Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast

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Calcineurin is a conserved Ca\(^{2+}\)/calmodulin-dependent protein phosphatase that plays a critical role in Ca\(^{2+}\) signaling. We describe new components of a calcineurin-mediated response in yeast, the Ca\(^{2+}\)-induced transcriptional activation of FKS2, which encodes a \(\beta\)-1,3 glucan synthase. A 24-bp region of the FKS2 promoter was defined as sufficient to confer calcineurin-dependent transcriptional induction on a minimal promoter in response to Ca\(^{2+}\) and was named CDRE (for calcineurin-dependent response element). The product of CRZ1 (YNL027w) was identified as an activator of CDRE-driven transcription. Crz1p contains zinc finger motifs and binds specifically to the CDRE. Genetic analysis revealed that crz1\(^{D}\) mutant cells exhibit several phenotypes similar to those of calcineurin mutants and that overexpression of CRZ1 in calcineurin mutants suppressed these phenotypes. These results suggest that Crz1p functions downstream of calcineurin to effect multiple calcineurin-dependent responses. Moreover, the calcineurin-dependent transcriptional induction of FKS2 in response to Ca\(^{2+}\), \(\alpha\)-factor, and Na\(^{+}\) was found to require CRZ1. In addition, we found that the calcineurin-dependent transcriptional regulation of PMR2 and PMC1 required CRZ1. However, transcription of PMR2 and PMC1 was activated by only a subset of the treatments that activated FKS2 transcription. Thus, in response to multiple signals, calcineurin acts through the Crz1p transcription factor to differentially regulate the expression of several target genes in yeast.

[Key Words: S. cerevisiae; calcineurin; calcium signaling; transcriptional activation; cell wall maintenance; ion homeostasis]

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Changes in intracellular Ca\(^{2+}\) concentration signal a variety of physiological responses in many different cell types (Clapham 1995). The amplitude and duration of dynamic Ca\(^{2+}\) signals contribute to the diversity in signaling of this single ion. One mechanism by which Ca\(^{2+}\) acts is by binding to and activating calmodulin. As an intracellular Ca\(^{2+}\) receptor, calmodulin activates a number of target enzymes such as calmodulin-dependent protein kinases and phosphatases. One of these targets is the serine/threonine-specific protein phosphatase calcineurin that acts as an effector of Ca\(^{2+}\) signaling by regulating the phosphorylation state of proteins (Klee et al. 1988).

Calcineurin activity is critical for many Ca\(^{2+}\)-regulated processes, including T-cell activation (Clipstone and Crabtree 1992; O'Keefe et al. 1992) and neutrophil chemotaxis (Hendley et al. 1992; Lawson and Maxfield 1995). The function of calcineurin in different cell types has been assessed in part by examining the effects of FK506 and cyclosporin A, immunosuppressive drugs that specifically inhibit this phosphatase (Liu et al. 1991a). Inhibition of calcineurin by these drugs prevents activation of NFAT, a transcription factor that is necessary for the proliferation of T cells (Clipstone and Crabtree 1992; O'Keefe et al. 1992). Specifically, dephosphorylation of NFAT by calcineurin allows translocation of this transcription factor from the cytoplasm to the nucleus where it induces expression of a number of cytokine genes (Jain et al. 1993; Northrop et al. 1993). In other cell types, calcineurin has been implicated in the control of ion homeostasis. For example, calcineurin regulates the Na\(^{+}\)/K\(^{+}\) ATPase in renal tubule cells (Aperia et al. 1992) and the NMDA receptor in neurons (Lieberman and Mody 1994; Tong et al. 1995).

The calcineurin enzyme functions as a heterodimer of catalytic (A) and regulatory (B) subunits that have been highly conserved through evolution. The catalytic subunit contains a carboxy-terminal autoinhibitory domain, and Ca\(^{2+}\)-calmodulin binding activates the enzyme by relieving this inhibition (Hubbard and Klee 1989). Truncations of the catalytic subunit that remove the autoinhibitory domain result in a constitutively active enzyme that no longer requires Ca\(^{2+}\) (Hubbard and Klee 1989). In
the yeast Saccharomyces cerevisiae, calcineurin catalytic subunits are encoded by CNA1 and CNA2 (Cyert et al. 1991; Liu et al. 1991b), and the regulatory subunit is encoded by CNB1 (Kuno et al. 1991; Cyert and Thorner 1992). The physiological role of calcineurin in yeast has been examined by characterizing cells that lack functional calcineurin, that is, cna1 cna2 mutants, cnb1 mutants, or cells incubated with FK506 or cyclosporin A.

Yeast calcineurin is essential under specific environmental conditions. During prolonged incubation with pheromone, calcineurin is necessary to maintain viability; however, the nature of this requirement is not well understood (Moser et al. 1996; Withee et al. 1997). Calcineurin-deficient cells also grow poorly in the presence of high concentrations of certain ions, including Mn²⁺, Na⁺/Li⁺, and OH⁻ (Nakamura et al. 1993; Mendoza et al. 1994; Farcaeanu et al. 1995; Pozos et al. 1996). These ion sensitivities can be explained, at least in part, by altered levels of several ion transporters. Calcineurin is required for transcriptional induction of PMR2, which encodes a Na⁺/ATPase (Rudolph et al. 1989; Haro et al. 1991), and PMC1 and PMR1, which encode Ca²⁺ATPases (Rudolph et al. 1989; Cunningham and Fink 1994, 1996; Mendoza et al. 1994).

Calcineurin also regulates transcription of another gene, FKS2. FKS2 and its homolog, FKS1, encode catalytic subunits of a major, cell wall synthetic enzyme, β-1,3 glucan synthase. fks1 fks2 double mutants are inviable, and cells lacking FKS1 and calcineurin are also inviable because of insufficient expression of FKS2 (Douglas et al. 1994; Eng et al. 1994; Garrett-Engele et al. 1995). In addition, FKS2 mRNA levels increase when cells are incubated with either Ca²⁺ or mating pheromone, and under both of these conditions, transcriptional activation is completely dependent on calcineurin (Mazur et al. 1995). However, transcriptional regulation of FKS2 is complex and is also regulated by calcineurin-independent mechanisms (Mazur et al. 1995). For example, during growth at elevated temperatures, FKS2 transcription increases, and this induction is the result of the independent and additive effects of both calcineurin and the PKC1-regulated cell integrity pathway (C. Zhao, U. Jung, P. Garrett-Engele, T. Roe, M. Cyert, and D. Levin, in prep.). Furthermore, the expression of FKS2 is induced, independently of calcineurin, by growth on non-dextrorotatory carbon sources and at stationary phase (Mazur et al. 1995; C. Zhao, U. Jung, P. Garrett-Engele, T. Roe, M. Cyert, and D. Levin, in prep.). In this study we further characterize the mechanism by which calcineurin activates transcription of FKS2 and identify a transcription factor, Crz1p, that mediates calcineurin-dependent changes in gene expression.

Results

Dissection of the FKS2 promoter identifies a region that is necessary for calcineurin-dependent transcriptional induction

FKS2 mRNA levels increase in response to Ca²⁺ and mating pheromone (α-factor) in a calcineurin-dependent fashion (Mazur et al. 1995). We previously determined that a lacZ reporter gene containing ∼900 bp of FKS2 promoter sequence displays calcineurin-dependent Ca²⁺-induced expression, whereas a construct containing ∼700 bp does not (C. Zhao, U. Jung, P. Garrett-Engele, T. Roe, M. Cyert, and D. Levin, in prep.). Here, we have extended that analysis and found that gene fusions containing 762 bp or more of FKS2 promoter sequence exhibited increased expression of β-galactosidase in response to Ca²⁺ (Fig. 1). We observed a four- to sixfold increase in transcription in response to Ca²⁺ that was calcineurin dependent because it was eliminated by the addition of FK506, a specific inhibitor of calcineurin (Fig. 1). No such induction was observed with the reporter containing only 705 bp or with the vector lacking any FKS2 regulatory sequences (pLG178; data not shown). We also found that the same constructs that displayed a calcineurin-dependent induction in response to Ca²⁺ were able to support a three- to fivefold calcineurin-dependent induction in response to α-factor (Fig. 1). Correspondingly, α-factor-induced expression was observed for the 762-bp construct but not the 705-bp construct (Fig. 1). From these results we conclude that the region from ∼762 bp to ∼705 bp is necessary for both Ca²⁺- and α-factor-induced calcineurin-dependent transcription.

Identification of the calcineurin-dependent response element

To further define the region mediating calcineurin-dependent expression, reporter genes containing heterologous promoters were constructed (see Materials and Methods). No calcineurin-dependent expression was ob-

![Figure 1](image-url)
A 60-bp sequence was subdivided into overlapping 24-bp regions, creating four reporter gene constructs A–D (24):cyc1::lacZ. Only one 24-bp region, contained in construct A (24):cyc1::lacZ, was able to support calcineurin-dependent Ca\(^{2+}\)-induced expression (4.4 ± 0.9-fold induction; Table 1) that was similar in magnitude to that observed for the larger 60-bp construct. We named this element, which is composed of the sequence CAC-CAGTCGGTGGCTGTGCGCTTG, the CDRE (calcineurin-dependent response element). Analysis of the CDRE failed to find any matches to consensus binding sites defined previously for yeast transcription factors (Prezridge 1991).

Multiple copies of the CDRE increase calcineurin-dependent transcriptional activation

We found that multimerization of the CDRE increased the sensitivity of the reporter gene. In contrast to the 2- to 5-fold calcineurin-dependent Ca\(^{2+}\)-induced expression exhibited by one copy of the CDRE, 15-fold and 69-fold inductions were observed in cells carrying heterologous promoter constructs that contained two and four tandem copies of the CDRE, respectively (Fig. 2). Additionally, the most sensitive reporter gene, containing four tandem copies of the CDRE (4×CDRE::lacZ), supported an approximately threefold increase in expression in response to α-factor that was calcineurin dependent (Fig. 2). No α-factor-induced expression was observed with the other constructs.

We also constructed a derivative of the CDRE (mutCDRE; see Materials and Methods) that was not able to promote calcineurin-dependent transcriptional regulation. When tandem repeats of mutCDRE were placed upstream of a minimal promoter, no calcineurin-dependent increase in expression was observed with either Ca\(^{2+}\) or α-factor treatment (Fig. 2).

Identification of high-copy plasmids that allow expression of the CDRE reporter gene in the absence of calcineurin

We used the calcineurin mutant strain containing the 4×CDRE::lacZ reporter (ASY 461) to identify gene products that activate CDRE-mediated expression in the absence of calcineurin. We introduced two different multicopy genomic libraries into ASY 461 cells and identified plasmids that conferred CDRE-dependent gene expression (see Materials and Methods). Four classes of plasmids were isolated. One class of plasmids contained a copy of CNB1, encoding the calcineurin regulatory subunit. These plasmids complemented the cnb1Δ mutation in the parent strain, thus restoring calcineurin activity and CDRE-driven gene expression. A second class of plasmids contained truncations of CNA2, encoding the calcineurin catalytic subunit. Evidently, enough catalytic activity was supported by truncated Cna2p even in the absence of Cnb1p to test positive in our sensitive reporter system. The third class of plasmids consisted of a single isolate. Specific activation of the reporter by this plasmid was attributable to a single open reading frame (ORF), YMR030w, that is predicted to encode a protein of 43 kD of molecular mass with no significant homology to any other protein. Cells deficient for YMR030w still displayed CDRE-dependent transcriptional activation (data not shown); therefore, this ORF was not studied further. The fourth class of plasmids contained at least part of a previously uncharacterized ORF, YNL027w. YNL027w is predicted to encode a protein of 76 kD of molecular mass containing a polyglutamine tract, which commonly acts as a transcriptional activation domain (Perutz 1994), and two zinc finger domains of the Cys2-His2 type, which mediate protein-nucleic acid interactions in many transcription factors (Evans and Hollenberg 1988; Desjarlais and Berg 1992) (Fig. 3). In addition, YNL027w contains a third putative

### Table 1. Calcineurin-dependent induction of heterologous reporter genes in response to Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>fks2::cyc1::lacZ reporter(^a) (bp)</th>
<th>FKS2 sequence</th>
<th>β-Galactosidase (U/mg protein)(^b)</th>
<th>Calcineurin-dependent induction(^c) (ratio: (+\text{Ca}^{2+}/\text{+Ca}^{2+})FK)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(+\text{Ca}^{2+})</td>
<td>(+\text{Ca}^{2+})FK</td>
</tr>
<tr>
<td>60</td>
<td>−762 to −705</td>
<td>56.4 ± 2.6</td>
<td>11.6 ± 0.5</td>
</tr>
<tr>
<td>A (24)</td>
<td>−762 to −738</td>
<td>13.6 ± 0.6</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>B (24)</td>
<td>−750 to −726</td>
<td>3.1 ± 0</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>C (24)</td>
<td>−738 to −714</td>
<td>3.1 ± 0.2</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>D (24)</td>
<td>−726 to −705</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.2</td>
</tr>
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</table>

\(^a\)The 60-bp, A, B, C, and D reporters are contained on plasmids pAM S327, pAM S342, pAM S344, pAM S455, and pAM S456, respectively.

\(^b\)β-Galactosidase activity per milligram of protein was assayed from cells grown in synthetic medium supplemented as indicated with 200 mM CaCl\(_2\) (Ca\(^{2+}\)) and 1 µg/ml of FK506 (FK). Data are the averages of extracts assayed in triplicate from one representative experiment, and the standard deviation is representative of the error between these samples.

\(^c\)Calcineurin-dependent induction is presented as the average of the largest possible ratio and the smallest possible ratio, with the error indicating the span between the two numbers.
zinc finger that is less well conserved and only contains one cysteine and one histidine (Fig. 3). We renamed YNL027w CRZ1 for calcineurin-responsive zinc finger protein. This same gene was identified independently by Matheos et al. and named TCN1 (Matheos et al., this issue).

CRZ1 encodes an essential component of the CDRE-mediated transcriptional induction

A diploid strain, ASY650, heterozygous for a null allele of CRZ1 (crz1::loxP-kanMX-loxP) was sporulated. All haploid segregants from this diploid were viable, and cells carrying the CRZ1 disruption allele (ASY472) showed no obvious defects in growth or morphology. Thus, CRZ1 is not an essential gene. However, crz1Δ mutant cells were not able to activate the 4×-CDRE::lacZ reporter in the absence (ASY589) or in the presence of calcineurin (ASY587). When incubated with Ca2+, the crz1Δ mutant (ASY587), the cnb1Δ mutant (ASY461), and the cnb1Δ crz1Δ double mutant (ASY589) showed similar low levels of CDRE-mediated expression (4–7 β-galactosidase U/mg of protein) in contrast to the high level of CDRE-mediated expression (183 ± 2 β-galactosidase U/mg of protein) observed with wild-type cells (ASY459) (data not shown). These results indicate that CRZ1 is an essential component of calcineurin-dependent CDRE-mediated gene expression.

Crz1p binds specifically to the CDRE

The similarity of Crz1p to other transcription factors (Fig. 3) and the observation that this protein is a required component of calcineurin-dependent CDRE-mediated transcriptional induction suggest that Crz1p may directly bind the CDRE. To test this possibility, we performed gel-shift experiments. Cell extracts were incubated with a 32P-labeled 24-bp double-stranded oligonucleotide corresponding to the CDRE sequence and analyzed by fractionation on nondenaturing polyacrylamide gels. Cell extracts prepared from wild-type cells contained an activity that bound to the oligonucleotide and retarded its migration in the gel, resulting in the formation of a new band (Fig. 4, lane 2, band A). This DNA-binding activity was absent from crz1Δ cells (Fig. 4, lane 3). These results demonstrate that Crz1p is required for assembly of a CDRE-binding activity.

Further experiments demonstrated that Crz1p binds to the CDRE directly and specifically. First, we created an epitope-tagged version of Crz1p by inserting a DNA segment encoding the hemagglutinin antigen (HA) epitope into CRZ1 near the 5′ end of the coding region (Wilson et al. 1984). The epitope-tagged gene, HA–CRZ1, when expressed from a centromere-based plasmid (pAMS451) fully complemented the CDRE-mediated transcription defect and the ion sensitivities of a crz1Δ mutant strain (see below) indicating that the epitope tag did not impair the function of Crz1p (data not shown). More CDRE-binding activity was observed for cells overexpressing
HA–Crz1p than for wild-type cells (Fig. 4, lanes 4 vs. lane 2). This increase in binding activity was attributable to the high level of HA–Crz1p expression and was equivalent to that observed for cells overexpressing native Crz1p (data not shown). To examine the specificity of the observed binding to CDRE, we added an excess of unlabeled CDRE or mutCDRE oligonucleotides to the binding reaction. The CDRE was able to compete for binding (Fig. 4, lane 8), but the mutCDRE had no effect (Fig. 4, lane 9). Thus, the observed Crz1p-dependent binding activity is specific for the intact CDRE sequence. To ascertain whether Crz1p is a component of this CDRE-binding activity, we added anti-HA antibody to the binding reaction containing extract from cells expressing HA-tagged Crz1p. Addition of anti-HA antibodies to this binding reaction resulted in the disappearance of most of band A and the appearance of a new band of slower mobility, band B (Fig. 4, lane 5). This supershift was specific for HA-tagged Crz1p, as no shift was observed for cells containing native Crz1p (data not shown). Addition of anti-myc antibody (Evan et al. 1985), which does not recognize HA-tagged Crz1p, had no effect (Fig. 4, lane 6). These observations establish that Crz1p is a component of the CDRE-specific DNA-binding activity. The remainder of band A that was not supershifted by the addition of anti-HA antibody may represent a distinct protein–CDRE complex. However, as mentioned above, no CDRE-binding activity was detected for crz1Δ cells. Therefore, a more likely explanation is that the epitope present at the amino terminus of the protein is removed from a fraction of HA–Crz1p such that it is not recognized by the antibody but still retains DNA-binding activity.

**Figure 4.** Crz1p binds to the CDRE. Extracts of strains YPH499 containing YEp351 ("WT"), ASY472 containing YEp351 ("crz1Δ"), and ASY472 containing pAM S446 ("crz1Δ, YEpHA–CRZ1") were analyzed by DNA mobility retardation analysis using the CDRE as probe (see Materials and Methods). The plasmid YEp(HA–CRZ1) carries a gene encoding a version of Crz1p with the HA epitope inserted near the amino terminus of the protein. (Lanes 5–7) Equal amounts of α-HA antibody, α-myc antibody, or antibody dilution buffer were added 5 min before gel loading; (lanes 8,9) a 100-fold molar excess of unlabeled CDRE or mut-CDRE oligonucleotide to probe was added before addition of extract.

crz1Δ phenotypes are similar to those of calcineurin mutants

We identified CRZ1 by its capacity to modify one calcineurin mutant phenotype, the defect in CDRE-mediated transcription. To further investigate the relationship between CRZ1 and calcineurin, we analyzed crz1Δ cells for other phenotypes exhibited by calcineurin mutants. Calcineurin mutants are more sensitive to Mn²⁺, Li⁺, and high pH than wild-type cells (Nakamura et al. 1993; Mendoza et al. 1994; Farcasanu et al. 1995; Pozos et al. 1996). They also die during prolonged treatment with α-factor (Moser et al. 1996; Withee et al. 1997). The growth of crz1Δ cells was also impaired by Mn²⁺ and Li⁺; however, crz1Δ cells were less sensitive to these ions than were calcineurin mutant cells (Fig. 5A). When grown on plates containing lower concentrations of either Mn²⁺ or Li⁺, cells lacking both CRZ1 and calcineurin, crz1Δ cnb1Δ (ASY475), displayed the same sensitivity to these treatments as the cnb1Δ single mutant (data not shown). In addition, crz1Δ cells exhibited a survival defect when incubated with α-factor that is less severe than that of cnb1Δ cells (Fig. 5B). Furthermore, as observed for the ion sensitivities described above, crz1Δ cnb1Δ and cnb1Δ cells exhibited equivalent α-factor survival defects (Fig. 5B). These observations, together with the effect of the crz1Δ allele on CDRE reporter activity, indicate that CRZ1 and calcineurin function in the same pathway to regulate both ion homeostasis and CDRE-mediated transcription. However, unlike calcineurin mutant cells, crz1Δ cells do not exhibit sensitivity to high pH (data not shown). In addition, crz1Δ cells exhibit Ca²⁺-sensitive growth, whereas calcineurin mutant cells are Ca²⁺ tolerant (data not shown; see Discussion).

Overproduction of CRZ1 suppresses calcineurin mutant phenotypes

We also characterized the properties of cells overproducing Crz1p. Expression of CRZ1 from a high-copy plasmid YEp(CRZ1) was able to partially suppress the Mn²⁺ and Li⁺ sensitivities of calcineurin mutant cells (Fig. 6A). YEp(CRZ1) also increased the viability of calcineurin-deficient cells during prolonged α-factor treatment (Fig. 6B). Furthermore, overproduction of CRZ1 in wild-type cells increased the tolerance of these cells to Mn²⁺ and Li⁺ (data not shown) and increased survival during prolonged incubation with pheromone (Fig. 6B).

Crz1p functions as a general mediator of calcineurin-dependent transcriptional induction

We analyzed the effect of the crz1Δ allele on FKS2 mRNA levels by Northern blot analysis. We found that CRZ1 is necessary for the previously observed calcineurin-dependent increase in FKS2 expression in response to
both α-factor and Ca²⁺ (Fig. 7). These observations confirmed that CRZ1 is required not only for expression of CDRE-driven reporter genes but also for calcineurin-dependent regulation of genomic FKS2. In addition, we observed that FKS2 mRNA levels increase in response to Na⁺ and that this response was largely calcineurin dependent (Fig. 7). This calcineurin-dependent induction of FKS2 expression in response to Na⁺ also required CRZ1 (Fig. 7). Thus, CRZ1 is required for the calcineurin-dependent induction of FKS2 in response to α-factor, Na⁺, or Ca²⁺ treatment.

The ion sensitivities of calcineurin mutants are in part attributable to decreased expression of several genes encoding ion transporters (Mendoza et al. 1994; Cunningham and Fink 1996). Because crz1Δ mutant cells similarly exhibit ion sensitivities, we examined whether CRZ1 is required for the calcineurin-dependent transcriptional induction of two genes that encode P-type ATPases, PMR2 and PMC1 (Rudolph et al. 1989; Haro et al. 1991; Cunningham and Fink 1994). CRZ1 is required for the calcineurin-dependent induction of a PMR2 reporter gene (pmr2::lacZ-pFR70; Mendoza et al. 1994) in...
response to Ca\(^{2+}\) as well as Na\(^+\) (Fig. 8A). The crz1D mutation had no effect on the Na\(^+\)-induced expression of pmr2::lacZ that is calcineurin independent. In addition, CRZ1 was required for the calcineurin-dependent expression of a PMC1 reporter gene (pmc1::cyc1::lacZ-pAMS381) in response to Ca\(^{2+}\). No increase in \(\beta\)-galactosidase expression was observed for PMC1 in response to Na\(^+\) (Fig. 8B) or with PMR2 or PMC1 reporters in response to \(\alpha\)-factor (data not shown).

Discussion

Identification of a promoter element, the CDRE, and a novel transcription factor, Crz1p, that function downstream of calcineurin to activate transcription in response to Ca\(^{2+}\)

Calcineurin is required for the transcriptional induction of FKS2 in response to Ca\(^{2+}\) (Mazur et al. 1995; C. Zhao, U. Jung, P. Garrett-Engele, T. Roe, M. Cyert, and D. Levin, in prep.). We identified a region within the FKS2 upstream sequence from −762 bp to −705 bp that is both necessary and sufficient for this calcineurin-dependent transcriptional induction (see Fig. 1). Furthermore, from within this region, we defined a 24-bp element, the CDRE, that supports calcineurin-dependent transcriptional induction in response to Ca\(^{2+}\) (see Table 1; Fig. 2). We performed a genetic screen and identified a novel transcription factor, Crz1p, that when overexpressed bypasses a requirement for calcineurin inactivation of a CDRE-containing reporter gene. This transcription factor is identical to Tcn1p (Matheos et al., this issue). In addition, we find that crz1Δ cells exhibit sensitivities to Mn\(^{2+}\), Na\(^+/\)Ca\(^{2+}\) response to Ca\(^{2+}\) as well as Na\(^+\) (Fig. 8A). The crz1Δ mutation had no effect on the Na\(^+\)-induced expression of pmr2::lacZ that is calcineurin independent. In addition, CRZ1 was required for the calcineurin-dependent expression of a PMC1 reporter gene (pmc1::cyc1::lacZ-pAMS381) in response to Ca\(^{2+}\). No increase in \(\beta\)-galactosidase expression was observed for PMC1 in response to Na\(^+\) (Fig. 8B) or with PMR2 or PMC1 reporters in response to \(\alpha\)-factor (data not shown).

Calcineurin is required for the transcriptional induction of PMR2, PMC1, and PMR1 as well as other genes in response to Ca\(^{2+}\). Preliminary analysis using DNA microarray technology indicates that many genes exhibit calcineurin-dependent changes in gene expression that also require CRZ1 (T. Roe, J. DeRisi, A. Stathopoulos, P. Brown, and M. Cyert, in prep.). Here, we show that Crz1p is required for calcineurin-dependent transcription of PMR2 and PMC1 (see Fig. 8). Calcineurin-dependent transcription of PMR1 has also been shown to require Crz1p (Matheos et al., this issue). In addition, we found that crz1Δ cells exhibit sensitivities to Mn\(^{2+}\), Na\(^+/\)Ca\(^{2+}\) response to Ca\(^{2+}\) as well as Na\(^+\) (Fig. 8A). The crz1Δ mutation had no effect on the Na\(^+\)-induced expression of pmr2::lacZ that is calcineurin independent. In addition, CRZ1 was required for the calcineurin-dependent expression of a PMC1 reporter gene (pmc1::cyc1::lacZ-pAMS381) in response to Ca\(^{2+}\). No increase in \(\beta\)-galactosidase expression was observed for PMC1 in response to Na\(^+\) (Fig. 8B) or with PMR2 or PMC1 reporters in response to \(\alpha\)-factor (data not shown).

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Calcineurin is required for the transcriptional induction of PMR2, PMC1, and PMR1 as well as other genes in response to Ca\(^{2+}\). Preliminary analysis using DNA microarray technology indicates that many genes exhibit calcineurin-dependent changes in gene expression that also require CRZ1 (T. Roe, J. DeRisi, A. Stathopoulos, P. Brown, and M. Cyert, in prep.). Here, we show that Crz1p is required for calcineurin-dependent transcription of PMR2 and PMC1 (see Fig. 8). Calcineurin-dependent transcription of PMR1 has also been shown to require Crz1p (Matheos et al., this issue). In addition, we found that crz1Δ cells exhibit sensitivities to Mn\(^{2+}\), Na\(^+/\)Ca\(^{2+}\) response to Ca\(^{2+}\) as well as Na\(^+\) (Fig. 8A). The crz1Δ mutation had no effect on the Na\(^+\)-induced expression of pmr2::lacZ that is calcineurin independent. In addition, CRZ1 was required for the calcineurin-dependent expression of a PMC1 reporter gene (pmc1::cyc1::lacZ-pAMS381) in response to Ca\(^{2+}\). No increase in \(\beta\)-galactosidase expression was observed for PMC1 in response to Na\(^+\) (Fig. 8B) or with PMR2 or PMC1 reporters in response to \(\alpha\)-factor (data not shown).

Calcineurin is required for the transcriptional induction of FKS2 in response to Ca\(^{2+}\) (Mazur et al. 1995; C. Zhao, U. Jung, P. Garrett-Engele, T. Roe, M. Cyert, and D. Levin, in prep.). We identified a region within the FKS2 upstream sequence from −762 bp to −705 bp that is both necessary and sufficient for this calcineurin-dependent transcriptional induction (see Fig. 1). Furthermore, from within this region, we defined a 24-bp element, the CDRE, that supports calcineurin-dependent transcriptional induction in response to Ca\(^{2+}\) (see Table 1; Fig. 2). We performed a genetic screen and identified a novel transcription factor, Crz1p, that when overexpressed bypasses a requirement for calcineurin inactivation of a CDRE-containing reporter gene. This transcription factor is identical to Tcn1p (Matheos et al., this issue). In addition, we find that crz1Δ cells exhibit sensitivities to Mn\(^{2+}\), Na\(^+/\)Ca\(^{2+}\) response to Ca\(^{2+}\) as well as Na\(^+\) (Fig. 8A). The crz1Δ mutation had no effect on the Na\(^+\)-induced expression of pmr2::lacZ that is calcineurin independent. In addition, CRZ1 was required for the calcineurin-dependent expression of a PMC1 reporter gene (pmc1::cyc1::lacZ-pAMS381) in response to Ca\(^{2+}\). No increase in \(\beta\)-galactosidase expression was observed for PMC1 in response to Na\(^+\) (Fig. 8B) or with PMR2 or PMC1 reporters in response to \(\alpha\)-factor (data not shown).

Figure 7. CRZ1 is necessary for calcineurin-dependent transcriptional induction of the FKS2 gene in vivo. YPH499 ("WT") and ASY472 ("crz1Δ") cells were incubated for 4 hr in low pH YPD with or without FK506 and inducing treatments. Total RNA isolated from these cells was subjected to Northern blot and hybridized successively to FKS2 and ACT1 probes. FKS2 mRNA levels were normalized to ACT1 mRNA levels and are from one representative experiment with mRNA from cells that were either untreated (open bars) or treated with 10 µg/ml of \(\alpha\)-factor (shaded bars), 0.8 M NaCl (hatched bars), or 200 mM CaCl\(_2\) (solid bars). The ACT1-normalized FKS2 mRNA level in untreated wild-type cells was arbitrarily designated a value of 1.

Figure 8. CRZ1 is a general mediator of calcineurin-dependent transcriptional regulation. (A) Wild-type cells containing a plasmid-based pmr2::lacZ reporter gene (pFR70; Marquez and Serrano 1996) were grown at 30°C for 4 hr with or without FK506 in either YPD (open bar), synthetic media containing 200 mM CaCl\(_2\) (solid bar), or YPD containing 0.8 M NaCl (hatched bar). Wild-type cells containing a plasmid-based pmc1::cyc1::lacZ reporter gene (pAMS381) were grown at 30°C for 4 hr with or without FK506 in either YPD (open bar), synthetic media containing 200 mM CaCl\(_2\) (solid bar), or YPD containing 0.8 M NaCl (hatched bar). \(\beta\)-Galactosidase activities are shown for extracts from cells (YPH499) from one representative experiment. Each extract was assayed in triplicate, and the S.D. is representative of the error between these samples.
Crz1p functions downstream of calcineurin to control transcriptional activation in response to multiple environmental signals

Calcineurin is required not only for transcriptional inductions that result when cells are grown in high levels of Ca\(^{2+}\) but also those that result in response to at least three other environmental signals: Na\(^{+}\); elevated temperature, and mating pheromone (\(\alpha\)-factor). Several observations indicate that calcineurin-dependent activation of Crz1p occurs under all of these conditions. First, when cells are incubated in Na\(^{+}\), the expression of at least two genes, FKS2 and PMR2, is increased in a calcineurin-dependent manner (see Fig. 7; Mendoza et al. 1994); we show that Crz1p is required for both of these responses (see Figs. 7 and 8A). Second, elevated temperature induces calcineurin-dependent transcriptional FKS2 (C. Zhao, U. Jung, P. Garrett-Engele, T. Roe, M. Cyert, and D. Levin, in prep.), and preliminary experiments with CDRE reporter genes suggest that Crz1p also mediates this response (A. Stathopoulos, unpubl.). Finally, in response to pheromone, only FKS2 is known to increase in a calcineurin-dependent fashion (Mazur et al. 1995), and we find that this response requires CRZ1 as well (see Fig. 7). Furthermore, like calcineurin, Crz1p is required for cell viability during prolonged treatment with \(\alpha\)-factor (see Fig. 5B), and overexpression of CRZ1 can suppress the survival defect of calcineurin mutants during incubation with pheromone (see Fig. 6B). These results indicate that Crz1p-mediated transcriptional regulation contributes to cell viability under these conditions. However, fks2 mutant cells do not exhibit a survival defect when incubated with pheromone (Mazur et al. 1995). Therefore, additional genes, as yet unidentified, must also be transcriptionally regulated by Crz1p and calcineurin in response to \(\alpha\)-factor (see Fig. 9).

Differential gene expression results from multiple environmental signals and is mediated by both calcineurin and Crz1p

Surprisingly, although calcineurin and Crz1p are both activated in response to a variety of environmental signals (i.e., Ca\(^{2+}\), \(\alpha\)-factor, and Na\(^{+}\)), the genes transcriptionally regulated by Crz1p are differentially induced by subsets of these signals. FKS2 is induced by Ca\(^{2+}\), \(\alpha\)-factor, and Na\(^{+}\); whereas PMR2 is induced only by Ca\(^{2+}\) and Na\(^{+}\), and PMC1 is induced only by Ca\(^{2+}\) (see Figs. 7 and 8). Although calcineurin-dependent regulation of PMR2 and PMC1 requires CRZ1, it is unclear whether Crz1p is directly regulated in a Crz1p/CDRE-dependent manner, suggest that the mechanism of calcineurin- and Crz1p-dependent transcriptional regulation may vary under different environmental conditions. We initially observed that reporters containing the FKS2 promoter sequence exhibit calcineurin-dependent transcriptional induction in response to both Ca\(^{2+}\) and \(\alpha\)-factor and that a 57-bp region from −762 bp to −705 bp is necessary for both of these responses. However, whereas only 24 bp of this region, the CDRE, was sufficient to confer Ca\(^{2+}\)-induced expression on a reporter gene, even the entire 57 bp was not sufficient to confer \(\alpha\)-factor-dependent regulation on a reporter gene (see Table 1; Fig. 2). Although we do observe a small calcineurin-dependent induction of the 4×CDRE reporter gene when cells are treated with \(\alpha\)-factor (see Fig. 2), this response may not reflect true \(\alpha\)-factor-dependent signaling but may instead result from the rise in intracellular Ca\(^{2+}\) known to occur during pheromone treatment (Iida et al. 1990). Thus, our observations suggest that though the CDRE responds somewhat to \(\alpha\)-factor, the full induction of FKS2 expression in response to \(\alpha\)-factor requires additional regulatory se-
quences. Similarly, with Na+ treatment, although a substantial increase in FKS2 mRNA levels occurs, no induction of CDRE-driven constructs is observed (A. Stathopoulos, unpubl.), suggesting the requirement of additional sequences for this response as well. We conclude that although the CDRE is sufficient for transcriptional induction in response to Ca\(^{2+}\), it may act in conjunction with binding sites for other factors to mediate responses to α-factor and Na+ (see Fig. 9).

How does calcineurin regulate the ability of Crz1p to activate transcription?

The mechanism by which calcineurin regulates the activity of Crz1p remains to be determined. Calcineurin may regulate the binding affinity of Crz1p for the CDRE or the nuclear translocation of this protein. However, we currently favor a different model in which calcineurin regulates the ability of Crz1p to function as a transcriptional activator. Preliminary experiments with a chimeric protein containing Crz1p fused to the Gal4p DNA-binding domain support this model. This fusion protein, which also contains a functional nuclear localization sequence, exhibits calcineurin-dependent transcriptional activation of a reporter gene (UAS GAL4) that is induced in a Gal4p-specific manner (A. Stathopoulos, unpubl.). These results suggest that Crz1p contains a calcineurin-dependent transcriptional activation domain. Preliminary two-hybrid experiments failed to detect an interaction between full-length Crz1p and the calcineurin catalytic subunit (A. Stathopoulos, unpubl.). Therefore, more extensive analysis is required to determine whether calcineurin directly dephosphorylates Crz1p or whether instead the calcineurin-dependent regulation of Crz1p is indirect.

A subset of calcineurin-dependent events are mediated by Crz1p

Comparison of the phenotypes exhibited by calcineurin and crz1Δ cells demonstrates that only a subset of calcineurin functions are mediated through Crz1p (see Fig. 9). Calcineurin mutants exhibit a greater degree of sensitivity to Mn\(^{2+}\) and Li\(^{+}\) and a more pronounced α-factor viability defect than crz1Δ cells (see Fig. 5). Thus, calcineurin must also carry out functions that are independent of Crz1p that affect these phenotypes. Similarly, fks1Δ mutants are inviable in the absence of calcineurin activity, whereas these cells are viable though severely growth impaired in the absence of Crz1p (A. Stathopoulos, unpubl.). Furthermore, calcineurin mutants display two phenotypes that are not shared by crz1Δ cells. First, calcineurin mutants are sensitive to high pH (Nakamura et al. 1993), whereas crz1Δ cells are not. Second, calcineurin mutants and cnb1Δ crz1Δ double mutants are Ca\(^{2+}\) tolerant (Tanida et al. 1996; Withee et al. 1997), whereas crz1Δ single mutants are Ca\(^{2+}\) sensitive. An inability to induce PMC1 expression, these cells are Ca\(^{2+}\) tolerant because of decreased cytosolic Ca\(^{2+}\) levels (Tanida et al. 1996). Thus, calcineurin also functions independently of Crz1p to inhibit Ca\(^{2+}\) sequestration.

We have demonstrated that Crz1p is a component of one or more calcineurin-dependent pathways controlling transcriptional activation and that calcineurin carries out additional functions that are not mediated by Crz1p. The identification and characterization of the CDRE and Crz1p should facilitate the identification of additional components of these and other calcineurin-dependent signal transduction pathways. This analysis should more clearly define the roles of the multifunctional calcineurin phosphatase in yeast and other eukaryotic cells.

Materials and methods

Media and general methods

Yeast media and culture conditions were those recommended (Sherman et al. 1986) except that nutritional supplements in synthetic media were added at twice the specified level and 3.5 grams of ammonium chloride per liter was substituted for ammonium sulfate as indicated. In addition, low pH YP media was adjusted to pH 5.0 by adding succinate (10 gram/liter). Where indicated, α-factor (Star Biochemicals) or the chloride salt of certain ions were added to media at the specified level. A stock solution of FK506 (Fugisawa, Inc.; 20 mg/ml in 90% ethanol-10% Tween 20) was also added to liquid media to a final concentration of 1 µg/ml where noted.

All procedures involving recombinant DNA in S. cerevisiae and Escherichia coli were performed using standard techniques (Ausubel et al. 1987). DNA was introduced into yeast cells by lithium acetate transformation (Ausubel et al. 1987) and into bacteria by electroporation (Ausubel et al. 1987). Double-stranded DNA templates used for sequencing were prepared according to the manufacturer’s instructions (Promega Wizard Miniprep). The Sequenase version of using the P7T DNA polymerase and nonradioactive nucleotides were obtained from U.S. Biochemical Corporation. [α-32P]dATP was obtained from Amer sham. Sequencing reactions were conducted according to the manufacturer’s instructions.

Plasmids

A series of fks2::lacZ reporter genes was constructed from cyc1::lacZ reporters pLG178 and pLG312 (Guarente and Mason 1983). The fks2::lacZ reporter plasmids pAMS312, pAMS317, and pAMS319 were constructed by removing a region of CYC1 sequence from pLG178 with XhoI and BamHI and inserting PCR products flanked by XhoI and BamHI sites containing various segments of the FKS2 gene upstream sequence (−910/+6, −762/+6, and −705/+6 bp, respectively). pAMS327 was constructed by removing the CYC1 upstream activating sequence (UAS) from pLG312 with Smal and Xhol and inserting a PCR product flanked by blunt and SalI sites containing the FKS2 sequence from −762 bp to −705 bp. Plasmids pAMS342, pAMS344, pAMS545, and pAMS546 were constructed by removing the UAS sequence from pLG312 with Smal and Xhol and inserting annealed complementary oligonucleotides flanked by blunt and SalI sites that contained the FKS2 sequence from −762 bp to −738 bp, −750 bp to −726 bp, −738 bp to −714 bp, and −726 bp to −705 bp, respectively. Plasmids pAMS363 and pAMS366 contain two and four tandem
repeats, respectively, of the CRE flanked by Xhol and Sall sites (XS-CRE: 5'-TCGACACCATCGTGGCTCGCTTGCGCTTGCGCAACGGACGATCGAGCATGTCGCTTTG-3' and 3'-GTGGTACGACGCAAGCGTCGAGTCCCGAAAGTCCGATCGAGCATGTCGCTTTG-5'). Two and four tandem repeats of the CRE were constructed by iterative insertion of the CRE into the Xhol site of pBluescript (Stratagene) creating plasmids pAMS346 and pAMS347, respectively. A Xhol-Sall fragment containing the tandem repeats plus pBluescript polylinker sequence was inserted into the Xhol site of pLG1178. Plasmid pAMS364 was constructed exactly as pAMS366 except four tandem copies of mutCDRE (XS-mutCDRE: 5'-TCGACCTCTGTTGCACGGT-GAGCCCTAGC-3' and 3'-GAGACACCTTGAGACTCGG-GATCGAGCATGTCGCTTTG-5'), constructed by iterative insertion into the Xhol site of pBluescript to create plasmid pAMS350, were used.

Another series of CRE reporter genes was constructed from the gal::lacZ integrating reporter plJL638 (Li and Herskowitz 1993). Plasmids pAMS367 and pAMS369 were created by iterative insertion of a double-stranded oligonucleotide flanked by BamHI and BglII sites containing the CRE sequence or mutCDRE sequence, respectively, into the BglII site of pAMS433, constructing exactly as pAMS366 except four tandem copies of the CRE (XS–CRE: 5'-CCACCCGCTTCGTGGTACGACGCAAGCGTCGAGTCCCGAAAGTCCGATCGAGCATGTCGCTTTG-3' and 3'-GTGGTACGACGCAAGCGTCGAGTCCCGAAAGTCCGATCGAGCATGTCGCTTTG-5'). Plasmid pAMS341 and pAMS354 were constructed by iterative insertion of the CDRE into the EcoRI sites at which the CRE sequences are flanked by the EcoRI sites of pAMS433 and pAMS435, respectively. Both PCR products were amplified from the unique EcoRI and XhoI sites of pAMS433 and pAMS435, placing the epitope between the twelfth and thirteenth codons of Crz1p, creating pAMS446. Plasmid pAMS364 was constructed by iterative insertion of a double-stranded oligonucleotide flanked by BamHI and BglII sites containing the CRE sequence or mutCRE sequence, respectively, into the BamHI and BglII sites of pITMUS 28 (E.B.

Plasmid pAMS343 containing CRZ1 flanked by BamHI and Sall sites was isolated by gap repair in yeast strain DD12 of EcoRI-digested plasmid pAMS417. PCR fragments (575 and 600 bp) were amplified from the 5' and 3' regions of CRZ1 using genomic yeast DNA as a template and were flanked by BamHI and EcoRI or EcoRI and Sall sites, respectively. Both PCR products were inserted into BamHI- and Sall-digested pRS315 (Sikorski and Hieter 1989) to create plasmid pAMS417. A 3.2-kb BamHI–Sall fragment from pAMS433, containing CRZ1, was inserted into the BamHI and Sall sites of YEp351 to create pAMS435. A 130-bp PCR fragment containing a double-strand oligonucleotide flanked by BamHI and EcoRI sites was amplified from plasmid pAMS345 to create pAMS446. BamHI–Sall (3.2-kb) fragments from pAMS345 and pAMS347 were inserted into pRS316 (Sikorski and Hieter 1989) creating pAMS452 and pAMS450, respectively.

A pmc1::cycl1::lacZ reporter construct, pAMS381, was created by first removing the UAS sequence from pLG3312 with SmaI and Xhol and then inserting a Stu–Xhol PCR fragment containing the PMC1 sequence from −568 to −207 relative to the initiation codon.

Yeast strains

The yeast strains used in this study are listed in Table 2 and were constructed through transformation or isogenic crosses by using standard techniques (Sherman et al. 1986). Each reporter gene construct (pAMS367, pAMS369, plJL638) was integrated at the URA3 locus as described (Li and Herskowitz 1993) into both strains YPH499 and DD12 to create reporter strains ASY459, ASY460, ASY461, ASY462, ASY465, and ASY466. The cyr1::loxP–kanMX–loxP cassette in pHU6 (Guldener et al. 1996) and also contain 40 bp of homology to the DNA flanking the CRE1 coding sequence. Correct integration of the cyr1::loxP–kanMX–loxP cassette was determined by homologous recombination in yeast of a PCR-amplified fragment that was transformed into yeast cells (Guldener et al. 1996). This PCR-amplified fragment was generated with primers that recognize the loxP–kanMX–loxP cassette and the CRE1 coding sequence. Correct integration of the cyr1::loxP–kanMX–loxP cassette was confirmed by PCR.

β-Galactosidase assays

Quantitative assay  Exponentially growing cells (OD600 of 0.8–1.2) were incubated at 21°C in synthetic media containing ammonium chloride for 6 hr unless otherwise noted. When indicated, salts, α-factor and/or FK506 were added to the media at the start of this incubation. Cells were then harvested, and the cell pellet was frozen at −20°C. Cell extracts were prepared essentially as described in Withee et al. (1997). Protein concentration of the extracts was determined using the Bio-Rad Bradford assay kit, with dilutions of bovine serum albumin used to generate the standard curve. The β-galactosidase activity was determined at room temperature using the substrate ONPG (O-nitrophenyl-β-D-galactopyranoside, Sigma) as described (Miller 1972) and are given in units of nanomoles ONPG converted per minute per milligram of protein.

<table>
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<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>YPH499</td>
<td>MATa ura3-52 lys2-801 ade2-101 trp5-Δ63 his3-Δ200 leu2-Δ1</td>
<td>Sikorski and Hieter 1989</td>
</tr>
<tr>
<td>YPH500</td>
<td>MATα ura3-52 lys2-801 ade2-101 trp5-Δ63 his3-Δ200 leu2-Δ1</td>
<td>Sikorski and Hieter 1989</td>
</tr>
<tr>
<td>YPH501</td>
<td>MATα YPH499 x YPH500</td>
<td>Sikorski and Hieter 1989</td>
</tr>
<tr>
<td>MCY3</td>
<td>same as YPH501 except CNB1/cnb1::LEU2</td>
<td>Cyert and Thorner 1992</td>
</tr>
<tr>
<td>DD12</td>
<td>same as YPH499, except cnb1::hisG</td>
<td>Cyert and Thorner 1992</td>
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<td>same as YPH499, except ura3-52::URA3-4×-CRE::gal1::lacZ</td>
<td>this study</td>
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<td>ASY472</td>
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<tr>
<td>ASY589</td>
<td>same as ASY461, except crz1::loxP–kanMX–loxP</td>
<td>this study</td>
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</table>
Qualitative assay. Colonies were scored for β-galactosidase activity essentially as described (Hannon et al. 1993) with minor modifications. Supported nitrocellulose filters (Schleicher & Schuell) were used instead of Whatman 50 filters. Positive colonies showed blue color in 15 min to 16 hr (overnight), whereas negatives remained white even after an overnight incubation.

Genetic screen to identify activators of CDRE-dependent expression

Under standard growth conditions, wild-type cells (ASY 459) expressed enough β-galactosidase from the 4× CDRE::lacZ reporter to make colonies blue when incubated with its chromogenic substrate, X-gal. This expression was calcineurin dependent, because calcineurin mutant cells (ASY 461) incubated under these same conditions remained white. We confirmed that the lacZ reporter was functional because plasmids containing CNB1, thus complementing the cnb1A mutation, allowed for ASY 461 to activate the reporter and turn blue. No β-galactosidase production was observed for strains containing the 4× mutCDRE::lacZ reporter (ASY 460 and ASY 462).

Two high-copy genomic libraries, 2J351 (Hill et al. 1986; Engbercht et al. 1990) and Y2HL (James et al. 1996), were screened for plasmids that allowed ASY 461 to form blue colonies. Plasmids were harvested from blue colonies and then amplified in E. coli TOP10. These plasmids were then used to retransform the original strain (ASY 461), a strain containing the mutCDRE reporter (ASY 462), and a strain containing a lacZ reporter with no upstream activating sequence (ASY 466). Because strains ASY 462 and ASY 466 were not able to support calcineurin-dependent transcriptional induction, they served as negative controls to eliminate positives that induced expression independently of an intact CDRE. Only plasmids were further characterized that specifically induced expression in strain ASY 461 but not in strains ASY 462 or ASY 466 and therefore conferred CDRE-dependent gene expression in the absence of calcineurin. The ends of the genomic inserts were sequenced, and this sequence was used to scan the yeast genome to identify all ORFs contained within the insert.

DNA mobility retardation assays

The synthetic double-stranded XS-CDRE oligonucleotide (described above) was used as a probe in all binding experiments. XS-CDRE was end-labeled and passed over a G25 Sephadex column (Sigma) to separate out unincorporated nucleotides. XS-CDRE was end-labeled and passed over a G25 Sephadex column (Sigma) to separate out unincorporated nucleotides. XS-CDRE was end-labeled and passed over a G25 Sephadex column (Sigma) to separate out unincorporated nucleotides.

References


Calcinurin acts through CRZ1


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Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast

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