains one of the two possible binding sites for Nrg1, a transcription factor which, very recently, has been proposed to negatively regulate *ENA1* transcription under the influence of Rim101 (25). However, we observed that the increase in *ENA1* expression characteristic of *ppz1* mutants was completely blocked by incubation of the cells with the calcineurin inhibitor FK506; by mutation of the *CNB1* gene, encoding the regulatory subunit of calcineurin; or by the absence of the calcineurin-activated Crz1/Tcn1 transcription factor (Fig. 3), which is responsible for most, if not all, of the transcriptional effects of calcineurin (50). Furthermore, we show (Fig. 3, right) that a reporter construct containing the CDRE sequence found in *FKS2*, which does not contain the CCCCT or CCCTC sequences proposed as Nrg1 binding sites (35), was also over-expressed in a *ppz1* strain. These findings are consistent with the hypothesis that the effect of Ppz1 on the regulation of *ENA1* expression is not related to Nrg1 function. Mapping of the *ENA1* promoter activity in a *ppz1 ppz2* mutant revealed an additional functionally relevant target region, designated ARR2 by Serrano et al. (43). Transcription from this region is not blocked by FK506, indicating that it is not influenced by calcineurin activity. This region was identified recently as being

responsible for a substantial, calcineurin-insensitive component of the alkaline response of *ENA1* (43). Therefore, the complete absence of Ppz activity results in increased *ENA1* expression by modulation of the activity of at least two different sites in its promoter.

The results presented in this paper suggest that Ppz1 might act as an upstream negative regulator of the calcineurin pathway (see Fig. 9 for a schematic depiction). The observations that a *ppz1* mutant is sensitive to calcium ions, as would be expected for a strain with higher-than-normal calcineurin activity, and that this sensitivity is fully abolished by deletion of *cnb1* are in agreement with this model. It should be noted that a previous report failed to identify a *ppz1* mutant as calcium sensitive (42). These authors based their work in a genetic background (W303-1A-derived strains) different from those used in this work. We have also tested calcium tolerance for *ppz1* and *ppz1 ppz2* mutants in the W303-1A background, and we were able to observe a slight calcium-sensitive phenotype in the *ppz1* mutant that was exacerbated in the double mutant (data not shown).

It has recently been shown that calcineurin function can be modulated by a conserved family of proteins termed calcipressins, represented in budding yeast by a single gene, *RCN1* (17, 23). It is not evident from the literature how Rcn1 acts on calcineurin. For instance, overproduction has been shown to inhibit calcineurin function, and bacterially expressed Rcn1

inhibits calcineurin *in vitro* (23). In contrast, a null mutant exhibits reduced calcineurin activity and, like calcineurin mutants, presents increased sensitivity to sodium and lithium cations (15, 17, 22, 23). Recently, K. Cunningham's laboratory (John Hopkins University) has suggested a model in which Rcn1 stimulates calcineurin when phosphorylated at a given site, while a nonphosphorylated form of Rcn1 would have a potent inhibitory activity. These authors have also identified the Mck1 protein kinase, a member of the glycogen synthase kinase-3 family, as the major Rcn1 kinase activity in yeast (K. Cunningham, personal communication).

We have found that expression from the *ENAI* promoter is somewhat lower in an *rcn1* mutant than in a wild-type strain (6.5 ± 0.4 versus 8.9 ± 0.4 Miller units) and that lack of Rcn1 in a *ppz1* mutant only marginally decreases the activity of the promoter (Fig. 5). In contrast, deletion of *MCK1* in a *ppz1* background decreases the activity of the promoter to wild-type levels. We consider that these results provide support for the model of Mck1/Rcn1-mediated regulation of calcineurin activity mentioned above. Lack of Rcn1 would represent the absence of an inhibitor (when dephosphorylated) but also that of a possible activator of calcineurin (when phosphorylated by Mck1). This might have little influence on *ENAI* expression in a *ppz1* mutant, in which calcineurin activity would already be abnormally increased due to the absence of the Ppz1 phosphatase. However, lack of Mck1 would result in the incapacity to phosphorylate Rcn1, and this unphosphorylated form of the protein would strongly inhibit the activity of calcineurin, possibly overriding the positive effect due to the lack of Ppz1. It should be noted that our results rule out the possibility of Ppz1 acting as an Rcn1 phosphatase (which would account for the calcineurin-dependent activation of the *ENAI* promoter in the *ppz1* mutant), as in this scenario the absence of Ppz1 should not result in increased *ENAI* promoter activity in an *rcn1* strain. A role for Mck1 in sodium tolerance was postulated some time ago (36). These authors observed that an *mck1* mutant was more sensitive to high sodium concentrations and that expression of *ENAI* was reduced under 0.5 M NaCl stress. In addition, overexpression of *MCK1* was able to somewhat improve the growth of a strain lacking both catalytic subunits of calcineurin, which would suggest a calcineurin-independent mechanism. However, in this experiment the *MCK1* gene was expressed from the strong *ADHI* promoter, and therefore, conditions were far from physiological.

Recent work (49) has shown that the *ppz1 ppz2* mutant has a more alkaline cytosolic pH, and it was postulated that the increased basal activity of the *ENAI* promoter may be the result of this alkalization. In this study, we show that the induction of the *ENAI* promoter in the double mutant maps to two different regions; a pH-responsive region (-573 to -490) and the calcineurin-dependent CDRE (-751 to -667). Interestingly, several lines of evidence demonstrate that, in a single *ppz1* mutant, the CDRE is necessary and sufficient for the observed *ENAI* induction and that alterations in intracellular pH are not involved in the changes in gene expression observed in this mutant. First, only promoters of calcineurin-responsive genes, such as *ENAI* or *PHO89*, show increased activity in a *ppz1* mutant, while several other alkaline pH-responsive genes do not. Second, the CDRE-containing region of *ENAI* is not particularly sensitive to alkaline pHs. Thus, the

possibility that lack of Ppz1 might result in a slight increase in pH that would be to drive transcription from this element seems unlikely. In fact, the increase in expression of *ENAI* in a *ppz1* mutant is quantitatively similar at different ambient pHs (from pH 5.8 to 7.2 [data not shown]). Third, the increased activity of the *ENAI* promoter due to the lack of Ppz1 is largely conserved in a *trk1 trk2* mutant (Fig. 8), ruling out the possibility of intracellular alkalization as a result of enhanced Trk activity in the absence of Ppz1. In addition, we confirm and extend here the previous observation (49) that the absence of both the Ppz1 and Ppz2 phosphatases results in increased expression of alkali-inducible genes. This increased expression is not at all affected by calcineurin inhibition in some cases, while in other cases it displays a significant calcineurin-dependent component, suggesting that the absence of both phosphatases triggers regulatory mechanisms that are not elicited by the simple loss of Ppz1 function.

An interesting question is how much the increased expression of *ENAI* contributes to the salt-tolerant phenotype of a *ppz1* mutant. As shown in Fig. 4B, a *crz1* mutant is less sensitive to lithium or sodium than a *cnb1* mutant, supporting the notion that calcineurin plays a role(s) in salt tolerance that does not involve Crz1-mediated transcriptional regulation. Interestingly, deletion of *CRZ1* in a *ppz1* background reduces tolerance to lithium and sodium cations, although the effect is less potent than that observed upon deletion of *CNBI*. As we show that increased *ENAI* expression observed in the absence of Ppz1 is dependent on the presence of Crz1, it could be hypothesized that the difference in tolerance between a *ppz1* and a *ppz1 crz1* mutant would be due to the incapacity of the latter to increase *ENAI* expression levels. Similarly, a higher *ENAI* expression would explain the FK506-sensitive increase in sodium and lithium tolerance provided by disruption of *PPZ1* in a *trk1 trk2* strain (Fig. 8), suggesting a role for calcineurin as a downstream effector of Ppz1. In addition to regulating *ENAI* expression, it is known that, in the presence of high NaCl concentrations, calcineurin is able to regulate the Trk potassium transporters, positively influencing the ability of Trk to convert to the high-affinity state which allows for better discrimination of potassium from sodium (32, 33). Therefore, a likely possibility that would contribute to the marked salt tolerance phenotype of a *ppz1* mutant would be that the absence of Ppz1 would activate calcineurin and consequently influence the cation selectivity of the Trk transporters. Of course, this model does not rule out an alternative, calcineurin-independent regulation of Trk function.

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