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Genome-wide Analysis of Gene Expression Regulated by the Calcineurin/Crz1p Signaling Pathway in *Saccharomyces cerevisiae**

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In *Saccharomyces cerevisiae*, the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, is activated by specific environmental conditions, including exposure to Ca^{2+} and Na^+, and induces gene expression by regulating the Crz1p/Tcn1p transcription factor. We used DNA microarrays to perform a comprehensive analysis of calcineurin/Crz1p-dependent gene expression following addition of Ca^{2+} (200 mM) or Na^+ (0.8 M) to yeast. 163 genes exhibited increased expression that was reduced 50% or more by calcineurin inhibition. These calcineurin-dependent genes function in signaling pathways, ion/small molecule transport, cell wall maintenance, and vesicular transport, and include many open reading frames of previously unknown function. Three distinct gene classes were defined as follows: 28 genes displayed calcineurin-dependent induction in response to Ca^{2+} and Na^+, 125 showed calcineurin-dependent expression following Ca^{2+} but not Na^+ addition, and 10 were regulated by calcineurin in response to Na^+ but not Ca^{2+}. Analysis of *crz1*Δ cells established Crz1p as the major effector of calcineurin-regulated gene expression in yeast. We identified the Crz1p-binding site as 5′-GNGGC(G/T)CA-3′ by *in vitro* site selection. A similar sequence, 5′-GAGGCTG-3′, was identified as a common sequence motif in the upstream regions of calcineurin/Crz1p-dependent genes. This finding is consistent with direct regulation of these genes by Crz1p.

Calcineurin, a highly conserved Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase, plays an important role in coupling Ca^{2+} signals to cellular responses (reviewed in Ref. 1). Calcineurin is a heterodimeric enzyme consisting of a catalytic (A) subunit and a tightly associated, Ca^{2+}-binding, regulatory (B) subunit. The activity of calcineurin is inhibited rapidly and specifically *in vivo* by the immunosuppressive drugs FK506 and cyclosporin A (reviewed in Ref. 2). In mammals, calcineurin plays a key role in many Ca^{2+}-regulated processes including T-cell activation (3, 4), neutrophil chemotaxis (5, 6), apoptosis (7), cardiac hypertrophy (8), memory (9), and angiogenesis (10). For many of these physiological responses, calcineurin exerts its effects by regulating members of the nuclear factor of activated T cells (NFAT)[1] family of transcription factors. Calcineurin directly dephosphorylates NFAT transcription factors, causing their activation and translocation to the nucleus (reviewed in Ref. 11).

In the yeast *Saccharomyces cerevisiae*, calcineurin is activated under specific environmental conditions, such as exposure to high extracellular levels of Ca^{2+} or Na^+, elevated temperature, and prolonged incubation with α-factor (Fig. 1) (12). Under many of these conditions, calcineurin is required to maintain cell viability. One consequence of calcineurin-dependent signaling is increased expression of *FKS2*/*GSC2*, which encodes a β-1,3-glucan synthase (13). A 24-bp region of the *FKS2* promoter, termed the CDRE (calcineurin-dependent response element), is necessary and sufficient to direct Ca^{2+}-induced calcineurin-dependent gene expression (14). The *CRZ1*/*TCN1*/*HAL8* transcription factor was identified as an activator of CDRE-driven transcription as well as a substrate phosphoprotein for calcineurin (14–17). Crz1p contains a zinc finger motif for DNA binding and binds specifically to the CDRE. *crz1* mutants display similar phenotypes to those of calcineurin mutants, and in calcineurin mutants these phenotypes are suppressed by *CRZ1* overexpression (14, 16). Thus, Crz1p functions downstream of calcineurin to effect calcineurin-dependent responses. Calcineurin controls Crz1p activity by regulating its subcellular localization (17). When calcineurin-dependent signaling is low, Crz1p is phosphorylated and resides primarily in the cytosol. Once calcineurin is activated, however, it dephosphorylates Crz1p, causing its rapid translocation to the nucleus. This change in localization is the result of increased nuclear import and decreased nuclear export of Crz1p (18, 19). Thus, although Crz1p and NFAT display only limited sequence similarity, their modes of regulation by calcineurin are remarkably similar.

In addition to *FKS2*, transcription of several other genes is regulated in a calcineurin/Crz1p-dependent manner. These genes, *PMC1*, *PMR1,* and *ENA1*/*PMR2*, all encode P-type ATPases that are required for distinct aspects of ion homeostasis. *PMC1* encodes a Ca^{2+}-ATPase responsible for Ca^{2+} sequestration in vacuole (20), and *PMR1* similarly transports Ca^{2+} and Mn^{2+} into the Golgi apparatus (21–23). Ca^{2+} induces

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[1] The abbreviations used are: NFAT, nuclear factor of activated T-cells; CDRE, calcineurin-dependent response element; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol.

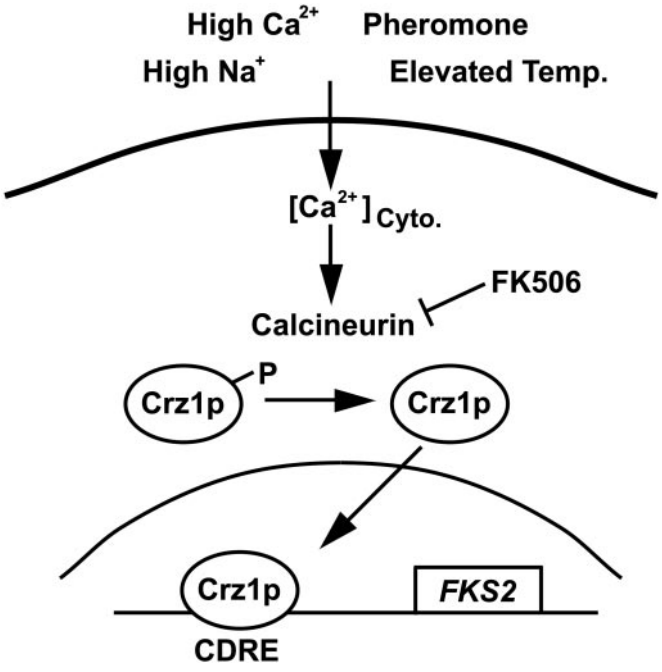


FIG. 1. **Outline of the calcineurin/Crz1p signaling pathway in *S. cerevisiae*.** Calcineurin activity is activated by specific environmental conditions (high levels of Ca^{2+} and Na^+, elevated temperature, and pheromone (α-factor)). Its activity is inhibited rapidly and specifically *in vivo* by the immunosuppressive drug FK506. Calcineurin dephosphorylates Crz1p, thereby regulating its localization and activity. Transcription of *FKS2*, which encodes a β-1,3-glucan synthase, is activated in response to Ca^{2+}. A 24-bp region of the *FKS2* promoter, termed the CDRE, is sufficient to direct Ca^{2+}-induced gene expression, and this transcription requires both calcineurin and Crz1p. *Cyto*, cytosol.

transcription of both *PMC1* and *PMR1* in a calcineurin/Crz1p-dependent manner, and expression of these genes is required for yeast growth under high Ca^{2+} conditions (24). *ENA1* encodes a plasma membrane Na^+/Li-ATPase that is required for growth in the presence of high concentrations of these ions (25). *ENA1* expression is induced by Ca^{2+} and Na^+ in a calcineurin/Crz1p-dependent manner and by osmotic stresses through the high osmolarity-induced (HOG) mitogen-activated protein kinase pathway (26, 27).

In this report, we present a comprehensive identification of yeast genes whose expression is regulated in a calcineurin-dependent manner. We activated calcineurin signaling *in vivo* by exposing yeast cells to high extracellular levels of Ca^{2+} or Na^+ and used DNA microarrays to analyze the resulting changes in gene expression in the presence and absence of FK506. In this way we identified genes whose expression is dependent on calcineurin and also characterized the transcriptional output of calcineurin-dependent signaling under two different environmental conditions. Furthermore, we examined the transcriptional response of *crz1*Δ cells under these same conditions. These studies confirm that Crz1p is the major, and possibly the only, effector of calcineurin-regulated gene expression in yeast. We also establish a DNA-binding site for Crz1p, 5′-GNGGC(G/T)CA-3′, using methylation interference analysis and *in vitro* site selection. Analysis of the upstream regions of calcineurin-dependent genes identified a common sequence motif that closely matches this site. This observation is consistent with direct regulation of calcineurin-dependent genes by Crz1p.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

The wild-type strain used in this study was *S. cerevisiae* DBY7286 (*MAT***a** *ura3-52 GAL2*) (28). YHY804 (*MAT***a** *ura3-52 GAL2 crz1*::*KANMX4*) was constructed by one-step gene replacement method mentioned integrating *kanMX* in the *CRZ1* locus in the strain DBY7286 as described previously (14, 29). The *Escherichia coli* GST-Crz1p expression plasmid, pRSP121, was made by inserting a *Hin*dIII/*Sal*I fragment containing the *CRZ1* open reading frame into pGEX4T-3 (Amersham Biosciences) digested with *Hin*dIII and *Xho*I (19).

Medium

YPD (1% yeast extract, 2% bactopeptone, and 2% dextrose) was used. Where noted, YPD containing either 0.4 M $CaCl_2$ or 1.6 M NaCl was mixed with an equal volume of YPD to achieve final concentrations of 0.2 M $CaCl_2$ and 0.8 M NaCl. YPD containing 0.4 M $CaCl_2$ was buffered to pH 5.0 with 7.5 mM succinate to prevent precipitation of $CaPO_4$. Where noted, a 20 mg/ml stock solution of FK506 (Fujisawa, Inc., Deerfield, IL), in ET (90% ethanol and 10% Tween) or ET was also added to YPD at a 1:20,000-fold dilution (FK506 final concentration, 1 μg/ml).

Culture Conditions

Experiments 1 and 2: Ca^{2+}, Ca^{2+} + FK506 Time Course—Cells were grown at 30 °C in YPD medium (buffered to pH 5.0 with 7.5 mM succinate) containing ET solution to a density of 0.6×10^7 cells/ml (A_{600} = 0.6). The culture was divided in two. One part was supplemented with ET at 30 °C for 15 min, and the other part was supplemented with FK506 (1.0 μg/ml final concentration) in ET at 30 °C for 15 min. Cells were collected for the t = 0-min sample. Next, an equal volume of YPD medium (pH 5.0) containing ET and 0.4 M $CaCl_2$ was added to the ET-treated culture (final concentration, 0.2 M $CaCl_2$). An equal volume of YPD medium (pH 5.0) containing FK506 (1.0 μg/ml) and 0.4 M $CaCl_2$ was also added to the FK506-treated culture (final concentration, 0.2 M $CaCl_2$). Cells were collected by centrifugation at 5, 15, 30, and 60 min, frozen at −80 °C, and processed for RNA extraction (see below).

Experiment 3: Ca^{2+} + FK/Ca^{2+}—Cells were cultured with or without FK506 treatment and exposed to $CaCl_2$ as described for experiments 1 and 2. Samples were collected from the drug-treated and control cultures at 15 and 30 min after Ca^{2+} addition.

Experiment 4: crz1/CRZ1:Ca^{2+}—Wild-type strain DBY7286 and the *crz1*Δ strain YHY804 were cultured and exposed to $CaCl_2$ as described for Experiments 1 and 2. Samples were collected at 15 and 30 min after Ca^{2+} addition.

Experiments 5 and 6: Na^+, Na^+ + FK506 Time Course—Cells were grown in YPD medium (pH 5.0) at 30 °C to a density of 0.6×10^7 cells/ml (A_{600} = 0.6). The culture was divided in two. One part was supplemented with ET at 30 °C for 15 min, and the other part was supplemented with FK506 (1.0 μg/ml) in ET at 30 °C for 15 min. Cells were collected for the t = 0-min sample. Next, an equal volume of YPD medium (pH 5.0) containing ET solution and 1.6 M NaCl was added to the ET-treated culture (final concentration 0.8 M NaCl). An equal volume of YPD medium (pH 5.0) containing FK506 (1.0 μg/ml) and 1.6 M NaCl was also added to the FK506-treated culture (final concentration, 0.8 M NaCl). Samples were collected 15, 30, 45, and 60 min after Na^+ addition.

Experiment 7: Na^+ + FK/Na^+—Cells were cultured with or without FK506 treatment and exposed to 0.8 M NaCl as described for Experiments 5 and 6. Samples were collected from the drug-treated and control cultures at 30 and 45 min after Na^+ addition.

Experiment 8: crz1/CRZ1:Na^+—Wild-type strain DBY7286 and the *crz1* mutant strain YHY804 were cultured and exposed to NaCl as described for Experiments 5 and 6. Samples were collected at 30 and 45 min after Na^+ addition.

RNA Isolation

Total RNA was extracted by the hot acid phenol method as described (30). mRNA was purified from total RNA using the Micro-FastTrack 2.0 kit (Invitrogen). Quantitation of RNA was carried out by UV spectroscopy. cDNA was synthesized from mRNA by reverse transcription and incorporated Cy3-dUTP and Cy5-dUTP (Amersham Biosciences) into the cDNA. The fluorescently labeled product was recovered and used as a hybridization probe as described previously (31).

Microarray Analysis

The time course experiments were performed by directly comparing the abundance of mRNA relative to the t = 0 sample as shown in Table I. mRNA from the t = 0 sample was labeled with Cy3 (represented as *green* color), and mRNA from treated samples, harvested at the indicated times, was labeled with Cy5 (represented as *red* color). mRNA levels of the *crz1* mutant strain were compared with those of the isogenic wild-type strain as shown in Table I. mRNA from the *crz1*

TABLE I
List of DNA microarray experiments

Experiment name	Sampling time	Cy3-labeled sample	Cy5-labeled sample
	min		
1) Ca^{2+}	5, 15, 30, 60	0 min	Indicated time
2) Ca^{2+} + FK506	5, 15, 30, 60	0 min	Indicated time
3) Na^+	15, 30, 45, 60	0 min	Indicated time
4) Na^+ + FK506	15, 30, 45, 60	0 min	Indicated time
5) Ca^{2+} + FK506/Ca^{2+}	15, 30	Ca^{2+}	Ca^{2+} + FK506
6) Na^+ + FK506/Na^+	30, 45	Na^+	Na^+ + FK506
7) *crz1*/*CRZ1*:Ca^{2+}	15, 30	wt[a] + Ca^{2+}	*crz1* + Ca^{2+}
8) *crz1*/*CRZ1*:Na^+	30, 45	wt + Na^+	*crz1* + Na^+

[a] wt indicates wild type.

mutant strain was labeled with Cy5, and mRNA from the isogenic wild-type strain was labeled with Cy3. Yeast open reading frame DNA microarrays were prepared, processed, and analyzed as described previously (31). The microarrays were scanned with a Gene Pix 4000 scanner (Axon instruments, Foster City, CA), and the Gene Pix 4000 software package was used to locate spots in the microarray. Each microarray experiment was performed in duplicate. Data analysis was performed using the software GeneCluster and TreeView (32). For most data sets, results from two independent microarrays were averaged, and a gene was considered to have consistently altered expression if its average fold change in duplicate arrays was more than two. A subset of our supplemental data is available on our website (www.stanford.edu/group/cyert/microarrays.html).

Crz1p DNA Binding Analysis

GST-Crz1p expressed from pRSP121 in *E. coli* strain BLR was purified from French press extracts using glutathione-agarose beads (Amersham Biosciences) according to the manufacturer's instructions. The CDRE probe was generated by annealing synthetic oligonucleotides (CDREB, 5′-TCGACAAGCGCACAGCCACCGACTGGTG-3′ and CDRET, 5′-GATCCACCAGTCGGTGGCTGTGCGCTTG-3′) and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase as recommended by the manufacturer (New England Biolabs, Beverly, MA). Full-length products were purified from a denaturing polyacrylamide gel (5 M urea, 15% acrylamide, 1× TBE), and each labeled oligonucleotide was then annealed to its unlabeled compliment by heating to 100 °C and slowly cooling the mixture (1.5 pmol of CDRET, 1.5 pmol of CDREB, 50 mM NaCl, 2 mM $MgCl_2$). Methylation of 0.5 pmol of CDRE was carried out in dimethyl sulfate reaction buffer (50 mM sodium cacodylate, 1 mM EDTA, pH 8, 0.5% dimethyl sulfate) for 5 min. EMSA was then carried out as described below using 0.5 pmol of methylated CDRE and 30 pmol of GST-Crz1p. The bands corresponding to the "bound" and "free" CDRE were identified by autoradiography and isolated from the gel. Cleavage at methylated bases was carried out by resuspending the samples in 10 mM sodium phosphate, pH 7, and incubating for 15 min at 90 °C, followed by the addition of NaOH to 100 mM and incubating an additional 30 min. The fragments were then separated on a denaturing polyacrylamide gel (20% acrylamide, 1× TBE, 5 M urea) and visualized by autoradiography.

The EMSA was carried out in 20 μl of gel shift buffer (1 mM $MgCl_2$, 10 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 2 mg/ml leupeptin) containing 0.5 pmol of methylated CDRE, 30 pmol of GST-Crz1p, and 3 μg of poly(dI-dC) (Amersham Biosciences). After 10 min of incubation at room temperature, the reaction was run on a native 4% polyacrylamide TBE gel, dried down, and visualized by autoradiography.

Binding Site Selection, Amplification Analysis

GST-Crz1p purified from *E. coli* (see above) was incubated with a partially degenerate pool of DNA sequences. Sequences that bound GST-Crz1p were then isolated using EMSA, amplified by PCR, and enriched by carrying out three more cycles of EMSA followed by PCR. Finally, the selected sequences were subcloned and sequenced. Experimental details of each step are as follows.

Generation of Random Sequences—A 60-nucleotide oligonucleotide, 5′-CAGTCAGTTCAAAGCTTCAT(N)$_{20}$AAGTCTAGAACTGACTAGTC-3′ (where N is any of the four bases and the *Hin*dIII and *Xba*I sites in the constant regions that were included for subcloning are underlined) was made double-stranded by PCR using primers complementary to the constant regions (primer 1, 5′-GACTAGTCAGTTCTAGACTT-3′, and primer 2, 5′-CAGTCAGTTCAAAGCTTCAT-3′) and 20 cycles under the following conditions: 45 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C. The PCR was carried out using *Taq* DNA polymerase under conditions recommended by the manufacturer (New England Biolabs) except that 1 pmol of 5′-^{32}P-labeled primer 2 was included in the reaction. Primer 2 was labeled using T4 polynucleotide kinase (New England Biolabs) as recommended by the manufacturer and [γ-^{32}P]ATP (Amersham Biosciences). The double-stranded product (dsR60) was gel isolated from a native 8% polyacrylamide/TBE gel.

Electrophoretic Mobility Shift Assay—The first round of EMSA was performed in 50 μl of gel shift buffer (see above) to which 10 pmol of dsR60, 3 μg (29 pmol) of pure GST-CRZ1p, and 3 μg of poly(dI-dC) were added. After 10 min of incubation at room temperature, the bound molecules were separated from the free on a native 4% acrylamide/TBE

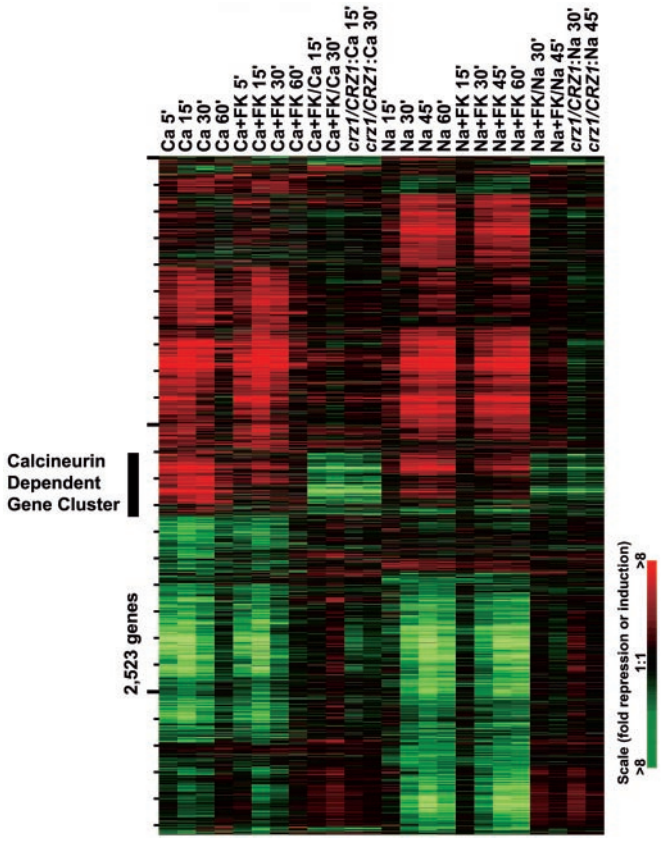
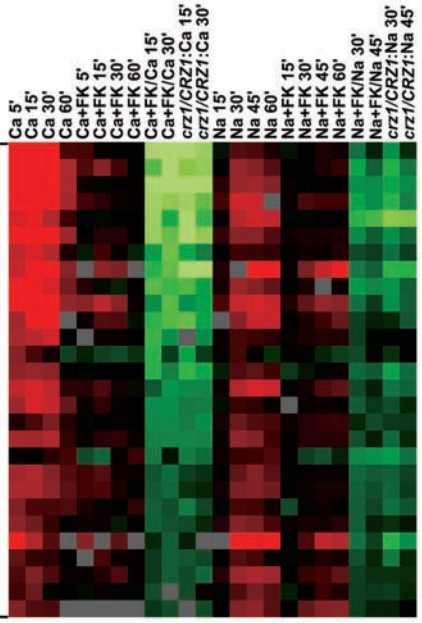


FIG. 2. **An overview of yeast gene expression following exposure to Ca^{2+} or Na^+.** The expression profiles of 2,523 genes that exhibited a ≥2-fold change in at least two of 24 array experiments are shown. One major cluster of genes displaying calcineurin-dependent expression is noted. Each *column* represents an individual experiment, labeled as in Table I and described under "Experimental Procedures." Each *row* represents the expression ratios for a particular gene under all the conditions listed. *Red* represents a higher level of expression in the Cy5-labeled sample compared with the Cy3-labeled sample (see Table I). The degree of color saturation represents the magnitude of the expression ratio, as indicated by the scale bar. *Black* indicates no detectable difference in expression levels; *gray* denotes a missing observation. Genes that share similar expression profiles were grouped by correlation clustering (32) and displayed using the TreeView program.

A

Calcineurin-dependent genes regulated by both Ca^{2+} and Na^{+}

ORF	Name	Function	CNCa	CRZCa	CNNa	CRZNa
YOR385W		unknown	30.1	29.4	3.6	5.1
YOL016C	*CMK2*	Ca/calmodulin-dependent protein kinase	22.7	14.2	6.4	8.2
YLR194C		unknown	22.4	27.7	5.2	6.5
YMR316W	*DIA1*	unknown	15.3	15.0	6.3	7.8
YLR414C		unknown	15.1	21.8	10.0	13.1
YBR005W		unknown	14.8	19.3	4.5	4.9
YDL234C	*GYP7*	GTPase-activating protein	11.7	6.5	2.3	3.5
YHR139C	*SPS100*	spo.-specific cell wall maturation protein	11.2	34.2	3.8	7.9
YMR316C-A		unknown	10.9	10.4	5.6	5.3
YOR220W		unknown	10.7	5.9	3.6	5.0
YNL093W	*YPT53*	GTP-binding protein, rab family	10.6	11.2	2.1	2.6
YER185W		unknown	9.3	4.7	2.1	1.1
YOL014W		unknown	7.5	6.1	2.3	1.8
YNL192W	*CHS1*	chitin synthase 1	7.2	8.9	3.3	6.1
YHR097C		unknown	5.4	4.5	2.3	3.7
YNR059W	*MNT4*	mannosyltransferase	4.8	4.0	2.8	2.4
YJL172W	*CPS1*	carboxypeptidase	4.5	4.8	2.3	2.0
YJL171C		unknown	4.3	4.3	2.5	3.3
YGR189C	*CRH1*	cell wall protein	3.8	3.9	5.1	5.7
YGL165C		unknown	3.4	2.1	3.2	2.9
YGL166W	*CUP2*	transcriptional activator	3.2	1.8	2.9	3.1
YPL057C	*SUR1*	synthesis of mannosylated sphingolipids	2.9	3.9	2.9	3.5
YGR121C	*MEP1*	ammonia permease	2.9	2.3	2.8	1.8
YOL084W	*PHM7*	unknown	2.9	1.5	3.6	8.6
YOR219C	*STE13*	dipeptidyl aminopeptidase	2.8	2.5	2.5	3.9
YEL060C	*PRB1*	vacuolar protease B	2.8	3.2	2.1	1.8
YNL196C	*SLZ1*	unknown	2.3	2.3	2.1	2.1
YDR535C		unknown	2.3	2.3	2.1	1.5



B

Calcineurin-dependent genes regulated by Ca^{2+}

ORF	Name	Function	CNCa	CRZCa
YGR213C	*RTA1*	involved in 7-aminocholesterol resist.	20.3	19.3
YCR007C		unknown	13.3	9.6
YLL057C		unknown	11.7	4.6
YLR054C		unknown	9.8	N.D.
YNR064C		unknown	7.9	5.3
YGL006W	*PMC1*	Ca ATPase	7.0	4.6
YKL218C	*SRY1*	serine racemase homolog in yeast	6.4	4.6
YOL158C	*ENB1*	ferric enterobactin transporter	6.4	4.1
YBR296C	*PHO89*	Na/Pi symporter	6.3	N.D.
YMR096W	*SNZ1*	stationary phase-induced gene family	5.5	5.0
YBR287W		unknown	5.4	4.4
YPL149W	*APG5*	involved in autophagy	5.3	4.0
YER184C		unknown	5.1	5.8
YGR212W		unknown	5.0	3.7
YOR134W	*BAG7*	GTPase activating protein	4.9	6.6
YBR182C	*SMP1*	transcription factor	4.7	1.9
YLR136C	*TIS11*	zinc finger protein	4.6	2.0
YKL159C	*RCN1*	regulator of calcineurin	4.5	4.0
YLR120C	*YPS1*	GPI-anchored aspartic protease	4.3	3.9
YNL043C		unknown	4.3	4.8
YBR071W		unknown	4.2	3.9
YGL053W	*PRM8*	pheromone-regu. membrane protein	4.2	3.7
YML118W	*NGL3*	DNase/RNase	4.0	2.9
YAR027W	*UIP3*	Ulp1 Interacting Protein 3	4.0	3.2
YML125C		protein required for cell viability	3.9	2.6
YHR138C		unknown	3.9	3.2
YMR016C	*SOK2*	transcription factor	3.8	3.3
YPR198W	*SGE1*	transporter	3.8	3.5
YOR209C	*NPT1*	nicotinate phosphoribosyltransferase	3.6	4.0
YGR268C		unknown	3.5	2.8
YMR095C	*SNO1*	unknown	3.5	2.5
YNL044W	*YIP3*	interacts with Ypt proteins	3.4	5.2
YGR144W	*THI4*	involved in thiamine biosynthesis	3.4	2.6
YMR305C	*SCW10*	glucosidase	3.4	2.2
YOL163W		unknown	3.3	2.9
YER050C	*RSM18*	structural protein of ribosome	3.2	N.D.
YOL162W		unknown	3.2	2.4
YMR007W		unknown	3.1	2.3
YDL012C		unknown	3.1	2.9
YGL156W	*AMS1*	vacuolar alpha mannosidase	3.1	3.8
YBR284W		unknown	3.1	2.3
YJL094C	*KHA1*	putative K/H antiporter	3.1	2.4
YMR020W	*FMS1*	putatitive amine oxidase	3.0	3.3
YPL056C		unknown	3.0	4.1
YDR129C	*SAC6*	actin cross-linking	3.0	2.3
YDR038C	*ENA5*	Na ATPase	3.0	3.2
YPR197C		unknown	3.0	2.5
YNL092W		unknown	2.9	1.9
YDR040C	*ENA1*	P-type ATPase	2.9	3.6
YJR078W		unknown	2.9	2.6
YPL233W		protein required for cell viability	2.9	2.9
YMR062C	*ECM40*	amino acid N-acetyltransferase	2.9	1.3
YDL010W		unknown	2.9	2.4

(Continued)

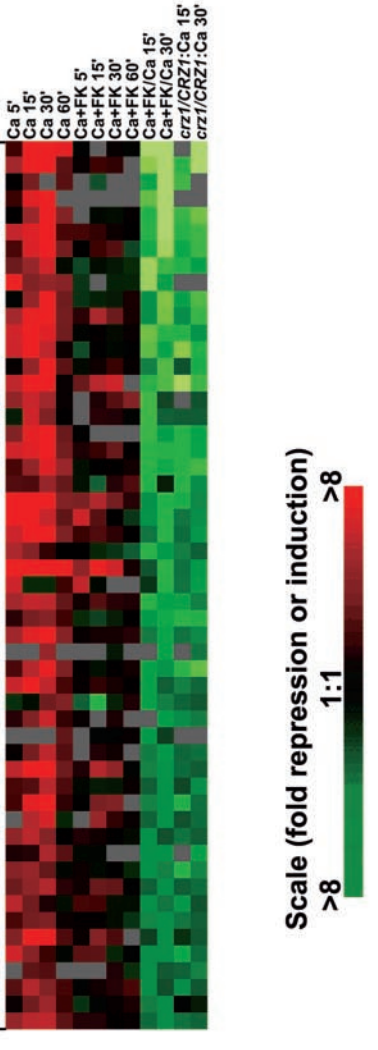


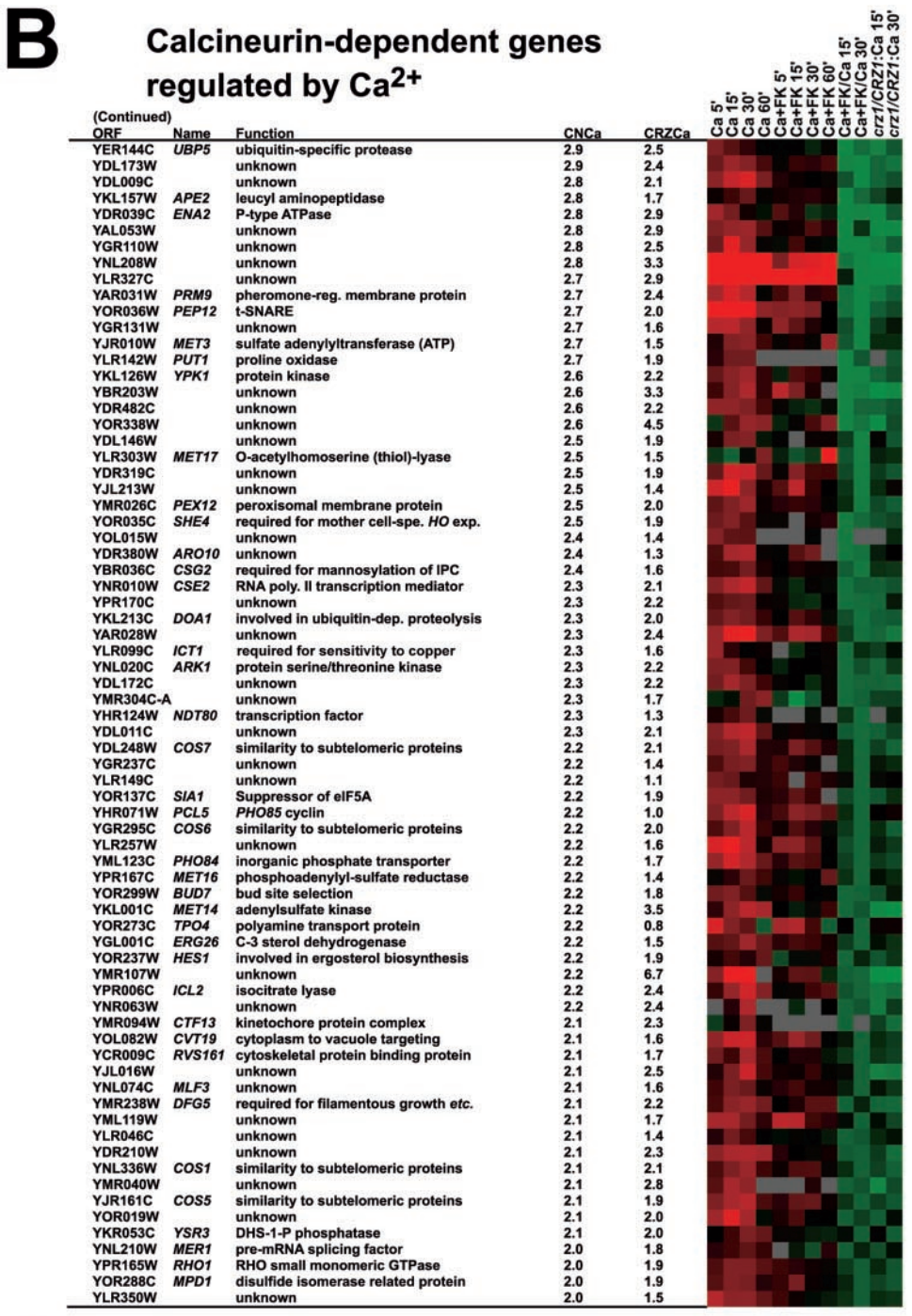
FIG. 3. **Calcineurin-dependent genes.** *A*, list of genes with calcineurin dependence score ≥2 following exposure to both Ca^{2+} and Na^{+} (CNCa and CNNa ≥2). The Crz1p dependence score (CRZCa and CRZNa) is also shown. (See text for explanation of dependence scores.) Expression data for each gene are displayed using TreeView (see legend for Fig. 2). Gene names and functions were downloaded from the Saccharomyces Genome Data base (genome-www.stanford.edu/Saccharomyces/). *B*, genes with calcineurin dependence score ≥2 following exposure to Ca^{2+} only. *C*, genes with calcineurin dependence score ≥2 following exposure to Na^{+} only.

B

Calcineurin-dependent genes regulated by Ca^{2+}

(Continued)

ORF	Name	Function	CNCa	CRZCa
YER144C	*UBP5*	ubiquitin-specific protease	2.9	2.5
YDL173W		unknown	2.9	2.4
YDL009C		unknown	2.8	2.1
YKL157W	*APE2*	leucyl aminopeptidase	2.8	1.7
YDR039C	*ENA2*	P-type ATPase	2.8	2.9
YAL053W		unknown	2.8	2.9
YGR110W		unknown	2.8	2.5
YNL208W		unknown	2.8	3.3
YLR327C		unknown	2.7	2.9
YAR031W	*PRM9*	pheromone-reg. membrane protein	2.7	2.4
YOR036W	*PEP12*	t-SNARE	2.7	2.0
YGR131W		unknown	2.7	1.6
YJR010W	*MET3*	sulfate adenylyltransferase (ATP)	2.7	1.5
YLR142W	*PUT1*	proline oxidase	2.7	1.9
YKL126W	*YPK1*	protein kinase	2.6	2.2
YBR203W		unknown	2.6	3.3
YDR482C		unknown	2.6	2.2
YOR338W		unknown	2.6	4.5
YDL146W		unknown	2.5	1.9
YLR303W	*MET17*	O-acetylhomoserine (thiol)-lyase	2.5	1.5
YDR319C		unknown	2.5	1.9
YJL213W		unknown	2.5	1.4
YMR026C	*PEX12*	peroxisomal membrane protein	2.5	2.0
YOR035C	*SHE4*	required for mother cell-spe. *HO* exp.	2.5	1.9
YOL015W		unknown	2.4	1.4
YDR380W	*ARO10*	unknown	2.4	1.3
YBR036C	*CSG2*	required for mannosylation of IPC	2.4	1.6
YNR010W	*CSE2*	RNA poly. II transcription mediator	2.3	2.1
YPR170C		unknown	2.3	2.2
YKL213C	*DOA1*	involved in ubiquitin-dep. proteolysis	2.3	2.0
YAR028W		unknown	2.3	2.4
YLR099C	*ICT1*	required for sensitivity to copper	2.3	1.6
YNL020C	*ARK1*	protein serine/threonine kinase	2.3	2.2
YDL172C		unknown	2.3	2.2
YMR304C-A		unknown	2.3	1.7
YHR124W	*NDT80*	transcription factor	2.3	1.3
YDL011C		unknown	2.3	2.1
YDL248W	*COS7*	similarity to subtelomeric proteins	2.2	2.1
YGR237C		unknown	2.2	1.4
YLR149C		unknown	2.2	1.1
YOR137C	*SIA1*	Suppressor of eIF5A	2.2	1.9
YHR071W	*PCL5*	*PHO85* cyclin	2.2	1.0
YGR295C	*COS6*	similarity to subtelomeric proteins	2.2	2.0
YLR257W		unknown	2.2	1.6
YML123C	*PHO84*	inorganic phosphate transporter	2.2	1.7
YPR167C	*MET16*	phosphoadenylyl-sulfate reductase	2.2	1.4
YOR299W	*BUD7*	bud site selection	2.2	1.8
YKL001C	*MET14*	adenylsulfate kinase	2.2	3.5
YOR273C	*TPO4*	polyamine transport protein	2.2	0.8
YGL001C	*ERG26*	C-3 sterol dehydrogenase	2.2	1.5
YOR237W	*HES1*	involved in ergosterol biosynthesis	2.2	1.9
YMR107W		unknown	2.2	6.7
YPR006C	*ICL2*	isocitrate lyase	2.2	2.4
YNR063W		unknown	2.2	2.4
YMR094W	*CTF13*	kinetochore protein complex	2.1	2.3
YOL082W	*CVT19*	cytoplasm to vacuole targeting	2.1	1.6
YCR009C	*RVS161*	cytoskeletal protein binding protein	2.1	1.7
YJL016W		unknown	2.1	2.5
YNL074C	*MLF3*	unknown	2.1	1.6
YMR238W	*DFG5*	required for filamentous growth *etc.*	2.1	2.2
YML119W		unknown	2.1	1.7
YLR046C		unknown	2.1	1.4
YDR210W		unknown	2.1	2.3
YNL336W	*COS1*	similarity to subtelomeric proteins	2.1	2.1
YMR040W		unknown	2.1	2.8
YJR161C	*COS5*	similarity to subtelomeric proteins	2.1	1.9
YOR019W		unknown	2.1	2.0
YKR053C	*YSR3*	DHS-1-P phosphatase	2.1	2.0
YNL210W	*MER1*	pre-mRNA splicing factor	2.0	1.8
YPR165W	*RHO1*	RHO small monomeric GTPase	2.0	1.9
YOR288C	*MPD1*	disulfide isomerase related protein	2.0	1.9
YLR350W		unknown	2.0	1.5



C

Calcineurin-dependent genes regulated by Na^{+}

ORF	Name	Function	CNNa	CRZNa
YLR417W	*VPS36*	defective in vacuolar protein sorting	3.3	3.3
YPR159W	*KRE6*	beta-glucan synthase	2.9	2.9
YOL011W	*PLB3*	phospholipase B	2.5	2.2
YBR004C		unknown	2.3	2.7
YPL221W	*BOP1*	bypass of PAM1	2.2	3.6
YGL183C	*MND1*	needed for Meiotic Nuclear Divisions	2.2	1.0
YJL165C	*HAL5*	Protein kinase homolog	2.1	2.8
YCR068W	*CVT17*	lipase	2.1	1.0
YDR422C	*SIP1*	protein kinase complex component	2.0	1.0
YGL052W		unknown	2.0	2.0

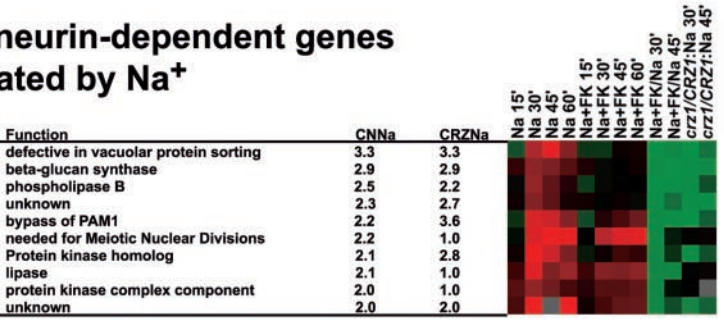


FIG. 3—*continued*

gel, and the bound fraction was localized by comparison with a parallel lane in which the CDRE sequence localized within a 60-bp DNA fragment served as probe. The bound fraction was electroeluted from the gel slice, ethanol-precipitated, and amplified by PCR as described above. Rounds 2–4 were carried out like round one except that 6 pmol of dsR60 were used in the EMSA.

Subcloning and Sequencing—Following PCR amplification of round 4, the selected molecules were digested with *Hin*dIII and *Xba*I and subcloned into pBluescript SK+ (Stratagene, La Jolla, CA) digested with the same enzymes. The resulting clones were sequenced using BigDye (PerkinElmer Life Sciences) and analyzed at the Stanford PAN Facility (Stanford University, Stanford, CA).

Conserved Motif Identification Analysis

For each gene that was expressed ≥4-fold more in wild-type than *crz1*Δ cells in at least one experiment (40 total), 800 bp of DNA sequence upstream of the ATG was analyzed using MEME (www.sdsc.edu/MEME/meme/website/meme.html) (33) and Regulatory Sequence Analysis tools (embnet.cifn.unam.mx/~jvanheld/rsa-tools/) (34) to identify common motifs. Crz1p-binding sequences identified through site selection were also analyzed using MEME.

RESULTS

Global Analysis of Calcineurin-dependent Gene Expression in Yeast—The goal of these studies was to identify, in a comprehensive manner, the genomic targets of the calcineurin/Crz1p signaling pathway. To this end, we exposed yeast to either 200 mM Ca^{2+} or 0.8 M Na^+, and we used DNA microarrays to examine the gene expression of all of the open reading frames nominally encoded by the *S. cerevisiae* genome (see "Experimental Procedures"). The DNA microarray experiments performed in this study are summarized in Table I. First, to identify genes regulated by Ca^{2+} or Na^+, we characterized the pattern of gene expression at several times from 5 to 60 min after exposure of yeast to these ions. In each case the Ca^{2+} or Na^+-treated sample was compared with the same culture prior to ion addition. Second, to identify the specific subset of genes that are regulated by the calcineurin signaling pathway, we carried out the same time course analysis using cells treated with FK506, a specific inhibitor of calcineurin, prior to the addition of either Na^+ or Ca^{2+} (see "Experimental Procedures"). Third, we directly compared the transcriptional response of FK506-treated cells to that of non-drug-treated cells after Ca^{2+} or Na^+ addition. Finally, to examine the role of the Crz1p transcription factor, we compared the transcriptional response of a *crz1* strain (YHY804) to that of its isogenic wild-type parent (DBY7286) after treatment with Ca^{2+} or Na^+. Data collected from all of these experiments were organized by hierarchical clustering. This method groups genes according to their similarity in expression pattern, such that genes that display the most similar patterns throughout the data set are arranged together (32). The clustered data were then graphically displayed using the TreeView program (Fig. 2) (32) (see "Experimental Procedures").

An overview of the DNA microarray results is presented in Fig. 2. Of the 6,166 genes that could be scored, expression of 2,523 (41%) was altered at least 2-fold in two or more experiments. These genes were selected for further analyses. Some general patterns are apparent from visual inspection of these data. First, cells exposed to Ca^{2+} showed significant changes in gene expression by 5 min (974 genes). The number of genes showing ≥2-fold changes reached a maximum at 15 min (1509 genes) and then declined slightly at 30 min (1176 genes) and further at 60 min (287 genes). Thus, samples taken 15 and 30 min after Ca^{2+} addition showed the greatest overall change in gene expression. For this reason, when comparing FK506-treated *versus* non-treated cells and *crz1 versus* wild-type cells after exposure to Ca^{2+}, we restricted our analysis to these time points. In contrast, the number of genes whose transcript levels changed by ≥2-fold within 15 min of transfer to high Na^+ medium was considerably smaller (189 genes). More changes were observed by 30 min (1219 genes), and overall changes in gene expression peaked at 45 min (1493 genes) and then decreased by 60 min (1265 genes). Thus, in further characterizations of Na^+-treated cells, we analyzed changes only at the 30- and 45-min time points.

Inspection of this full data set reveals several characteristics of the calcineurin-dependent gene cluster. First, only a small subset of the genes whose transcript levels change in response to Ca^{2+} or Na^+ is calcineurin-dependent (Fig. 2). Second, under these conditions, calcineurin mainly mediates activation of gene expression rather than repression, as the most prominent differences in FK506-treated samples reflect decreased expression levels. Third, there is notable similarity in the gene expression patterns of FK506-treated cells and *crz1* cells, suggesting that Crz1p is required for most calcineurin-dependent changes in gene expression. Finally, the magnitude and number of calcineurin-dependent changes in gene expression is significantly greater in response to Ca^{2+} treatment than Na^+ treatment, although this is not the case for the overall changes as noted above. These points will be discussed further below. Our entire data set is available at www.stanford.edu/group/cyert/microarray.html.

Identification of Genes Induced by Ca^{2+} or Na^+ in a Calcineurin-dependent Manner—We used two criteria to identify genes whose induction by Ca^{2+} or Na^+ was dependent on calcineurin. First, we identified genes whose expression was increased more than 2-fold by Ca^{2+} or Na^+ in the time course experiments (experiments 1 and 3, see Table I). In total, 934 genes were induced more than 2-fold by Ca^{2+} at either 15 or 30 min, and 931 total genes were induced more than 2-fold by Na^+ at 30 or 45 min. Second, we assessed the extent to which the expression of each of these genes was reduced by calcineurin inhibition by direct comparison of FK506-treated and non FK506-treated cells exposed to Ca^{2+} or Na^+ (experiments 5 and 6, see Table I). Specifically, we defined the Green/Red expression ratio from the Ca^{2+} + FK506/Ca^{2+} or Na^+ + FK506/Na^+ DNA microarrays as a calcineurin dependence score (CNCa, and CNNa, respectively, see Fig. 3), where the green-labeled sample corresponds to non-drug-treated cells and the red-labeled sample corresponds to FK506-treated cells. For these analyses we sampled two different time points, 15 and 30 min for Ca^{2+} and 30 and 45 min for Na^+, and the larger of these two values was used to represent the calcineurin dependence of each gene. Genes with a calcineurin dependence score of ≥2 were considered to be calcineurin-regulated.

By using these criteria we identified a set of 163 genes whose expression we term calcineurin-dependent (Fig. 3). 153 genes displayed increased expression in response to Ca^{2+} that was reduced 2–30-fold in cells treated with FK506. 38 genes displayed calcineurin-dependent induction in response to Na^+ and showed a 2–10-fold decrease in expression under conditions of calcineurin inhibition. Thus, the calcineurin-dependent transcriptional program differs in Ca^{2+}- and Na^+-treated cells. The calcineurin-dependent genes fell into 3 different groups: 28 genes were more than 2-fold dependent on calcineurin under both conditions (Ca^{2+} and Na^+) (Fig. 3*A*). Another group of genes (125) displayed calcineurin-dependent activation of expression only in response to Ca^{2+} addition (Fig. 3*B*). A final group of genes (10) displayed calcineurin-dependent gene activation only in response to Na^+ addition (Fig. 3*C*). These calcineurin-regulated genes include some known to function in signaling pathways, ion homeostasis/small molecule transport, cell wall maintenance, and vesicular transport, as well as many open reading frames of unknown function (see "Discussion").

The genes identified here as calcineurin-dependent include previously identified transcriptional targets of calcineurin such as *PMC1* and *ENA1*. However, other genes also known to show calcineurin-dependent gene expression, such as *FKS2* and *PMR1*, were not identified using these criteria. Thus, the statistical thresholds used in our data analyses allow identification of many, but not all, genes with relevant *in vivo* regulation by calcineurin.

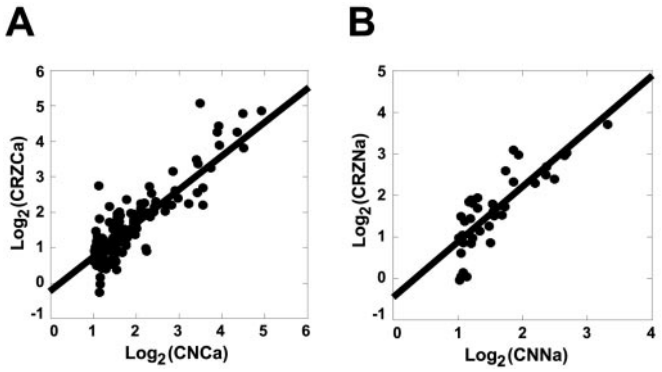


FIG. 4. **Induction of calcineurin-regulated genes depends on Crz1p.** *A*, scatter plot comparing the calcineurin dependence score for Ca^{2+} and the Crz1p dependence score for Ca^{2+}. Log_2(CNCa) and log_2(CrzCa) values for all genes with a calcineurin dependence score of ≥2 are plotted. *B*, scatter plot comparing the calcineurin dependence score for Na^+ and the Crz1p dependence score for Na^+. Log_2(CNNa) and log_2(CrzNa) values for all genes with a calcineurin dependence score of ≥2 are plotted.

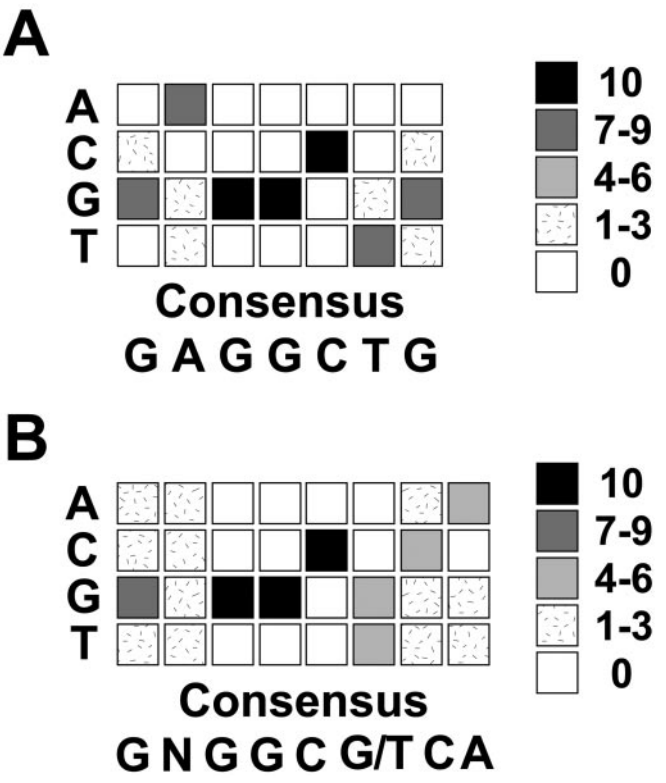


FIG. 5. **Crz1p binding consensus motif derived from analysis of calcineurin-dependent genes and *in vitro* site selection.** In each panel, the letter-probability matrix of the Crz1p-binding motif derived by MEME is shown. The scale, indicated by the degree of *shading*, corresponds to the probability of each possible base occurring at each position of the motif multiplied by 10 and rounded to the nearest integer. *A*, motif derived from analysis of 500 bp of upstream sequence of 40 genes identified in microarray experiments that displayed ≥4-fold Crz1-dependent expression following exposure to Ca^{2+} and/or Na^+. *B*, motif derived from analysis of 43 sequences identified by binding site selection-amplification (see "Experimental Procedures").

Requirement for Crz1p in Calcineurin-dependent Gene Regulation—Crz1p is the sole identified transcription factor in *S. cerevisiae* whose activity is regulated by calcineurin. We compared the transcriptional profiles of FK506-treated cells and *crz1*Δ cells to determine whether Crz1p was required for all calcineurin-dependent activation of gene expression. We compared the genomic expression patterns of *CRZ1* and *crz1*Δ strains after exposure to Ca^{2+} (15 and 30 min) or Na^+ (30, 45 min) (Figs. 2–4). For each calcineurin-dependent gene, the expression ratio in the *CRZ1 versus crz1*Δ strain at each of two time points was calculated. The higher of the two values is shown in Fig. 3 as a Crz1p dependence score (Crz1Ca, Crz1Na). 71% (116/163) of calcineurin-dependent genes had a Crz1p dependence score ≥2, and genes that had a lower Crz1p dependence score (*i.e.* less than 2) were among those that showed the lowest calcineurin dependence. Consistent with this observation, visual comparison of gene expression patterns revealed that calcineurin-dependent genes were similarly perturbed by FK506 treatment and mutational inactivation of *crz1* (Fig. 2). Finally, direct comparison of the calcineurin dependence score of 153 calcineurin-regulated genes during Ca^{2+} treatment (log_2CNCa) to the Crz1p-dependence score (log_2CrzCa) showed a linear relationship (Fig. 4*A*). Similarly, direct comparison of the calcineurin dependence score of 38 calcineurin-regulated genes during Na^+ treatment (log_2CNNa) to the Crz1p dependence score (log_2CrzNa) showed a linear relationship (Fig. 4*B*). These data strongly indicate that Crz1p is the major effector of calcineurin-regulated gene expression in yeast.

Promoter Analysis of Crz1p-dependent Genes—If the calcineurin-dependent genes identified above are regulated directly by Crz1p, they should contain a DNA-binding site for Crz1p. The promoters of 44 genes showing 4-fold or greater Crz1p-dependent expression after either Ca^{2+} or Na^+ treatment were analyzed using the MEME program to identify potential common regulatory sequences in these genes (33). This analysis identified a statistically significant motif present in the promoters of Crz1p-dependent genes, 5′-GAGGCTG-3′ (Fig. 5*A*). This motif shows considerable similarity to a sequence, 5′-GTGGCTG-3′, found in the CDRE, a 24-bp element to which Crz1p binds (see below). Studies presented below confirm that the motif identified in promoters of calcineurin/Crz1p-dependent genes corresponds to the core Crz1p DNA-binding site. This finding is consistent with direct regulation of these genes by Crz1p.

Experimental Determination of Crz1-binding Site, Methylation Interference Analysis of GST-Crz1p Binding to the CDRE—The CDRE is a 24-bp DNA element derived from the *FKS2* promoter that is necessary and sufficient for calcineurin/Crz1p-mediated transcriptional activation (14). Previous studies established that Crz1p is required for and is a component of a CDRE binding activity identified in yeast extracts by EMSA (14). To characterize further Crz1p binding to DNA, we first determined that purified GST-Crz1p produced in *E. coli* binds directly to the CDRE as monitored by EMSA (data not shown, see "Experimental Procedures"). Next we used methylation interference to identify specific bases within the CDRE required for Crz1p binding (see "Experimental Procedures"). We observed that methylation of guanine and adenine residues in the central portion of the CDRE, 5′-GGTGGCTGTGC-3′, interfered with Crz1p binding (Fig. 6). These results establish that Crz1p forms specific DNA contacts in this region of DNA. Equivalent results were obtained when the source of Crz1p used in the experiment was from yeast extracts (data not shown).

Binding Site Selection-Amplification Analysis—To determine the sequence specificity of Crz1p binding to DNA, we used a binding site selection-amplification assay (35). A pool of oligonucleotides containing a core 20-bp region of random sequence was used as the binding substrate (see "Experimental Procedures"). After four rounds of binding and amplification, the selected oligonucleotides could be detected by gel mobility shift assay (data not shown). The amplified products were cloned, and 45 individual clones were sequenced (data not

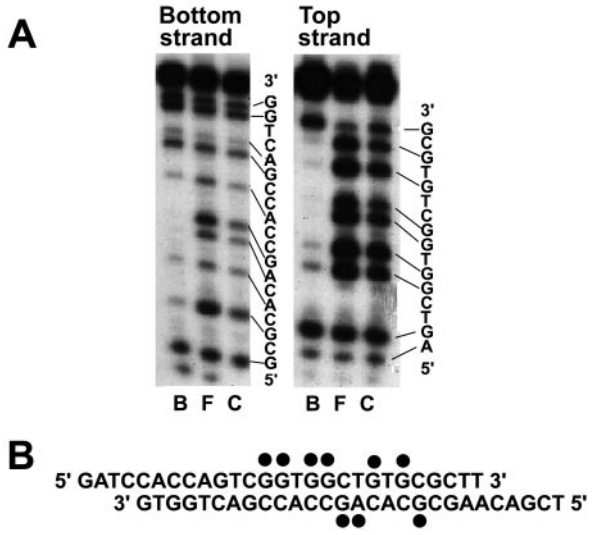


FIG. 6. **Methylation interference analysis of Crz1p DNA binding specificity.** *A*, the bottom strand (*left*) and top strand (*right*) of the CDRE sequence of *FKS2* were used as probes in a methylation interference experiment. DNA probes were modified to a limited degree with dimethyl sulfate and incubated with Crz1p. The unbound fraction of probe DNA was separated from the protein-bound probe fraction by a gel retardation assay. The DNA probe present in the unbound and bound fractions was excised, cleaved, and electrophoresed on a sequencing gel, and the dried gel was autoradiographed. *B*, cleavage products of DNA probe molecules that bound protein; *F*, cleavage products of unbound DNA probe; *C*, control lane in which probe was incubated in the absence of protein and processed in the same manner as the other samples. The DNA sequence is displayed on the right side of each panel. *B*, methylation interference pattern. The methylation interference data are taken from *A*. Sites of strong methylation interference are indicated by *filled circles*.

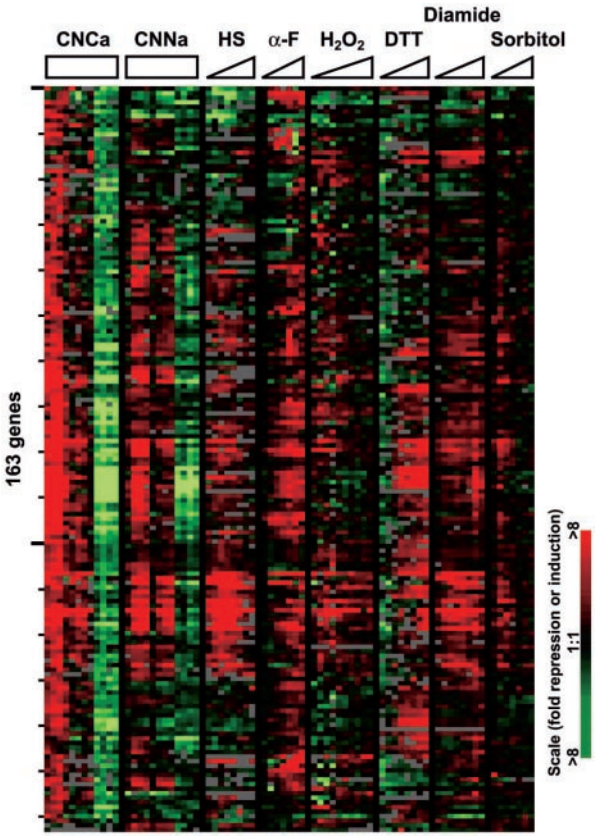


FIG. 7. **Expression profiles of 163 highly calcineurin-dependent genes in response to environmental stress.** Data from the experiments described here were compiled with published data and analyzed using Cluster and TreeView (32). Experiments labeled "CNCa" and "CNNa" are as described in Table I and are presented in the same order as in Fig. 2. Heat shock, H_2O_2, diamide, and sorbitol data were collected as described previously (37), and expression in response to α-factor was determined as described previously (36).

shown). From these 45 clones, a consensus sequence was identified using the MEME program (33). As shown in Fig. 5*B*, this analysis determined the core consensus DNA-binding site for Crz1p as 5′-GNGGC(G/T)CA-3′. 43/45 sequences identified in the analysis contained one or more copies of this sequence. G and T at the 6th position were equally represented. This binding site is consistent with the DNA contact region observed in the DNA methylation experiments. Furthermore, we found that CDRE oligonucleotides that contained any base substitution in "GGCT" portion of the binding site eliminated binding of GST-Crz1p as determined by EMSA, with the exception of the T to G change (data not shown).

Activation of Calcineurin-dependent Genes by Additional Environmental Conditions—We compared the gene expression data we compiled for highly calcineurin-dependent genes with previously published data showing the response of yeast to elevated temperature, α-factor, H_2O_2, DTT, diamide, and sorbitol (36, 37). As shown in Fig. 7, subsets of the 163 genes identified as highly calcineurin-dependent were induced more than 2-fold in response to these different environmental conditions. Calcineurin signaling is active under conditions of elevated temperature and incubation with α-factor (13, 14, 38) and may also be active under the other conditions. Alternatively, expression of this set of genes may be activated under many different conditions and may be under the control of several distinct signaling pathways.

DISCUSSION

The goal of these studies was to conduct a genome-wide survey of calcineurin-regulated gene expression in yeast. Ca^{2+}/calcineurin-mediated gene expression is required for yeast cell viability during a variety of stress conditions, including exposure to several ions (reviewed in Ref. 12). Here we describe genomic targets of this regulation. Specifically, we identified 163 genes that displayed a ≥2-fold increase in expression following exposure to 200 mM Ca^{2+} or 0.8 M Na^{+} which was reduced by at least 50% by the calcineurin inhibitor, FK506. The products of these calcineurin-dependent genes participate in a variety of cellular processes, and 44% of the genes encode proteins of previously unknown function. Consistent with the role of calcineurin in providing tolerance to ionic stress, many of the genes encode proteins that function in small molecule transport or ion homeostasis. Examples of these include *MEP1*, *ENB1*, *PHO84*, *PHO89*, *KHA1*, and *TPO4* and the P-type ATPases *PMC1* and *ENA1*, *ENA2*, and *ENA5* (Fig. 3). Other genes whose expression is regulated by calcineurin/Crz1 contribute to cell wall synthesis/maintenance (*CHS1*, *CRH1*, *RHO1*, *SCW10*, and *KRE6*), lipid and sterol metabolism (*SUR1*, *CSG2*, *YSR3*, *ERG26*, *HES1*, and *PLB3*), and vesicle transport (*GYP7*, *YPT53*, *YIP3*, *PEP12*, *RVS161*, *SHE4*, *CVT17*, *CVT19*, and *VPS36*). Calcineurin-dependent regulation of these genes may help preserve cell wall and membrane function during stress and stimulate delivery of new components to the cell surface. The expression of several genes encoding degradative enzymes is also regulated by calcineurin (*CPS1*, *PRB1*, *APG5*, *YPS1*, *AMS1*, *UBP5*, and *APE2*) and may contribute to stress-induced remodeling of cellular components. Finally, activation of calcineurin/Crz1 leads to increased expression of several genes encoding components Ca^{2+}-dependent (*CMK2* and *RCN1*) and Ca^{2+}-independent signaling pathways (*YPK1*, *ARK1*, *SIP1*, *CUP2*, *SMP1*, *CSE2*, *TIS11*, *SOK2*, and *NDT80*). This regulation suggests possible mechanisms of feedback and cross-talk

between calcineurin and other signal transduction pathways.

Genetic and molecular studies previously identified a small number of calcineurin-regulated transcriptional targets: *FKS2*, which encodes $\beta-1,3$-glucan synthase; the P-type ATPases *PMC1*, *PMR1*, and *ENA1*; the calcineurin regulator *RCN1*; and the calcineurin-regulated transcription factor *CRZ1/TCN1* (13, 16, 24, 26, 39). Our studies failed to identify *FKS2*, *PMR1*, and *CRZ1* as calcineurin-dependent genes, because the magnitude of their transcriptional changes fell below the threshold levels defined as significant; the calcineurin dependence scores (CNCa) for these genes were 1.8, 1.1, and 1.8, respectively. Previous reports (14, 16) also determined *CRZ1*, *PMR1*, and *FKS2* expression to be less dependent on calcineurin than that of *PMC1* and *ENA1*, which were identified in our study. Nonetheless, it is important to note that the 163 genes classified here as calcineurin-dependent may somewhat underestimate the physiologically relevant gene expression changes controlled by Ca^{2+}/calcineurin-dependent signaling. An earlier genomic study identified 36 genes whose expression was decreased ≥2-fold by FK506 (40), and most of these genes were also identified by our analyses. In that study calcineurin-dependent signaling was activated by growing yeast in media containing 10 mM $CaCl_2$, which results in a relatively small induction of calcineurin-dependent transcription. This difference in culture conditions likely explains why we identified a larger number of transcriptional targets.

Calcineurin effects changes in gene expression by dephosphorylating the Crz1p transcription factor and regulating its activity and subcellular localization (17–19). In this report we characterized the DNA binding specificity of Crz1p. A 24-bp sequence in the promoter of *FKS2*, the CDRE, was shown previously (14) to be both necessary and sufficient to direct calcineurin- and Crz1p-dependent transcriptional activation. Here we identified residues within the CDRE that are critical for Crz1p binding using methylation interference. We also performed binding site selection and amplification to establish the sequence specificity of Crz1p DNA binding. These analyses establish the Crz1p binding sequence as 5′-GNGGC(G/T)CA-3′. Consistent with these findings, a recent study identified two Crz1p-binding sites in the promoter of the *ENA1* gene (41). One of these, 5′-GAAT**GGCT**G-3′ was a low affinity site and fits the Crz1p binding consensus less well than the other site, 5′-GG**GTGGCT**G-3′, to which Crz1p bound with high affinity. Interestingly, Ace2p and Swi5p, two transcription factors whose zinc fingers are very similar to those found in Crz1p, bind to a distinct core site, 5′-GCTGGT-3′ (14–16, 42, 43).

Mutational inactivation of either calcineurin or Crz1p causes similar growth defects, suggesting that many of the physiological functions of calcineurin are mediated by Crz1-dependent transcription. The analyses presented here show a strong correspondence between gene expression changes resulting from either calcineurin inhibition or Crz1p inactivation (Fig. 4). Specifically, the expression of 75/76 genes with a calcineurin dependence score of ≥3 was decreased 2-fold or more in *crz1*Δ cells compared with wild-type cells (Fig. 3). The one exception, *SMP1*, showed a 1.9-fold expression decrease in *crz1*Δ cells. Furthermore, of the 163 genes described here as calcineurin-dependent, only 4, *TPO4*, *MND1*, *CVT17,* and *SIP1*, fail to show Crz1p dependence. These observations indicate that Crz1p is the major and perhaps the only effector of calcineurin-dependent gene expression in yeast. Promoter analysis of genes determined to be Crz1p-dependent in the microarray studies identified a common sequence, 5′-GAGGCTG-3′ which is similar to the experimentally determined Crz1p-binding site, 5′-GNGGC(G/T)CA-3′. When these motifs are compared, their common elements specify a core Crz1p-binding sequence, 5′-GNGGC(G/T)-3′, which appears in the upstream regions of 146/163 calcineurin-dependent genes and is present in 1–6 copies per promoter. Thus, these genes are likely to be direct transcriptional targets of Crz1p.

Activation of calcineurin/Crz1p signaling by Ca^{2+} or Na^+ resulted in distinct transcriptional responses. After Na^+ addition, the expression of calcineurin-dependent genes increased more slowly than in response to Ca^{2+} and reached lower maximal levels of induction. Also different subsets of genes were expressed under the two conditions. Of 163 calcineurin-dependent genes, 28 were induced in response to both Ca^{2+} and Na^+ addition; 135 were induced only in response to Ca^{2+}, and 10 were induced only in response to Na^+. In particular, several genes, such as *RTA1*, *YCR007c*, *YLL057c*, *YLR054c*, *YNR064c*, and *PMC1*, displayed highly calcineurin-dependent expression following Ca^{2+} addition but were not calcineurin-regulated in response to Na^+ treatment (Fig. 3*B*). Distinct transcriptional responses to Ca^{2+} and Na^+ could be generated by activation of Crz1p in combination with different sets of additional transcription factors. Promoter analysis failed to uncover evidence of such auxiliary factors, however, because the Crz1p-binding consensus site was the sole common sequence motif identified for each class of calcineurin-dependent genes. Alternatively, different transcriptional outcomes could result from differences in the amount of active Crz1p generated under the two conditions. Several observations suggest that exposure to 200 mM Ca^{2+} leads to more extensive calcineurin/Crz1p signaling than does exposure to 0.8 M Na^+. First, addition of Ca^{2+} to growth media causes an immediate rise in the level of Ca^{2+} in the yeast cytosol, which peaks at 4–10-fold above resting levels and then declines over the following 1–2 min through vacuolar sequestration (44, 45). In contrast, addition of 0.8 M Na^+ causes a much smaller cytosolic Ca^{2+} transient, which also develops more slowly, reaching its peak value after 1 min (44). Second, calcineurin-dependent translocation of Crz1p to the nucleus differs substantially under these two conditions. Exposure to 200 mM Ca^{2+} causes complete nuclear translocation of Crz1p in 100% of cells within 10 min, whereas exposure to 0.8 M Na^+ causes only partial nuclear translocation, which also occurs more slowly (17). Thus, Na^+-treated cells may have a smaller pool of activated nuclear Crz1p, causing only a portion of the maximum possible Crz1p-binding sites to be occupied. The promoters of Crz1p-dependent genes that are efficiently expressed in response to both Na^+ and Ca^{2+} may have a higher affinity for Crz1p, possibly due to the sequence or distribution of their Crz1p-binding sites. In support of this idea, we noted that calcineurin-dependent genes displayed different induction kinetics following Ca^{2+} addition. Some genes displayed their peak expression at 5 min, with reduced expression at 15 and 30 min after Ca^{2+} addition, whereas others displayed higher levels of expression at 15 and 30 min than at 5 min (data not shown). The genes that were induced most rapidly following Ca^{2+} addition were those that were also induced in response to Na^+ treatment, consistent with the idea that their promoters have a higher affinity for Crz1p *in vivo*. This model, however, fails to explain the small number of calcineurin-dependent genes that were activated in response to Na^+ but not Ca^{2+} (Fig. 3*C*). These genes were only modestly calcineurin-dependent, and 3/10 were not Crz1p-dependent. Therefore, they may represent an exceptional class of genes that are regulated by calcineurin through more indirect mechanisms.

Our studies reveal that exposure of yeast to either 200 mM Ca^{2+} or 0.8 M Na^+ results in massive changes in the global pattern of yeast gene expression. Similar studies, using a variety of stress conditions, established that yeast undergo a common transcriptional response to stress, termed the environ-

mental stress response that involves ~10% of the yeast genome (37, 46). Much of this general response to stress is mediated by the Msn2p/Msn4p transcription factors. In contrast, we found that a small set of genes was under control of the calcineurin/Crz1p signal transduction pathway. Identification of calcineurin-dependent genes establishes a molecular fingerprint for calcineurin activity in yeast. Comparison of our data with other published data indicates that subsets of calcineurin-dependent genes are activated under several environmental conditions, including heat shock, exposure to α-factor, H_2O_2, diamide, sorbitol, and DTT treatment (Fig. 7) (36, 37). These observations confirm earlier studies showing that calcineurin-dependent signaling is activated by elevated temperature and prolonged exposure to α-factor (13, 14, 38) and suggest additional conditions that may turn on this pathway. Many calcineurin-dependent genes are also induced in response to alkali shock (46), which is consistent with the sensitivity of calcineurin mutants to alkali conditions (26, 47). A greater understanding of the physiological circumstances under which calcineurin-mediated signaling is active may lead to additional insights into the molecular mechanisms that govern this pathway *in vivo*.

In summary, these studies have identified an array of genes that are induced by Ca^{2+}/calcineurin/Crz1p-mediated signaling and establish that different environmental conditions trigger distinct transcriptional responses through this signal transduction pathway. In the future we plan to characterize further the products of these calcineurin-dependent genes and to elucidate their role in the yeast response to environmental stress.

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REFERENCES

1. Aramburu, J., Rao, A., and Klee, C. B. (2000) *Curr. Top. Cell. Regul.* **36,** 237–295
2. Liu, J. (1993) *Immunol. Today* **14,** 290–295
3. Clipstone, N. A., and Crabtree, G. R. (1992) *Nature* **357,** 695–697
4. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., and O'Neill, E. A. (1992) *Nature* **357**, 692–694
5. Hendey, B., Klee, C. B., and Maxfield, F. R. (1992) *Science* **258,** 296–299
6. Lawson, M. A., and Maxfield, F. R. (1995) *Nature* **377,** 75–79
7. Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999) *Science* **284,** 339–343
8. Molkentin, J., Lu, J., Antos, C., Markham, B., Richardson, J., Robbins, J., Grant, S., and Olson, E. (1998) *Cell* **93,** 215–228
9. Mansuy, I. M., Mayford, M., Jacob, B., Kandel, E. R., and Bach, M. E. (1998) *Cell* **92,** 39–49
10. Graef, I. A., Chen, F., Chen, L., Kuo, A., and Crabtree, G. R. (2001) *Cell* **105,** 863–875
11. Crabtree, G. R. (2001) *J. Biol. Chem.* **276,** 2313–2316
12. Cyert, M. S. (2001) *Annu. Rev. Genet.* **35,** 647–672
13. Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Clemas, J. A., Nielsen, J. B., and Foor, F. (1995) *Mol. Cell. Biol.* **15,** 5671–5681
14. Stathopoulos, A. M., and Cyert, M. S. (1997) *Genes Dev.* **11,** 3432–3444
15. Mendizabal, I., Rios, G., Mulet, J. M., Serrano, R., and de Larrinoa, I. F. (1998) *FEBS Lett.* **425,** 323–328
16. Matheos, D., Kingsbury, T., Ahsan, U., and Cunningham, K. (1997) *Genes Dev.* **11,** 3445–3458
17. Stathopoulos-Gerontides, A., Guo, J., and Cyert, M. S. (1999) *Genes Dev.* **13,** 798–803
18. Boustany, L., and Cyert, M. S. (2002) *Genes Dev.* **16,** 608–619
19. Polizotto, R., and Cyert, M. S. (2001) *J. Cell Biol.* **154,** 951–960
20. Cunningham, K. W., and Fink, G. R. (1994) *J. Cell Biol.* **124,** 351–363
21. Durr, G., Strayle, J., Plemper, R., Elbs, S., Klee, S. K., Catty, P., Wolf, D. H., and Rudolph, H. K. (1998) *Mol. Biol. Cell* **9,** 1149–1162
22. Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Dorman, T. E., LeVitre, J., Davidow, L. S., Mao, J. I., and Moir, D. T. (1989) *Cell* **58,** 133–145
23. Mandal, D., Woolf, T. B., and Rao, R. (2000) *J. Biol. Chem.* **275,** 23933–23938
24. Cunningham, K., and Fink, G. R. (1996) *Mol. Cell. Biol.* **16,** 2226–2237
25. Haro, R., Graciadebles, B., and Rodriguez-Navarro, A. (1991) *FEBS Lett.* **291,** 189–191
26. Mendoza, I., Rubio, F., Rodriguez-Navarro, A., and Pardo, J. M. (1994) *J. Biol. Chem.* **269,** 8792–8796
27. Marquez, J. A., and Serrano, R. (1996) *FEBS Lett.* **382,** 89–92
28. Ferea, T. L., Botstein, D., Brown, P. O., and Rosenzweig, R. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9721–9726
29. Guldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. (1996) *Nucleic Acids Res.* **24,** 2519–2524
30. Spellman, P. T., Sherlock, G., Zhang, M. Q., Iyer, V. R., Anders, K., Eisen, M. B., Brown, P. O., Botstein, D., and Futcher, B. (1998) *Mol. Biol. Cell* **9,** 3273–3297
31. DeRisi, J. L., Iyer, V. R., and Brown, P. O. (1997) *Science* **278,** 680–686
32. Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14863–14868
33. Bailey, T. L., and Elkan, C. (1994) *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **2,** 28–36
34. van Helden, J., Andre, B., and Collado-Vides, J. (2000) *J. Biol. Chem.* **16,** 177–187
35. Overdier, D. G., Porcella, A., and Costa, R. H. (1994) *Mol. Cell. Biol.* **14,** 2755–2766
36. Roberts, C. J., Nelson, B., Marton, M. J., Stoughton, R., Meyer, M. R., Bennett, H. A., He, Y. D., Dai, H., Walker, W. L., Hughes, T. R., Tyers, M., Boone, C., and Friend, S. H. (2000) *Science* **287,** 873–880
37. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) *Mol. Biol. Cell* **11,** 4241–4257
38. Zhao, C., Jung, U. S., Garrett-Engele, P., Roe, T., Cyert, M. S., and Levin, D. E. (1998) *Mol. Cell. Biol.* **18,** 1013–1022
39. Kingsbury, T. J., and Cunningham, K. W. (2000) *Genes Dev.* **14,** 1595–1604
40. Marton, M. J., DeRisi, J. L., Bennett, H. A., Iyer, V. R., Meyer, M. R., Roberts, C. J., Stoughton, R., Burchard, J., Slade, D., Dai, H., Bassett, D. E., Jr., Hartwell, L. H., Brown, P. O., and Friend, S. H. (1998) *Nat. Med.* **4,** 1293–1301
41. Mendizabal, I., Pascual-Ahuir, A., Serrano, R., and de Larrinoa, I. F. (2001) *Mol. Genet. Genom.* **265,** 801–811
42. Bohm, S., Frishman, D., and Mewes, H. W. (1997) *Nucleic Acids Res.* **25,** 2464–2469
43. Dohrmann, P. R., Voth, W. P., and Stillman, D. J. (1996) *Mol. Cell. Biol.* **16,** 1746–1758
44. Denis, V., and Cyert, M. S. (2002) *J. Cell Biol.* **156,** 29–34
45. Miseta, A., Kellermayer, R., Aiello, D. P., Fu, L., and Bedwell, D. M. (1999) *FEBS Lett.* **451,** 132–136
46. Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S., and Young, R. A. (2001) *Mol. Biol. Cell* **12,** 323–337
47. Nakamura, T., Liu, Y., Hirata, D., Namba, H., Harada, S., Hirokawa, T., and Miyakawa, T. (1993) *EMBO J.* **12,** 4063–4071