Direct Regulation of Genes Involved in Glucose Utilization by the Calcium/Calcineurin Pathway*3

Received for publication, October 19, 2007, and in revised form, February 13, 2008. Published, JBC Papers in Press, March 24, 2008, DOI 10.1074/jbc.M708683200

Amparo Ruiz‡, Raquel Serrano†, and Joaquín Ariño‡§1

From the ‡Departament de Bioquímica i Biologia Molecular and §Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Edifici V, Campus de Bellaterra, Cerdanyola, Barcelona 08193, Spain

Failure to use glucose as carbon source results in transcriptional activation of numerous genes whose expression is otherwise repressed. HXT2 encodes a yeast high affinity glucose transporter that is only expressed under conditions of glucose limitation. We show that HXT2 is rapidly and potently induced by environmental alkalinization, and this requires both the Snf1 and the calcineurin pathways. Regulation of calcineurin is mediated by the transcription factor Crz1, which rapidly translocates to the nucleus upon high pH stress, and acts through a previously unnoticed Crz1-binding element (calcineurin-dependent response element) in the HXT2 promoter (−507 GGGGCTG −501). We demonstrate that, in addition to HXT2, many other genes required for adaptation to glucose shortage, such as HXT7, MDH2, or ALD4, transcriptionally respond to calcium and high pH signaling through binding of Crz1 to their promoters. Therefore, calcineurin-dependent transcriptional regulation appears to be a common feature for many genes encoding carbohydrate-metabolizing enzymes. Remarkably, extracellular calcium allows growth of a snf1 mutant on low glucose in a calcineurin/Crz1-dependent manner, indicating that activation of calcineurin is sufficient to override a major deficiency in the glucose-repression pathway. We propose that alkalinization of the medium results in impaired glucose utilization and that activation of certain glucose-metabolizing genes by calcineurin contributes to yeast survival under this stress situation.

Glucose is the major carbon and energy source for most cells, and it is by far the preferred carbon source for the budding yeast Saccharomyces cerevisiae. In this organism glucose uptake is the limiting step in the utilization of this sugar, and the relevance of glucose to yeast metabolism is highlighted by the unusually large number of hexose transporter genes present in its genome (see Refs. 1 and 2 for reviews). These proteins transport their substrates by passive, gradient-dependent, energy-independent diffusion. At least six of them (Hxt1–7) have been shown to act as glucose transporters, whereas others are considered to transport galactose (Gal2) or other hexoses (Hxt5 and Hxt8–17) (1, 3).

These diverse glucose transporters exhibit different kinetic characteristics, and each of them appears particularly suited for a specific circumstance. For instance, Hxt1 is a low affinity, high capacity transporter, whereas Hxt2, Hxt6, and Hxt7 are examples of high affinity glucose transporters. Experimental evidence indicates that a strain lacking Hxt1–7 (often denominated as hxt null mutant) is unable to grow on glucose or other hexoses such as fructose or mannose (4, 5). Expression of HXT2, HXT6, and HXT7 allows growth of the hxt strain on low (0.1%) glucose, whereas other transporters are unable to do so (6). This confirms the role of these genes as high affinity glucose transporters, even though the major physiological role for glucose uptake under glucose shortage conditions can be attributed to Hxt2 (2).

The expression pattern of the different glucose transporters is clearly related to their intrinsic characteristics. For instance, HXT2 is expressed at low levels both in the absence of glucose and in the presence of high amounts of this sugar (when a high affinity transporter would not be needed), but its expression increases by 10-fold when low levels (0.1%) of glucose or fructose are available. This transcriptional regulation is physiologically relevant, and it is the result of a complex interaction (7) between at least two different pathways: the Snf3/Rgt2-Rgt1 and the Snf1-Mig1 pathways (see Refs. 8 and 9 for recent reviews). It has been recently pointed out that Rgt1 function could also be modulated by activation of the Gpr1/PKA2 path-way (10). The HXT2 promoter contains two Rgt1 binding sites and two Mig1 binding sites (11). Under complete glucose deficiency, Rgt1 can bind to the HXT2 promoter, repressing HXT2 expression. On the other hand, activation of the Snf1 protein kinase under these circumstances results in release of the Mig1 repressor and induction of HXT2. The interconnection of both pathways ensures that HXT2 is expressed only when the levels of extracellular glucose are low. In fact, Snf1 represents a key player in the process of adaptation to glucose shortage, because activation of this kinase allows de-repression not only of HXT2, but also of many genes required for gluconeogenesis, as well as utilization of ethanol, lactate, and other alternative carbon sources. Consequently, an Snf1-deficient strain cannot grow on.

* This work was supported in part by the Ministerio de Educacio´n y Ciencia, Spain and Fondo Europeo de Desarrollo Regional (Grant BFU2005-06388-C4-04-BMC to J. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

‡ Recipient of an Ajut de Suport a les Activitats dels Grups de Recerca (Grant 2005SGR-00542, Generalitat de Catalunya). To whom correspondence should be addressed: Tel.: 34-93-581-2182; Fax: 34-93-581-2006; E-mail: Joaquin.Ariño@UAB.ES.

§ The abbreviations used are: PKA, protein kinase A; CDRE, calcineurin-dependent response element; TAP5, 3-[(2-hydroxy-1,1-bis(hydroxymeth- yl)ethyl)amino]-1-propanesulfonic acid; GFP, green fluorescent protein; RT, reverse transcription; HA, hemagglutinin; WCE, whole cell extract.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Regulation of Glucose-metabolizing Genes by Calcineurin

low levels of glucose (0.05%) or non-fermentable carbon sources (9, 12, 13), because it cannot express genes required for survival under these conditions.

Glucose limitation is one of the many environmental challenges that yeast cells must cope with. For instance, S. cerevisiae grows better at acidic than at neutral or alkaline pH, and alkalization of the medium, even if moderate, represents a stress situation for this yeast. Exposure of budding yeast to sudden alkalization involves extensive gene remodeling that affects iron, copper, and phosphate homeostasis (14–16) and triggers a number of signaling pathways, including the Rim101-Nrg1, the Wsc1-Pkc1-Slt2 mitogen-activated protein kinase, and the calcium-activated calcineurin-Crz1 pathways (14, 16–21). Calcineurin is a Ser/Thr protein phosphatase that can be activated by a transient increase in cytosolic calcium (22, 23). In S. cerevisiae, the enzyme is composed of one of two possible catalytic subunits (CNA1 and CNA2) and one regulatory subunit, encoded by a single gene, CNB1. Activation of calcineurin has been recognized as being essential for survival under diverse stress conditions (24). One of the effects of calcineurin activation is the dephosphorylation of the transcription factor Crz1/Tcn1. Dephosphorylated Crz1 translocates to the nucleus and activates gene expression by binding to specific sequences, known as calcineurin-dependent response elements (CDREs), which have been identified in the promoters of several calcineurin-responsive genes (25–28). It has been proposed that activation of Crz1 accounts for most, if not all, calcineurin-dependent transcriptional remodeling (28).

In a recent report (19), we presented a genome-wide transcriptional analysis of the response of S. cerevisiae to severe alkaline pH stress and characterized the participation of the calcineurin pathway in gene expression remodeling induced by this circumstance. It was observed that a large number of genes involved in hexose transport and carbohydrate metabolism are induced after short exposure of the cells (10 min) to alkaline pH. The gene HXT2 attracted our attention for two reasons: 1) it accounted for one of the most potent responses to high pH and 2) its expression was significantly reduced in calcineurin-deficient (cnb1) mutants. The latter was a puzzling observation not only because there was no previous hint that expression of a hexose transporter of the HXT2 type might be regulated in a calcineurin-dependent fashion, but also because this gene had not been reported in a previous genome-wide analysis (28) as transcriptionally responsive to high extracellular calcium or sodium, conditions that are known to activate calcineurin. Therefore, we were interested in investigating the molecular basis of HXT2 induction under alkaline stress and the hypothetical role of calcineurin in this process. In this report we present evidence that, in addition to negative regulation by Rgt1 and Migl, the HXT2 gene can integrate positive inputs mediated by the calcium/calcineurin pathway. More importantly, we show that this is a common feature for other genes encoding carbohydrate-metabolizing enzymes, and we propose that calcineurin represents a novel regulatory mechanism required for survival under certain conditions involving impaired glucose utilization.

### EXPERIMENTAL PROCEDURES

**Yeast Strains and Growth Conditions**—Yeast strains used in this study are described in Table 1. Strain MAR225 was generated by transformation of strain EDN92 (crz1::kanMX4) with the snf1::LEU2 cassette recovered from plasmid pCC107::LEU2 (29) after cleavage with restriction enzymes BamHI and HindIII. Strains MAR240, MAR241, and MAR238 were made by transformation of hap2, hap3, and hap4 kanMX deletion mutants in the BY4741 background (30) with the snf1::LEU2 cassette mentioned above. Yeast cells were grown at 28 °C in YM medium (10 g/liter yeast extract, 20 g/liter peptone) containing in each case the specified amount of the carbon source or, when indicated, in synthetic minimal or complete minimal media (31).

**Plasmids**—Plasmids used in this work are listed in supplemental Table S1. Plasmid pBM2717 (32), containing the entire HXT2 promoter cloned in YEp357R, was a generous gift of S. Ozcan (University of Kentucky). The reporter plasmids pALD4-lacZ, pALD5-lacZ, and pALD6-lacZ were generated as follows. The ALD4, ALD5, and ALD6 upstream DNA regions containing −494 and +104, −817 and +32, or −493 and +39, respectively (relative to the starting ATG), were amplified by PCR with added EcoRI/HindIII restriction sites and cloned into the same sites in the YEp357R plasmid. The BamHI restriction site was added to the PCR product in order to optimize its efficiency to generate the appropriate restriction sites (see Table 1).

| Name       | Relevant genotype | Source/reference |
|------------|-------------------|------------------|
| DBY746     | MATa leu2-3,112 ura3-52 his3-D1 trep1-Δ39 | This work |
| RSC10      | DBY746 snf1::LEU2 | (60) |
| RSC40      | DBY746 cnb1::TRP1 | (60) |
| EDN92      | DBY746 crz1::kanMX4 | (14) |
| RSC31      | DBY746 cnb1::kanMX4 | (19) |
| RSC28      | DBY746 snf1::kanMX4 | (19) |
| RSC46      | DBY746 yvc1::kanMX4 | (19) |
| MAR225     | DBY746 crz1::kanMX4 snf1::LEU2 | This work |
| BY4741     | MATa his3-D1 leu2-3,112 mit15A ura3Δ | (30) |
| BY4741 hap2::kanMX4 | (30) |
| BY4741 hap3::kanMX4 | (30) |
| BY4741 hap4::kanMX4 | (30) |
| MAR240     | BY4741 hap2::kanMX4 snf1::LEU2 | This work |
| MAR241     | BY4741 hap3::kanMX4 snf1::LEU2 | This work |
| MAR238     | BY4741 hap4::kanMX4 snf1::LEU2 | This work |
| VW1A       | MATa leu2-3,112 ura3-52 trep1-289 his3-D1 MAL2-8c SUC2 hxt17A | (3) |
| JBY01      | VW1A hxt1::HXT1 | (58) |

### Table 1

S. cerevisiae strains used in this study
inserted into the KpnI and XhoI restriction sites of the pSLF-178K plasmid (34). The plasmids pCDRE2mut and pCDRE3 were constructed exactly as pCDRE2 except that the pairs of oligonucleotides 5′_CDRE2mut_HXT2 and 3′_CDRE2 mut_ HXT2, and 5′_CDRE3_HXT2 and 3′_CDRE3_HXT2 were employed, respectively. The mutation introduced in CDRE2 was the same as in the YEp357R-derived vector. All constructions were verified by sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems).

β-Galactosidase Activity Assay—Yeast cells were grown to saturation in the appropriate dropout media and then inoculated into YPD plus 4% glucose (YPD 4% glucose). Growth was resumed until the A660 was 0.5–0.7, and cultures were centrifuged for 5 min at 1620 × g. Cells were resuspended in YPD 4% glucose (no induction), YPD 4% glucose plus 50 mM TAPS adjusted to pH 8.0 (alkaline stress), YPD 0.05% glucose (low glucose), or YPD 4% glucose plus 0.2 mM CaCl2 (calcium treatment), and growth was resumed for 60 min. When chemical blocking of calcineurin was desired, 1.5 μg/ml FK506 (generously provided by Astellas Pharma) was added to the resuspension medium. In all cases, β-galactosidase activity was measured as described previously (35).

Subcellular Localization of Crz1—Wild-type DBY746 cells were transformed with the pLMB127 plasmid (a generous gift of M. Cyert, University of Stanford), which contains three tandem copies of GFP fused to the N terminus of Crz1 (36). Cells were grown (A660 of 0.9–1.0) on synthetic medium containing 4% glucose as the carbon source and in the absence of methionine to induce expression from the MET7 promoter present in the pLMB127 vector. Ammonium chloride was substituted for ammonium sulfate to reduce precipitation upon Ca2+ saturation in the appropriate dropout media and then inoculated into YPD plus 4% glucose (YPD 4% glucose). Growth was resumed until the A660 was 0.5–0.7, and cultures were centrifuged for 5 min at 1620 × g. Cells were resuspended in YPD 4% glucose (no induction), YPD 4% glucose plus 50 mM TAPS adjusted to pH 8.0 (alkaline stress), YPD 0.05% glucose (low glucose), or YPD 4% glucose plus 0.2 mM CaCl2 (calcium treatment), and growth was resumed for 60 min. When chemical blocking of calcineurin was desired, 1.5 μg/ml FK506 (generously provided by Astellas Pharma) was added to the resuspension medium. In all cases, β-galactosidase activity was measured as described previously (35).

Subcellular Localization of Crz1—Wild-type DBY746 cells were transformed with the pLMB127 plasmid (a generous gift of M. Cyert, University of Stanford), which contains three tandem copies of GFP fused to the N terminus of Crz1 (36). Cells were grown (A660 of 0.9–1.0) on synthetic medium containing 4% glucose as the carbon source and in the absence of methionine to induce expression from the MET7 promoter present in the pLMB127 vector. Ammonium chloride was substituted for ammonium sulfate to reduce precipitation upon Ca2+ addition.

Cultures (5 ml) were treated as follows: addition of 100 μl of 1 M KCl (control cells), addition of 100 μl of 1 M KOH (alkaline stress, pH 8.2), addition of 500 μl of 2 M CaCl2 (calcium treatment), or resuspension in 5 ml of the same medium containing 0.05% glucose (low glucose). Samples (250 μl) were taken at the appropriate times and fixed for 5 min by adding 13.5 μl of 37% formaldehyde. Cells were harvested, washed three times with Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and finally concentrated 10-fold before visualization. Cells were visualized with a fluorescein filter using a Nikon Eclipse E800 fluorescence microscope. Digital images were captured with an ORCA-ER 4742-80 camera (Hamamatsu) and Wasabi software.

Computational Search for Putative CRE in pH-induced, Glucose-repressed Genes—The combined list of 788 genes induced at least 2-fold by exposure to pH 8.0 (19) or 8.2 (21) was analyzed for genes induced by nutrient scarcity: low glucose (37) or diauxic shift (38). The resulting list of 107 genes was searched for putative CRE with a position-specific scoring matrix using the PATSER (version 3d) software available at the Regulatory Sequence Analysis Tools site (39). The parameters defined were: 800 upstream nucleotides (allowing overlapping with open reading frame) and a weight matrix based on the G(A/T)GGCTG sequence. Minimum score threshold was set to 6.0 (minimum score: −12.159, maximum score: 7.991). A control search was conducted using the same search parameters on 300 S. cerevisiae genes randomly selected by the system.

RNA Preparation and RT-PCR—For RNA preparation, yeast cells were grown on YPD to an optical density of 0.5–0.8 and split into aliquots. Cells were centrifuged and resuspended either in fresh YPD (non-stressed cells, pH 6.2) or YPD containing 50 mM TAPS (stressed cells, pH 8.0) for 10–30 min. When inhibition of calcineurin was desired, FK506 (final concentration of 1.5 μg/ml) was added to the medium 1 hr prior initiation of the alkaline treatment. Cultures were then centrifuged for 2 min at 1620 × g at 4°C, and total RNA was extracted by using hot phenol and glass beads as described previously (40) or the RiboPure-Yeast kit (Ambion). RNA quality was assessed by denaturing 0.8% agarose gel electrophoresis, and RNA quantification was performed by measuring A260 in a BioPhotometer (Eppendorf). RT-PCRs were performed using 200 ng of total RNA and the Ready-To-Go RT-PCR Beads kit (GE-Amersham Biosciences) for 25–30 cycles. Specific pairs of oligonucleotides (supplemental Table S2) were used to determine mRNA levels for HXT2, HXT7, GSY2, CIT2, HXK1, TPS1, MDH1, ALD4, and PHO89.

Chromatin Immunoprecipitation Assay—Chromatin cross-linking and immunoprecipitation were performed as previously described (20). Expression of N-terminally HA-tagged Crz1 was accomplished by transformation of strain EDN92 (crz1Δ) with the centromeric plasmid pAM5451 (pRS315-HA-Crz1) (25). Briefly, 50-ml cultures were grown up to A660 1.0 on YPD medium, and cells were exposed to alkaline stress (pH 8.0) or high calcium (CaCl2, 0.2 M) for 10 min as described for β-galactosidase activity assays. Then, cells were treated with 1% formaldehyde for 1 h at 24°C and quenched by addition of 100 mM glycine for 15 min at 24°C. Cells were collected and washed four times with ice-cold Tris-buffered saline, resuspended in 600 μl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin), and lysed with glass beads. The resulting extracts were sonicated to obtain chromatin fragments of 500–800 bp, centrifuged at 9300 × g for 3 min at 4°C, aliquoted, and stored at −80°C (whole cell extracts (WCEs)). For chromatin immunoprecipitation, 100 μl of Protein G-Sepharose (Amersham Biosciences) was coupled to 5 μg of a monoclonal mouse anti-HA antibody (Roche Applied Science). The anti-HA-Protein G-Sepharose complexes were incubated overnight with 200 μl of WCE at 4°C. Sepharose-protein complexes were transferred to 96-well filter plates (MultiScreen, Millipore) and washed at 4°C as follows: twice for 1 min with lysis buffer, twice with lysis buffer containing 500 mM NaCl, twice with washing buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and once with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Washes were discarded by centrifugation at 180 × g. Protein-DNA complexes were recovered from beads by incubation with 80 μl of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 10 min. The supernatant was removed (60 μl), 240 μl of elution buffer were added, and samples were incubated overnight at 65°C. WCE controls were prepared from untagged cells by mixing 15 μl of WCE with 240 μl of elution buffer and incubating overnight at 65°C. Formal-
Regulation of Glucose-metabolizing Genes by Calcineurin

diminishes the alkaline pH-dependent induction of As shown in Fig. 1, confirmed in experiments using the calcineurin inhibitor FK506. Calcineurin activation might contribute to the Snf1-independent burst (19) prompted us to consider the possibility that calcineurin in cells exposed to high pH stress, we introduced a reporter construct based in a translational fusion of the HXT2 promoter and the β-galactosidase gene into wild-type and snf1 cells. These cells were exposed to pH 8.0 for 1 h or shifted from high glucose to low glucose for the same period of time and the β-galactosidase activity was measured. The reporter confirmed the potent activation of HXT2 expression by high pH (Fig. 1A), comparable to that observed after shifting to low glucose (Fig. 1B). Interestingly, whereas lack of Snf1 fully blocked expression of HXT2 on low glucose, activation of the HXT2 promoter by high pH was only partially abolished in the snf1 strain, indicating the presence of additional regulatory events not triggered by external low glucose (Fig. 1, A and B). Although calcineurin has not been previously implicated in the regulation of HXT2, our previous results from DNA microarray analysis and the evidence that alkaline stress triggers an almost immediate cytosolic calcium burst (19) prompted us to consider the possibility that calcineurin activation might contribute to the Snf1-independent response of HXT2 to alkaline pH. This hypothesis was confirmed in experiments using the calcineurin inhibitor FK506. As shown in Fig. 1A, treatment of wild-type cells with FK506 diminishes the alkaline pH-dependent induction of HXT2 by ~40%. Remarkably, the transcriptional response of HXT2 is virtually absent in snf1 cells treated in a similar manner, suggesting that Snf1 and calcineurin define the major pathways for alkaline stress-induced HXT2 activation. This notion is reinforced by the observation (Fig. 1B) that, upon pH stress, expression from the HXT2 promoter is reduced ~50% in cells lacking CNB1 (encoding the regulatory subunit of calcineurin). Mutations of Rim101, a transcription factor relevant for induction of a number of alkaline stress-responsive genes (16, 18), did not affect the expression of the reporter (not shown). A role for calcineurin in HXT2 induction is further underscored by the fact that exposure of cells to 0.2 mM calcium chloride results in potent activation of the promoter. This activation is not affected by lack of Snf1, but it is fully blocked in the absence of calcineurin function (cnb1 mutant). In contrast, induction of HXT2 by low glucose was unaffected in the cnb1 strain (Fig. 1B).

The response of the HXT2 promoter to high pH and the partial dependence of this response on the Cnb1-mediated pathway were further confirmed by semi-quantitative RT-PCR (Fig. 1C). Therefore, the activation of HXT2 upon alkaline stress depends on parallel inputs mediated by the Snf1 protein kinase and the calcineurin phosphatase. This concept was confirmed by the observation that shifting cells to low glucose in the presence of 0.2 mM extracellular calcium resulted in additive induction of HXT2 (data not shown).

The involvement of calcineurin in the transcriptional response of HXT2 under high pH suggested that the HXT2 promoter could be a target for the Crz1 transcription factor. To test this possibility, we wanted to verify if alkalinization was able to promote entry of Crz1 into the nucleus and, in this case,
if this process was fast enough to justify the rapid accumulation of HXT2 mRNA (see Fig. 1 C). To this end, wild-type cells, transformed with a plasmid expressing a GFP-Crz1 fusion protein, were subjected to alkaline stress and the localization of the fluorescent protein was monitored by microscopy. As shown in Fig. 2A, Crz1 appeared in the nucleus shortly after alkaline treatment (5 min), similar to what is observed in calcium-treated cells. In contrast, the transcription factor maintained its cytosolic distribution in control cells (KCl-treated). The presence of Crz1 in nuclei was detected as early as 2.5 min after KOH treatment, and the factor remained there for ~30 min (not shown). After 60 min of alkaline treatment there were no longer traces of GFP-Crz1 in the nucleus. Switching cells from high (4%) to low (0.05%) glucose did not alter GFP-Crz1 distribution at any of the times tested (Fig. 2A). Therefore, alkalization of the medium results in fast entry of Crz1 into the nucleus, which is compatible with the rapid induction of HXT2.

As observed in Fig. 2B, exposure of cells lacking CRZ1 to high levels of extracellular calcium resulted in a complete loss of response of the HXT2 promoter. In addition, when cells lacking CRZ1 or CNB1 were exposed to high pH they displayed a quantitatively identical loss of HXT2 expression in comparison to wild-type cells, suggesting that the effect of calcineurin activation was entirely mediated by Crz1. It is known that alkaline pH-induced intracellular calcium burst is fully mediated by Cch1 and Mid1 (19), which are components of the voltage-gated high affinity calcium channel involved in calcium influx through the plasma membrane (41, 42). Therefore, we tested the responsiveness of HXT2 in cch1 and mid1 mutants. When subjected to alkaline stress, expression in these strains was very similar to that of cnb1 or crz1 cells. In contrast, in cells lacking the vacuolar Ca^{2+} channel Yvc1, expression from the HXT2 promoter did not decrease when compared with wild-type cells (Fig. 2B).

These observations link the Cch1/Mid1-mediated burst of calcium triggered by alkaline pH with the calcineurin and Crz1-mediated activation of HXT2 and strongly suggest that HXT2 is able to respond transcriptionally to calcium signaling. We considered that, if so, expression of HXT2 should be altered under other circumstances known to provoke increased cytosolic calcium levels. It is known that exposure to the α-factor pheromone results in a calcium burst (43, 44) and activation of calcineurin (45, 46). Similarly, rises in intracellular calcium and calcineurin activation occur after saline or hyperosmotic stress (47, 48). Therefore, to test this hypothesis wild-type cells transformed with the HXT2 reporter were treated with 50 nM α-factor for 90 min in the presence or the absence of the calcineurin inhibitor FK506. Under these circumstances, treatment with the pheromone resulted in a 2-fold increase in HXT2-driven expression, and this effect was fully blocked by FK506 (not shown). Similarly, when the DBY746 wild-type strain and its isogenic cnb1 derivative were subjected to 0.9 M NaCl for 4 h, expression from the HXT2 promoter increased in a fully CNB1-dependent manner (not shown). All these results clearly demonstrate that expression of the hexose transporter gene is under the control of the calcium/calcineurin pathway.

Identification of a Functional CDRE Element in the HXT2 Promoter—The results described above suggested that the HXT2 promoter should contain functional CDRE sequences. To identify possible CDREs we searched both strands in the upstream region of the HXT2 open reading frame for the GGC sequence, generally considered to be the invariant core of a Crz1-binding element. Three candidate sequences, named CDRE1–3, were selected, and reporters containing specific mutations within these sequences were constructed (Fig. 3A). Mutation of CDRE3 did not decrease the response of the promoter to alkaline pH, low glucose, or high calcium. Mutation of CDRE1 was also without effect when cells were treated with high pH or transferred to low glucose, although it resulted in a moderate decrease in the expression in cells exposed to high calcium. Finally, mutation of CDRE2 markedly decreased expression from the HXT2 promoter in wild-type cells exposed to high pH or transferred to low glucose, and completely blocked expression in cells treated with high calcium. The effect of the mutation of CDRE2 in cells transferred to low glucose is probably caused by the fact that CDRE2 partially overlaps (see Fig. 3A) with a previously characterized MIG1 binding site (11). This mutation virtually abolished the response of the promoter to high pH in snf1 cells. Therefore, it is likely that the sequence GGGGGCTG, present at positions...
Identification of a functional CDRE in the HXT2 promoter. In A: Upper panel, the sequence of the −650/−301 region of the HXT2 gene is shown. Previously defined Rgt1 and Mig1 binding sequences (11) are underlined and italicized, respectively. The three putative CDREs predicted in the promoter sequence (CDRE1–3) are in dark background. Lower panel, wild-type DBY746 cells (WT), as well as its cnb1 and snf1 derivatives, were transformed with plasmid pBM2717 (denoted here as pHXT2) or different versions (pHXT2CDRE1–3) in which the specific CDRE has been mutated (see “Experimental Procedures”). Cells were treated as indicated in the figure, and the response of the promoter was measured as ß-galactosidase activity. Data are mean ± S.E. from six experiments. B, DBY746 cells were transformed with the indicated plasmids and subjected to high pH and calcium stress as above. Data correspond to the ratio of ß-galactosidase activity between treated and untreated (NI) cells and correspond to the mean ± S.E. from six experiments.

The identification of a functional CDRE in the HXT2 promoter is shown. The sequence of the −507/−501 in the Crick strand of the HXT2 promoter, acts as a functional CDRE. The Crick strand was cloned into the transcriptionally inactive plasmid pSLFA-178K to yield pCDRE2(1x). In parallel, a mutated version pCDRE2(1x)mut, in which the sequence GGGCTG was replaced by an XbaI site (TCTAGA), was prepared. When wild-type cells containing these constructs were challenged with high pH or 0.2 M calcium, we observed that pCDRE2(1x) was able to drive a transcriptional response in both cases (albeit considerably weaker than that observed from the entire HXT2 promoter). Interestingly, neither the pCDRE2(1x)mut-mutated version, nor the same vector carrying one or three copies of CDRE3, were able to respond to high pH or calcium (Fig. 3B). These results confirm that CDRE2 acts as a functional calcineurin response element in the HXT2 promoter.

The identification of a functional CDRE in the promoter of HXT2 adds a new layer of complexity to the regulation of this gene and offers an example of calcium signaling playing a regulatory role in the metabolism of glucose in yeast. The fact that, despite the intense research carried out in this field for so many years, the involvement of calcineurin remained unnoticed can be explained by our observation that shifting the cells to low glucose medium, a common approach to characterize genes responsive to glucose scarcity, does not result in activation of calcineurin nor promotes translocation of Crz1 to the nucleus.

The data presented so far allow us to propose a model for activation of HXT2 in response to alkaline pH (Fig. 4). Full induction of HXT2 in response to high pH stress would involve activation of both the calcineurin and Snf1 pathways, a notion supported by the recent observation that alkalinization increases Snf1 catalytic activity (49). Activation of calcineurin would promote entry of Crz1 into the nucleus and subsequent binding to the CDRE defined above, thus promoting transcription. In parallel, activation of Snf1 would induce removal of the Mig1 repressor from the promoter. HXT2 expression is also controlled by the Rgt1 repressor (11), which in turn is regulated by the Snf3/Rgt2 sensors (8, 9). We have observed that deletion of Snf3, a membrane sensor required for full expression of HXT2 under glucose limitation (50), results in a decrease in the response of the HXT2 promoter when cells are challenged by alkaline pH (not shown). This would suggest that the effect of alkaline pH on HXT2 expression may involve regulation of Rgt1 function (Fig. 4). Interestingly, the effect of the snf3 mutation was not additive to that observed in cells lacking Snf1 (not shown), which could be explained by the previous observation that the Snf1/Mig1 pathway controls the expression of MTH1, which in turn is required for the repressor function of Rgt1 (7). As mentioned in the introduction, it has been proposed very
The list includes genes related to hexose metabolism that have been shown to be induced both by high pH stress (19, 21) and under glucose limiting conditions (37) or diauxic shift (38). The \textit{pH induction} heading correspond to the maximum increase in expression detected by Viladevall and coworkers (19) and/or Serrano et al. (21). \textit{Timing} refers to the time required for maximal induction after exposure to alkaline pH: E, early response (10–15 min); I, intermediate response (20–30 min); L, late response (45 min). A computer search of putative CDREs was made using the Regulatory Sequence Analysis Tools (RASAT) as indicated under “Experimental Procedures.” When more than one occurrence was found in a given promoter only those that reached the highest score are indicated.

| Open reading frame | Gene | pH induction | pH timing | CDRE | Functional features |
|-------------------|------|--------------|-----------|------|---------------------|
| **Hexose transport** | YMR011W | HXT2 | 21.1/26.6 | E/E | -507 GGGGCTG -501 | High affinity glucose transporter |
| | YDR343C | HXT6 | 7.8/3.4 | E/E | -617 GAGGCTC -611 | High affinity glucose transporter |
| | YDR343C | HXT7 | 9.1/5.3 | E/E | -487 GTGGCTG -481 | Galactose and glucose permease |
| | YLR081W | GAL2 | 4.4/- | E/- | -695 GAGGCTG -689 | Glucokinase |
| | YHR096C | HXT5 | 2.5/17.9 | E/E | -351 GAGGCCC -345 | Phosphoglucomutase |
| **Glycolysis** | YCL040W | GLK1 | 7.4/10.6 | E/E | -600 GGGGCTC -594 | Hexokinase isoenzyme 1 |
| | YFR053C | HXX1 | 10.7/10.9 | E/E | -205 GTGGCTG -199 | Phosphoglycerate mutase |
| | YGL253W | HXX2 | 4.1/- | E/- | -25 GAGGATC -19 | Glucose-6-phosphate isomerase |
| | YKL152C | GPM1 | 2.2/- | E/- | -380 GGGGCTC -374 | Phosphofructokinase |
| | YMR105C | PGM2 | 4.2/17.1 | E/E | -566 GTGGCTC -560 | Pyruvate kinase |
| | YOR347C | PYK2 | -2.8/ -E | -/E | -380 GGGGCTC -374 | Phosphofructokinase |

**Trichloroacetic acid cycle and related enzymes**

| Open reading frame | Gene | pH induction | pH timing | CDRE | Functional features |
|-------------------|------|--------------|-----------|------|---------------------|
| | YNR001C | CIT1 | -2.1/- | I/- | -621 GGGGCTG -615 | Citrate synthase |
| | YCR005C | CIT2 | 7/11.7 | I/E | -349 GCGGCTC -343 | Citrate synthase |
| | YNL037C | IDH1 | -2.6/- | I/- | -349 GCGGCTC -343 | Citrate synthase |
| | YOR363C | IDH2 | 3.8/- | I/E | -415 GTGGCTG -409 | Citrate synthase |
| | YDR148C | KGD2 | 2.4/- | E/- | -349 GCGGCTC -343 | Citrate synthase |
| | YDR178W | SDH4 | 2.7/3.1 | E/E | -598 GGGGCTC -592 | Mitochondrial malate dehydrogenase |
| | YGL062W | PYC1 | 3.3/6.7 | I/I | -598 GGGGCTC -592 | Cytoplasmic malate dehydrogenase |
| | YKL085W | MDH1 | 3/5.2 | E/E | -505 GGGGCTC -501 | Pyruvate kinase |
| | YOL126C | MDH2 | 5.7/2.6 | E/E | -505 GGGGCTC -501 | Pyruvate kinase |

**Alcohol and aldehyde metabolism**

| Open reading frame | Gene | pH induction | pH timing | CDRE | Functional features |
|-------------------|------|--------------|-----------|------|---------------------|
| | YMR169C | ALD3 | -9/3 | -/E | -243 GGGGCTG -237 | Cytoplasmic alcohol dehydrogenase |
| | YMR170C | ALD2 | -6/4 | -/E | -711 GTGGCTC -705 | Cytoplasmic alcohol dehydrogenase |
| | YMR083W | ADH3 | 2.2/- | E/- | -203 GTGGCTC -197 | Mitochondrial alcohol dehydrogenase |
| | YGL256W | ADH4 | -2.9/ | I/E | -203 GTGGCTC -197 | Mitochondrial alcohol dehydrogenase |
| | YOR374W | ALD4 | 8.1/9.0 | E/E | -467 GTGGACTC -461 | Mitochondrial alcohol dehydrogenase |
| | YPL061W | ALD6 | 7.5/3.4 | E/E | -431 GTGGCTC -425 | Mitochondrial alcohol dehydrogenase |

**Glycogen metabolism**

| Open reading frame | Gene | pH induction | pH timing | CDRE | Functional features |
|-------------------|------|--------------|-----------|------|---------------------|
| | YKL035W | UGP1 | 3.5/7.1 | I/E | -263 GAGGCTC -257 | UDP-glucose pyrophosphorylase |
| | YFR015C | GSY1 | 3.6/15.6 | E/E | -263 GAGGCTC -257 | Glycogen synthase |
| | YLR258W | GST2 | 2.5/5.2 | E/E | -494 GAGGCTC -488 | Glycogen synthase |

**Trehalose metabolism**

| Open reading frame | Gene | pH induction | pH timing | CDRE | Functional features |
|-------------------|------|--------------|-----------|------|---------------------|
| | YBR126C | TPS1 | 2.6/9.1 | E/E | -309 GGGGCTC -303 | Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex |
| | YDR074W | TPS2 | 3.2/7.1 | E/E | -634 GTGGCTC -628 | Phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex |
| | YMR261C | TPS3 | -3.1/- | -/E | -563 GTGGCTC -557 | Regulatory subunit of trehalose-6-phosphate synthase/phosphatase complex |

Table 2: Carbohydrate metabolism-related genes may be regulated by the calcium/calcineurin pathway

Regulation of Glucose-metabolizing Genes by Calcineurin

Recently (10) that a low level of PKA activity would result in regulation of Rgt1 function in a way that only high affinity hexose transporters (such as HXT2) would be induced. It is worth noting that intracellular acidification has been associated to increased CAMP-dependent PKA activity (51). Therefore, it is conceivable that the transient intracellular alkalization provoked by increasing extracellular pH could result in inhibition of PKA activity and thus further enhance HXT2 expression. Remarkably, Crz1 has been recently identified as a substrate for PKA. Phosphorylation of specific residues in Crz1 could inhibit translocation of the transcription factor to the nucleus, thus opposing the action of calcineurin (52). In this context, it is plausible that alkalization of the medium would regulate expression of Crz1-dependent genes by means of two synergistic mechanisms: 1) activation of calcineurin, which promotes dephosphorylation of Crz1, and 2) inhibition of PKA, thus decreasing Crz1 phosphorylation and lowering the threshold for calcineurin activation. Therefore, according to our model (Fig. 4) low levels of PKA would favor expression of HXT2 both by acting on Rgt1 and by facilitating activation of Crz1.

The Calcium/Calcineurin Pathway Directly Contributes to the Regulation of the Expression of Many Genes Related to Glucose Metabolism—The results presented so far demonstrate that HXT2, an important component of carbohydrate metabolism in yeast, is able to respond to increases in intracellular calcium leading to activation of the calcineurin/Crz1 system. We wondered whether this was an exceptional case or, on the contrary, it might be a common feature of other genes involved in carbohydrate metabolism, particularly of those induced by glucose limitation.

To this end we constructed, on the basis of the existing literature, a list of 107 genes that combine two characteristics: 1) increased expression under severe alkaline stress (19, 21) and 2) induction by glucose limitation or diauxic shift (37, 38). Their upstream regions were then searched for the presence of pos-
Regulation of Glucose-metabolizing Genes by Calcineurin

**FIGURE 5. Relevance of the calcium/calcineurin pathway on the regulation of the expression of genes involved in glucose metabolism.** A, wild-type DBY746 cells (WT) and the isogenic cnb1 or crz1 derivatives were transformed with plasmids pALD4, pALD5, and pALD6, which contain fusions of the respective promoters and the β-galactosidase gene. Cells were subjected to alkaline stress (pH 8.0) or high calcium (0.2 μM) and β-galactosidase activity was determined. Data are mean ± S.E. from 6–9 experiments, B, wild-type strain DBY746 was exposed to high pH (8.1) or high calcium (0.2 μM CaCl₂) for 10 min (HXT2, HXT7, HXK1, ALD4, and PHO89) or 15 min (CIT2, MDH1, GSY2, and TPS1) and total RNA extracted. Samples (200 ng) were subjected to semiquantitative RT-PCR and analyzed as described for Fig. 1C.

Possible CDRE elements using a position-specific scoring matrix and a score setting ≥ 6.0 (see “Experimental Procedures”). 108 hits were obtained, corresponding to 68 genes displaying one or more putative CDRE sequences (63.5% of the total number of genes). When a similar search was performed on 300 randomly selected yeast genes, only 42.7% could be considered as positives. We then limited the candidate genes to those encoding enzymes that participate in the major glucose utilization pathways, such as glucose uptake, glycolysis, tricarboxylic acid cycle, and so on. This yielded a final list of 32 genes, which is displayed in Table 2. As observed, 24 of these genes (75%) contain at least one putative CDRE sequence. Surprisingly, almost none of these genes appeared in a previous DNA-microarray-based genome-wide analysis of genes induced by calcium in a calcineurin-dependent fashion (28). However, a careful analysis of the original DNA microarray data revealed that >70% of the genes listed in Table 2 show increased expression in response to calcium. Furthermore, the time course of these responses was similar to those observed upon induction by alkaline pH, leaving open the possibility that these genes could be regulated by calcineurin.

Experimental support for our hypothesis was obtained by direct analysis of the expression of several genes listed in Table 2. ALD4 and ALD6 encode aldehyde dehydrogenases required for conversion of acetaldehyde to acetate and are examples of genes induced by short term alkaline stress (19). Wild-type cells were transformed with the pALD4 or pALD6 reporters and subjected to high pH stress or treated with 0.2 μM CaCl₂. As shown in Fig. 5A, β-galactosidase expression from ALD4 and ALD6 promoters was potently increased by both alkaline and calcium treatments. The response to high pH was partially abolished in cnb1 or crz1 cells, similarly to what was found for HXT2, indicating that the response of these genes to alkaline stress involves both calcineurin-dependent and -independent components. Consistent with our hypothesis, the effect of calcium was fully abolished in cells lacking calcineurin (cnb1) or Crz1. Expression of ALD5, a gene that is not induced by high pH, was not increased by calcium cations nor affected by deletion of CNB1 or CRZ1. The response to high calcium and alkaline pH for other relevant genes was monitored by RT-PCR. As observed (Fig. 5B), HXT7, which encodes a high affinity hexose transporter that is induced by low glucose, is also rapidly induced by exposure to alkaline pH and, to lesser extent, by high calcium. This induction was attenuated by either mutation of the CNB1 gene or incubation of wild-type cells with the calcineurin inhibitor FK506 (not shown). Induction by high calcium can also be demonstrated for HXK1, CIT2, MDH1, GSY2, and TPS1 (as well as confirmed for HXT2 and ALD4). The intensity of the responses is comparable to that of PHO89, a CDRE-containing gene previously known to be induced by high pH and calcium (14, 28), which is included as a reference. Further evidence for calcium/calcineurin-mediated activation of genes related to glucose metabolism was obtained from chromatin immunoprecipitation experiments. Yeast cells expressing an HA-tagged version of Crz1 were subjected to high pH or calcium stress for 10 min and processed for chromatin immunoprecipitation as described under “Experimental Procedures.” As observed in Fig. 6, high pH or calcium treatments did not promote binding of Crz1 to CDC26 or PHO84 promoters, which are included here as negative controls (note that, although PHO84 is induced by high pH, this occurs in a calcineurin-independent fashion (14)). In contrast, both treatments recruited Crz1 to the ENA1
Regulation of Glucose-metabolizing Genes by Calcineurin

Our finding that activation of the calcineurin pathway by extracellular calcium overrides the growth defect of a snf1 mutant on low glucose provides strong evidence that activation of this phosphatase can be a widespread mechanism to rapidly and positively regulate expression of genes required to survive under glucose shortage. It is worth noting that not all genes able to respond to low extracellular glucose are regulated by calcineurin. For instance, we have observed in the case of SLIC2, a glucose-repressed gene encoding invertase, that expression from this promoter is induced by high pH in a completely Snf1-mediated fashion and does not require calcineurin activation. Consequently, the SLIC2 promoter does not respond at all to the addition of calcium to the medium (data not shown). The identification of the possible target genes that, once activated by calcineurin, are sufficient to allow growth of a snf1 mutant on low glucose will represent a major challenge in the future. In this regard, we have performed some experiments to approach this issue. Because glucose transport across the plasma membrane is the limiting step for glucose metabolism, the possibility that the main calcineurin target could be genes encoding glucose-repressed, high affinity hexose transporters was consid-
Regulation of Glucose-metabolizing Genes by Calcineurin

FIGURE 7. Activation of calcineurin overrides the incapacity of a snf1 mutant for growing on low glucose or alternative carbon sources. A, wild-type strain DBY746 (+) and its snf1 derivative (−) were grown in the indicated media (the percentages indicate the amounts of carbon source added to the medium) in the absence or the presence of 100 mM CaCl2. Growth was monitored after 3 days (2% glucose) or 6 days (other conditions). B, the mentioned strains were grown on YP medium containing the indicated glucose concentrations in the absence or the presence of 1.5 μg/ml FK506, with or without added calcium. Growth was monitored after 3 days (2% glucose) or 5 days (0.05% glucose). C, wild-type strains DBY746 and snf1, crz1, and snf1 crz1 derivatives were grown under the indicated conditions for 4 days. Two dilutions (1:10) of the cultures are shown.

FIGURE 8. Effect of mutations on hexose transport and respiratory genes on the ability of calcineurin activation to sustain growth of a snf1 mutant on low glucose. A, strain VW1A and its derivative JBY01 (hxt2Δ HXT17), which lacks all 20 glucose transporters but constitutively expresses the low affinity glucose transporter HXT1, were incubated as indicated in Fig. 7, and growth was monitored after 4 days. B, BY4741 wild-type strain and its derivatives were grown as indicated for 4 days. Two dilutions (1:10) of the cultures are shown.

The evidence gathered indicates that, although HXT2 is potently induced in a calcineurin-dependent manner, growth of an snf1 mutant on low glucose in the presence of calcium cannot be attributed exclusively to the activation of HXT2, as it is also observed in an hxt2 snf1 strain (data not shown). The possibility that the effect of calcium in the absence of Snf1 could be explained by induction of a variety of hexose transporters was evaluated by monitoring growth of strain JBY01. This strain is devoid of all 20 known hexose transporters, whereas it maintains strong, constitutive expression of the low affinity transporter HXT1 (58). Consequently, this strain can grow on high glucose (100 mM) but shows slow growth on 5 mM glucose (58). As shown in Fig. 8A, growth of JBY01 cells on YP plus 0.05% added glucose (~2.8 mM) was undetectable in the absence of calcium, whereas addition of the cation (100 mM) effectively sustained growth. Because in the JBY01 strain expression of HXT1 is constitutive and no other glucose transporter is present, it can be concluded that the positive effect of calcineurin activation cannot be solely attributed to improved glucose transport and, consequently, additional downstream targets must exist. On the other hand, the glucose-repressed Hap2/3/4/5 CCAAT-binding complex acts as a transcriptional activator and global regulator of respiratory gene expression, and it is required for respiratory growth (see Ref. 59 for review). Interestingly, CIT1 and KGD2, which have been defined as targets for the Hap2/3/4/5 complex, are induced by high pH. These genes are likely to contain a CDRE sequence (Table 2) and in fact, the CIT1 promoter shows Crz1-binding capacity upon exposure to high pH and calcium stress (Fig. 6). We then considered the possibility that calcineurin activation may target respiratory genes. As shown in Fig. 8B, growth of Snf1−/− cells in the presence of calcium was almost fully blocked by deletion of HAP2, HAP3, or HAP4 genes. These results indicate that, although we have shown that some HAP-regulated genes are likely targets for calcineurin (CIT1 and CIT2), activation of calcineurin cannot replace the lack of a functional HAP complex in the absence of Snf1.

In essence, we propose that glucose utilization is probably impaired in yeast cells suddenly exposed to an increase in external pH. This notion is supported by several lines of evidence: 1) the large number of genes typically induced by low glucose that are also induced by high pH (19, 21); 2) the sensitivity to high pH of diverse mutants in genes required to respond to glucose scarcity, including snf1 and snf3 mutants (20, 49); and 3) the observation that tolerance to high pH can be enhanced by increasing the concentration of glucose in the medium.3 To confront this problem, the Snf1 kinase is activated (49) by still unknown mechanisms, and this contributes to the activation of the expression of genes required for survival under glucose

3 A. Ruiz and J. Ariño, unpublished work.
shortage. In parallel, alkaline pH triggers almost immediate entry of calcium from the medium, which results in activation of calcineurin (14) and leads to rapid entry of Crz1 into the nucleus. Our data show that a substantial number of genes required for glucose metabolism are able, after activation of calcineurin, to rapidly recruit Crz1 to their promoters. This would represent an additional positive input for their expression in response to alkaline pH stress. The activation of this panopoly of genes must be biologically relevant, because it is enough to sustain growth under limiting glucose availability even in the absence of the Snf1 kinase, which is considered to be a major regulator of carbohydrate metabolism. Therefore, calcineurin and Snf1 activation represent combined but independent strategies to cope with the likely alteration of glucose metabolism caused by alkaline pH stress.

Acknowledgments—We thank S. Ozcan, P. Sanz, E. Boles, and M. Cyert for plasmids and strains and D. Bernal, M. Platara, L. Viladevall, A. Gonzalez, R. Lahoz, A. Barcelo, and A. Casamayor for support. Thanks are given to E. Gonzalez de Antona, D. Powell, and A. Friedrich (Astellas Pharma) for kindly supplying the calcineurin inhibitor FK506 and to Lynne Yenush for revision of English usage. The excellent technical assistance of Anna Vilalta and Maria Jesus Alvarez is acknowledged.

REFERENCES

1. Ozcan, S., and Johnston, M. (1999) Microbiol. Mol. Biol. Rev. 63, 554–569
2. Boles, E., and Hollenberg, C. P. (1997) FEMS Microbiol. Rev. 21, 85–111
3. Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C. P., and Boles, E. (1999) FEBS Lett. 464, 123–128
4. Reifenberger, E., Freidel, K., and Ciriacy, M. (1995) Mol. Microbiol. 16, 157–167
5. Liang, H., and Gaber, R. F. (1996) Mol. Cell. Biol. 16, 1953–1966
6. Reifenberger, E. Boles, E., and Ciriacy, M. (1997) Eur. J. Biochem. 245, 324–333
7. Kaniak, A., Xue, Z., Macool, D., Kim, J. H., and Johnston, M. (2004) Eur. J. Biochem. 324, 620
8. Santangelo, G. M. (2006) Microbiol. Mol. Biol. Rev. 70, 253–282
9. Sanz, P. (2000) Front. Biosci. 5, 221–231
10. Santangelo, G. M. (2006) Microbiol. Mol. Biol. Rev. 70, 253–282
11. Sanz, P. (2000) Front. Biosci. 5, 221–231
12. Schneper, L., Duvel, K., and Broach, J. R. (2004) Curr. Opin. Microbiol. 7, 624–630
13. Gancedo, J. M. (1998) Microbiol. Mol. Biol. Rev. 62, 334–361
14. Serrano, R., Ruiz, A., Bernal, D., Chambers, J. R., and Arino, J. (2002) Mol. Microbiol. 46, 1319–1333
15. Serrano, R., Bernal, D., Simon, E., and Arino, J. (2004) J. Biol. Chem. 279, 19698–19704
16. Lamb, T. M., Xu, W., Diamond, A., and Mitchell, A. P. (2001) J. Biol. Chem. 276, 1850–1856
17. Aleup, P. M., Cunningham, K. W., and Estruch, F. (1997) Mol. Microbiol. 26, 91–98
18. Lamb, T. M., and Mitchell, A. P. (2003) Mol. Cell. Biol. 23, 677–686
19. Viladell, V., Serrano, R., Ruiz, A., Domenech, G., Giraldo, J., Barcelo, A., and Arino, J. (2004) J. Biol. Chem. 279, 43614–43624
20. Platara, M., Ruiz, A., Serrano, R., Palomino, A., Moreno, F., and Arino, J. (2006) J. Biol. Chem. 281, 36632–36642
21. Serrano, R., Martin, H., Casamayor, A., and Arino, J. (2006) J. Biol. Chem. 281, 39785–39795
22. Cyert, M. S. (2001) Annu. Rev. Genet. 35, 647–672
23. Aramburu, J., Rao, A., and Klee, C. B. (2000) Curr. Top. Cell Regul. 36, 237–295
24. Cyert, M. S. (2003) Biochem. Biophys. Res. Commun. 311, 1143–1150