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The role of the protein kinase A pathway in the response to alkaline pH stress in yeast

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Exposure of *Saccharomyces cerevisiae* to alkaline pH provokes a stress condition that generates a compensatory reaction. In the present study we examined a possible role for the PKA (protein kinase A) pathway in this response. Phenotypic analysis revealed that mutations that activate the PKA pathway (*ira1 ira2*, *bcy1*) tend to cause sensitivity to alkaline pH, whereas its deactivation enhances tolerance to this stress. We observed that alkalinization causes a transient decrease in cAMP, the main regulator of the pathway. Alkaline pH causes rapid nuclear localization of the PKA-regulated Msn2 transcription factor which, together with Msn4, mediates a general stress response by binding with STRE (stress response element) sequences in many promoters. Consequently, a synthetic STRE–LacZ reporter shows a rapid induction in response to alkaline stress. A *msn2 msn4* mutant is sensitive to alkaline pH, and transcriptomic analysis reveals that after 10 min of alkaline stress, the expression of many induced genes (47 %) depends, at least in part, on the presence of Msn2 and Msn4. Taken together, these results demonstrate that inhibition of the PKA pathway by alkaline pH represents a substantial part of the adaptive response to this kind of stress and that this response involves Msn2/Msn4-mediated genome expression remodelling. However, the relevance of attenuation of PKA in high pH tolerance is probably not restricted to regulation of Msn2 function.

Key words: alkaline stress, gene expression, Msn2, Msn4, protein kinase A (PKA), *Saccharomyces cerevisiae*, transcription factor.

INTRODUCTION

The regulation of the activity of the cAMP/PKA (protein kinase A) pathway plays a major role in the control of metabolism and cell proliferation in yeast cells, linked primarily to the available carbon source. In *Saccharomyces cerevisiae*, for instance, in response to a rapidly fermentable carbon source such as glucose the pathway activates the Cyr1 adenylate cyclase, which results in a transient increase in cAMP levels. PKA is a heterotetramer composed of two catalytic subunits and two regulatory subunits. The catalytic subunits can be encoded by three largely redundant genes (*TPK1*, *TPK2* and *TPK3*), whereas the regulatory subunits are encoded by a single gene (*BCY1*). Binding of a second messenger cAMP to the regulatory subunits results in dissociation of the complex and activation of PKA. Restoration of cAMP levels is controlled by the low- and high-affinity phosphodiesterases encoded by *PDE1* and *PDE2* respectively, which hydrolyse cAMP to AMP. Sequentially, PKA affects diverse downstream targets often at the gene transcription level, including the stimulation of cell growth and cell cycle progression, up-regulation of glycolysis, down-regulation of gluconeogenesis, and mobilization of glycogen and trehalose [1–5].

PKA can be activated in response to glucose by two parallel signalling pathways. The first involves the Ras1 and Ras2 small GTPases, which are activated by glucose uptake and phosphorylation. The active (GTP-bound) Ras proteins increase the activity of the adenylate cyclase. In turn, the GDP/GTP exchange on the Ras proteins is controlled by the GEFs (guanine-nucleotide-exchange factors) Cdc25 and Sdc25 (*S. cerevisiae* homologue of Cdc25). The reverse process is accelerated by the Ira proteins (encoded by the *IRA1* and *IRA2* genes), which act as Ras GAPs (GTPase-activating proteins) and maintain Ras in the GDP-bound inactive state. The second pathway involves Gpr1 (a putative G-protein-coupled receptor) and its Gα protein Gpa2. Both pathways converge to activate adenylate cyclase, resulting in the generation of cAMP [1–3].

Activation of PKA has a major impact on gene expression. Consequently, several transcription factors are among the known PKA targets. Two of those are Msn2 and Msn4, which mediate the transcription of the so-called STRE (stress response element)-controlled genes [6–8]. STRE-regulated genes are involved in important processes such as carbohydrate metabolism and growth regulation, as well as in adaption to diverse types of stress, including heat, DNA damage, and oxidative and osmotic stresses [9–12]. Under growth-promoting conditions (growth on glucose with the absence of stress), Msn2 and Msn4 are phosphorylated and reside in the cytosol. Upon glucose exhaustion or other stress conditions [13], they become hypophosphorylated and translocate to the nucleus, where they induce expression of the STRE-controlled genes. PKA plays a very important role inhibiting nuclear import of Msn2/Msn4, either through direct phosphorylation of their nuclear localization signal [13–15] or indirectly via the protein kinases Yak1 and Rim15.

S. cerevisiae grows far better at acidic than neutral or alkaline pH and consequently even a modest alkalinization of the medium represents a stress situation that is able to trigger a compensatory multifactorial response (for a review see [16]). Alkaline stress activates diverse signalling pathways, including the Rim101/Nrg1 [17,18], the calcium/calcineurin [19–21] and the Wsc1/Pkc1/Slt2 MAPK (mitogen-activated protein kinase) pathways [22]. Alkalinization of the environment also

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Table 1   Yeast strains used

Except otherwise indicated, single BY4741-derived *kanMX* disruptans that are not listed here correspond to the systematic gene disruption project [25].

Name	Relevant genotype	Source/reference
BY4741	MATa his3Δ1 leu2Δ met15Δ ura3Δ	[25]
MAR231	BY4741 *bcy1::kanMX4*	The present paper
W303-1A	*MATa ade2-1, can1-100, his3-112, leu2-3, trp1-1, ura3-1*	[52]
SC7	W303-1A *ira1::LEU2*	[42]
SC8	W303-1A *ira2::URA3*	[42]
PM903	W303-1A *ira1::LEU2 ira2::URA3*	[42]
WΔN1	W303-1A *cdc25::CDC25 aa907-1589/URA3*	[53]
WΔN2	W303-1A *cdc25::CDC25 aa1147-1589/URA3*	[53]
CCV35	W303-1A *pde1::kanMX4*	The present paper
PM942	W303-1A *pde2::URA3*	[27]
CCV36	W303-1A *pde1::kanMX4 pde2::URA3*	The present paper
MCY5278	W303-1A *msn2::kanMX4 msn4::hphMX4*	M. Carlson
CCV174	W303-1A *nrg1::nat1*	The present paper
CCV175	W303-1A *msn2::kanMX4 msn4::hphMX4 nrg1::nat1*	The present paper
tpk123 msn2 msn4	W303-1A *tpk1::URA3 tpk2::HIS3 tpk3::TRP1 msn2::HIS3 msn4::TRP1*	[14]
CCV37	W303-1A *snf1::LEU2*	The present paper
CCV38	W303-1A *msn2::kanMX4 msn4::hphMX4 snf1::LEU2*	The present paper
BY4742	MATα his3Δ1 leu2Δ met15Δ ura3Δ	[25]
DC90	BY4742 *tpk1::kanMX::HIS3 tpk2^{w3} tpk3::LEU2*	J.M. Thevelein
DBY746	MATa *ura3-52 leu2-3112 his3-1 trp1-239*	D. Botstein
AGS66	DBY746 *URA3*-STRE(7×)–*lacZ*	[29]

disturbs nutrient homoeostasis, as deduced from its impact on iron/copper and phosphate uptake/utilization pathways [19,23]. Work in our laboratory in previous years has shown that alkaline pH stress also has a profound impact on the expression of genes encoding glucose uptake and metabolism-related proteins, finding that exposure to high pH would mimic a situation of glucose starvation [20,21]. Given the strong link between carbohydrate metabolism and the PKA pathway, we speculated that alkaline stress might involve changes in the activity of this pathway and specifically that the correct adaptation to high pH could entail its down-regulation. Indeed, in the present paper we showed that alkaline pH stress causes a transient decrease in cAMP levels and that change in the activity of the PKA pathway alters tolerance to alkaline pH. The results of the present paper also indicate that the adaptive response to high pH involves PKA-regulated Msn2/Msn4-mediated gene remodelling.

MATERIAL AND METHODS

Yeast strains and growth conditions

Yeast cells (the strains are listed in Table 1) were grown at 28°C in YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose) or when carrying plasmids in synthetic complete medium [24] containing 2% glucose and lacking the appropriate selection requirements. Single *kanMX* deletion mutants on the BY4741 background, except MAR231, were generated in the context of the *Saccharomyces* Genome Deletion Project [25].

The MAR231 strain was made by transforming BY4741 with a *bcy1::kanMX4* cassette obtained from the KKY385 strain [26] by PCR amplification using the primers 5′-bcy1_disr (GAGGAGCATACGACTTCGGC) and 3′-bcy1_disr (CTGTCTTGTAGATCCTTTGG). The CCV35 and CCV36 strains were constructed as follows. A 1.6 kbp *pde1::kanMX4* cassette was amplified from genomic DNA of the BY4741 *pde1::kanMX4* strain with the oligonucleotides 5′-pde1 (CAAGGATCGTTACCCGGTA) and 3′-pde1 (GACTTATGT-TGGGATAGGGG). The purified DNA fragment was used to transform the W303 or PM942 strains [27], to yield the CCV35

and CCV36 strains respectively. The CCV37 and CCV38 strains were obtained transforming W303-1A wild-type and MCY5278 strains with a *SNF1::LEU2* disruption cassette [21] and the CCV174 and CCV175 strains were generated by transforming W303-1A and MCY5278 strains with the 2.1 kbp *nrg1::nat1* cassette from the plasmid pBS-nrg1::nat1 as described previously [28]. The AGS66 strain contained an integrated STRE(7×)–*lacZ* reporter system at the *URA3* locus [29].

Plasmids

The reporter plasmid p*HXK1*-lacZ was generated as follows. The *HXK1* upstream DNA region containing −624 and +39 relative to the starting ATG was amplified by PCR with added BamHI/PstI restriction sites and cloned into the same sites of YEp357 [30]. The plasmid YCp50-RAS2^{Ala18Val19} [31] expressed the *RAS2*^{Ala18Val19} hyperactive Ras2 allele [32] from the centromeric YCp50. The plasmid pG2CT-112.2 expressed the constitutively active *GPA2*^{R273A} allele from the episomal YEplac112 backbone [31]. The plasmid pAMS366 contains a tandem of four CDREs (calcineurin-dependent response elements) from the *GSC2*/*FKS2* gene fused to the lacZ reporter [33]. pPHO84-LacZ contains a PHO84-LacZ reporter fusion as described in [19]. pKC201 contains the *ENA1* promoter fused to *LacZ* [34,35].

Growth tests

The sensitivity of different yeast strains to alkaline pH was assayed by drop test on YPD plates containing 50 mM Taps adjusted with KOH at different pH values. Growth in liquid medium at high pH was performed in 5 ml cultures or in 96-well plates (250 μl), at 28°C in YPD medium buffered with 50 mM Taps and adjusted with KOH at the pH values indicated below. Growth was monitored by measuring the absorbance (A) at 660 nm. BY4742 and DC90 cells at an initial A_{660} of 0.001 were grown for 17 and 24 h respectively. The strains prepared from the W303-1A genetic background were grown from an initial A_{660} of 0.01 for

17 h (wild-type and MCY5278 strains) and for 21 h (CCV37 and CCV38 strains).

Measurement of cAMP levels

Measurements were made as described in [36]. Briefly, BY4741 cells were grown to an A_{660} value of approximately 1.0 in YPD at 28°C, and then the culture was centrifuged (5 min at 1220 **g** at room temperature) and resuspended in YPD containing 50 mM Taps (pH 8). Aliquots of 10 ml were filtered at the indicated times and extracted in the cold with 1 ml of 2 M perchloric acid. After neutralization with 1 ml of 1.8 M KOH and 0.4 M KHCO$_3$ and centrifugation (5 min at 1220 **g** at 4°C), the samples were purified with Amprep SAX minicolumns (Amersham), eluted with methanol/HCl and dried under vacuum. The assays were performed by a competitive binding method with the Amersham cAMP EIA (enzyme immunoassay) system. For calculation of yeast concentrations, one unit of absorbance at 660 nm was equivalent to 2.6 mg of wet weight/ml. In other experiments, the pH was raised to 8.2 by the addition of KOH (35 mM, final concentration). The control cells received the same concentration of KCl.

β-Galactosidase activity assay

Yeast cells were grown to saturation in the appropriate dropout medium and then inoculated into YPD medium (or YP plus 4% glucose, where indicated) at pH 5.5. Growth was resumed until A_{660} was 0.5–0.7 and the cultures were centrifuged at room temperature for 5 min at 1620 **g**. The cells were resuspended in YPD or YP 4% glucose where indicated (no induction) or YPD (or YPD 4% glucose) plus 50 mM Taps adjusted to pH 8.0 (alkaline stress), and growth was resumed for the indicated times. In all cases, β-galactosidase activity was measured as described previously [37].

Microscopy techniques

For the Msn2 subcellular localization experiments, the indicated strains were transformed with the plasmid pMSN2-GFP (given by Professor Francisco Estruch, University of Valencia, Valencia, Spain), a YCplac111-based vector that contains a C-terminal Msn2–GFP (green fluorescent protein) fusion [13]. The cells were grown in YPD or YP 2% ethanol until an A_{660} value of 0.8–1.0 was reached. The cultures (5 ml) were treated by either the addition of 100 μl of 1 M KCl (control cells, pH 5.5) or of 100 μl of 1 M KOH (alkaline stress, pH 8.0). Samples (500 μl) were taken at the appropriate times and fixed for 5 min by adding 30 μl of 37% formaldehyde. The cells were harvested, washed three times with PBS and concentrated 10-fold before visualization. In all cases the cells were visualized with a fluorescein filter using a Nikon Eclipse E800 fluorescence microscope (magnification, 1000×). Digital images were captured with an ORCA-ER 4742-80 camera (Hamamatsu) using Wasabi software. Intracellular distribution of Msn2–GFP was quantified by scoring at least in 200 cells per sample into one of three possible categories: cytoplasmic (fluorescence in the cytoplasm only), nuclear/cytoplasmic (fluorescence in the cytoplasm and nucleus) and nuclear (fluorescence in the nucleus only).

RNA purification, cDNA synthesis and DNA microarray experiments

For RNA purification, 50 ml of yeast culture (strains W303-1A and MCY5278) was grown at 28°C in YPD medium until an A_{660} value of 0.6–0.8 was reached, and then KOH or KCl was added from a concentrated stock solution (1 M) to reach a final concentration of 20 mM (pH 8.05 and pH 5.5 respectively). Yeast cells were harvested by filtration after 10 and 30 min and washed with ice-cold water; dried cells were kept at −80°C until RNA purification was performed. Total RNA was purified using a RiboPure-Yeast kit (Ambion) following the manufacturer's instructions. RNA quality was assessed by denaturing 0.8% agarose gel electrophoresis, and RNA quantification was performed by measuring absorbance at 260 nm in a BioPhotometer (Eppendorf). Transcriptional analyses were performed using DNA microarrays (printed at the Universitat Autònoma de Barcelona Genomics Facility) containing PCR-amplified fragments from 6014 *S. cerevisiae* open reading frames [20,38]. Fluorescent Cy3- and Cy5-labelled cDNA probes were prepared from 8 μg of purified total RNA by the indirect dUTP labelling method using a CyScribe post-labelling kit (Amersham Biosciences).

Pre-hybridization, hybridization and washes were carried out as recommended by The Institute for Genomic Research with minor modifications. Briefly, prehybridizations of the DNA microarrays were carried out at 42°C for 1 h in a solution containing 5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1% SDS and 1% BSA. For hybridization, dried Cy3 (indocarbocyanine)- and Cy5 (indodicarbocyanine)-labelled probes were resuspended in 35 μl of hybridization solution (50% formamide, 5× SSC and 0.1% SDS) each and mixed. Salmon sperm DNA (5 μg) was added to the mix before denaturation for 3 min at 95°C. DNA microarrays were hybridized in an ArrayBooster hybridization station (Sunergia Group) for 14 h at 42°C. The scanner ScanArray 4000 (Packard Instrument) was used to obtain the Cy3 and Cy5 images with a resolution of 10 μm. The fluorescent intensity of the spots was measured and processed using the GenePix Pro 6.0 software (Molecular Devices). Spots with either a diameter smaller than 120 μm or fluorescence intensity for Cy3 and Cy5 lower than 150 units were not considered for further analysis.

For each condition assayed, two independent experiments were performed and dye swapping was carried out for each experiment. Microarray data was deposited at the GEO (Gene Expression Omnibus; accession number GSE27925). The results from different experiments were combined, and the mean was calculated. A given gene was considered to be induced or repressed when the mean of the ratios (alkaline stress compared with no stress) was >2.0 or <0.50 respectively. Software from the GEPAS server (http://gepas.bioinfo.cipf.es/) was used to carry out clustering and other data analyses [39]. According to the expression of these genes in the *msn2 msn4* strain, different levels of dependence on these transcription factors were defined. Thus genes showing a mutant/wild-type ratio of 0.67 > X > 0.50 were considered 'weakly dependent', those with a ratio 0.50 > X > 0.25 were ranked as 'strongly dependent' (SD) and those with a ratio ⩽ 0.25 were defined as 'totally dependent'. Likewise, genes induced more than 2.5-fold in wild-type cells and considered not induced (i.e. the ratio of alkaline stress/no stress) <1.3) in *msn2 msn4* cells were also considered as totally dependent. This score is the same employed in our previous report on calcineurin dependence of high pH response [20].

RESULTS

Manipulation of upstream elements of the PKA pathway affects growth at alkaline pH

Our starting hypothesis was that adaptation to high pH stress should entail a decrease in the PKA pathway activity. Therefore we speculated that mutations increasing the activity of the
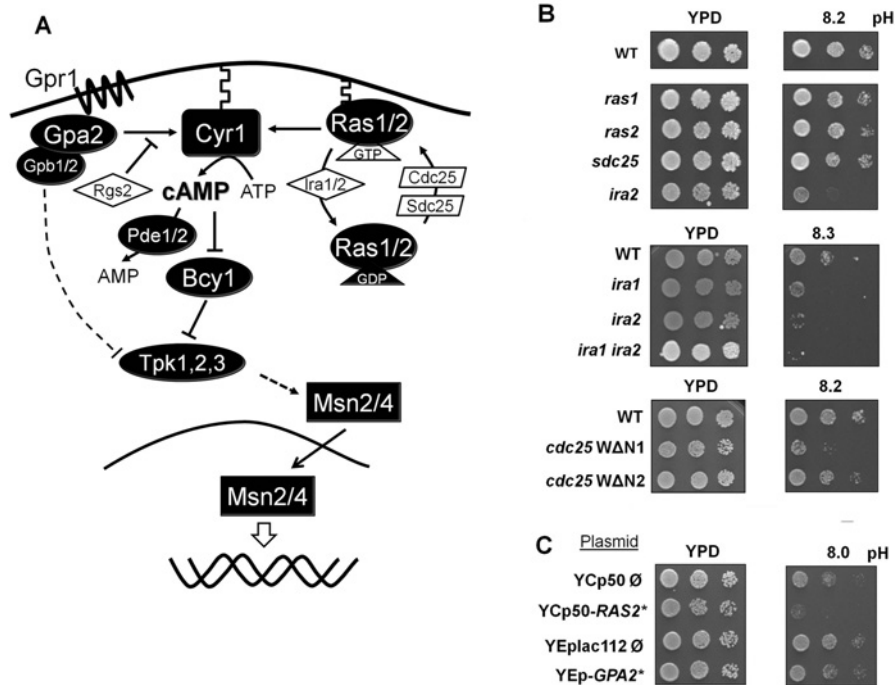
Figure 1   Effect of mutations in the upstream components of the PKA pathway on alkaline pH tolerance

(**A**) A simplified schematic depiction of the PKA signalling pathway. See the Introduction section for additional information. (**B**) Upper panels: three dilutions of cultures of wild-type BY4741 cells and the isogenic *kanMX* disruption derivatives *ras1, ras2, sds25* and *ira2* mutants were spotted on to YPD plates adjusted at the indicated pH values. Middle panels: wild-type strains W303-1A and its *ira1::LEU2* (strain SC7), *ira2::URA3* (SC8) *and ira1::LEU2 ira2::URA3* (PM903) derivatives were spotted as above. Lower panels: wild-type strains W303-1A and isogenic cells expressing the WΔN1 or WΔN2 alleles of *CDC25* were spotted. All plates were incubated for 3 days. (**C**) Wild-type strain W303-1A was transformed with the indicated plasmids. Positive clones were grown overnight in synthetic selective medium and spotted on to YPD plates adjusted at the indicated pH and growth was monitored after 2 days. YCp50-*RAS2** expresses a hyperactive allele (Ras2[Ala18Val19]) of the Ras protein. YEp-*GPA2** generates a constitutively active Gpa2[R273A] version of the protein.

pathway should result in a decreased tolerance to alkaline pH. We first tested strains lacking components of the Gpr1/Gpa2-sensing pathway such as *gpr1*, *gpa2*, *rgs2* (lacking a GAP of Gpa2), *gpb1* or *gpb2* (lacking the multistep regulator of cAMP/PKA signalling) (Figure 1A). However, none of these strains showed an altered tolerance to alkaline pH (results not shown). In contrast, deletion of *ira1* or *ira2*, the GAPs of Ras1 and Ras2, resulted in increased sensitivity. This phenotype was enhanced in the double *ira1 ira2* mutant (Figure 1B). Conversely, expression of the Cdc25 WΔN1 allele, which lacks the N-terminal domain of Cdc25 and elicits constitutive activation of Ras proteins, also results in poor growth at high pH (Figure 1B). All of these manipulations lead to increased Ras activity and hyperactivation of the PKA pathway. In contrast, the effect on high pH tolerance of the expression of the Cdc25 WΔN2 allele which does not lead to an increase in PKA activity was barely noticeable. These results were confirmed by expression of the hyperactive Ras allele *RAS2*[Ala18Val19] from a centromeric plasmid [31]. Among other phenotypes, expression of this allele decreased heat-shock tolerance. The expression of the *RAS2*[Ala18Val19] allele decreased alkaline pH tolerance (Figure 1C). In contrast, expression of a constitutively active form of Gpa2 (pG2CT-112.2) constructed by replacing the arginine residue at position 273 with an alanine residue [31] did not alter high pH tolerance.

Alkaline pH stress transiently decreases the levels of cAMP

cAMP levels determine the interaction between the regulatory subunit (Bcy1) and the catalytic subunits (Tpk) of PKA and are therefore crucial for the activity of the kinase. We have measured

the levels of cAMP after shifting the cells from pH 5.5 to medium buffered to pH 8.0 and found that this treatment drastically decreased the concentration of the second messenger cAMP in the first 5–15 min, followed by a recovery to the initial levels after 30 min of stress (Figure 2A). A similar result was obtained when KOH was added directly to the exponentially growing cultures to raise the pH. In this case the decrease in cAMP levels was apparent even only 2 min after stress (results not shown). Raising the pH of the liquid medium up to 8.2 did not result in detectable cell lysis, as determined by microscopic examination, viability counting or release of alkaline phosphatase activity to the medium.

The results of the present paper were consistent with the possibility that a drop in cAMP levels leading to down-regulation of PKA activity could be an adaptive strategy to confront high pH stress. Therefore we considered that mutations resulting directly in increased cAMP levels should be deleterious for cells subjected to alkaline pH stress. The *PDE1* and *PDE2* genes encode the yeast cAMP phosphodiesterase activity, which degrades cAMP to AMP. When the single mutants are grown on alkaline-pH plates, a growth defect can be observed for the *pde2* mutant (lacking the high-affinity isoform) that is exacerbated in the double *pde1 pde2* mutant strain (Figure 2B).

Lack of Bcy1, the regulatory subunit of PKA, leads to constitutive PKA activity, which results in a variety of phenotypes (impaired growth on different carbon sources, temperature sensitivity etc.; see [2] and references therein). Although the quantification of the effect is difficult because of the relatively poor growth of the *bcy1* mutant even under standard conditions, we can show that this strain exhibits a dramatic alkaline pH-sensitive phenotype (Figure 2C). The same effect was observed
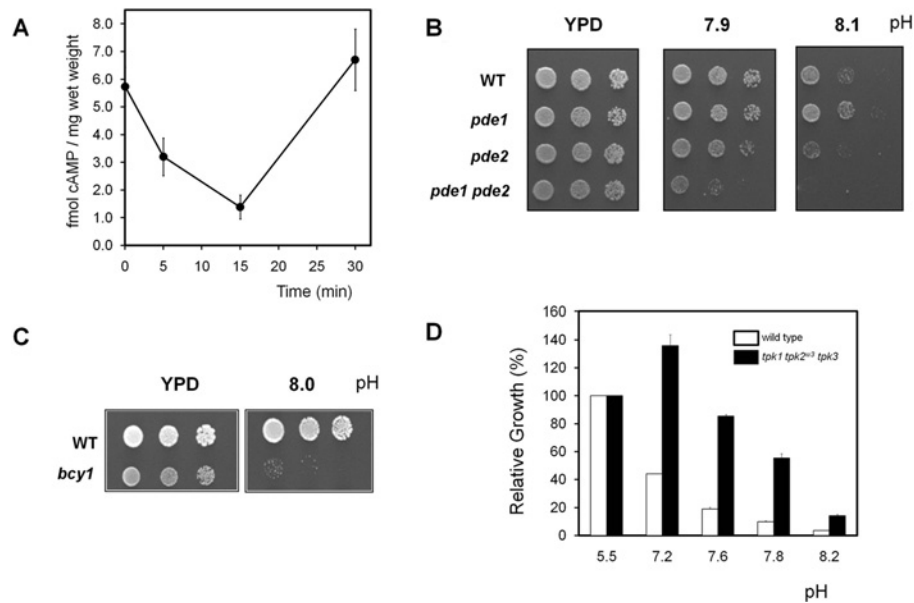
Figure 2   Changes in PKA pathway activity influences high pH tolerance

(**A**) Wild-type BY4741 cells were subjected to alkaline pH stress as described in the Materials and methods section and cultures were processed for cAMP determination. Results are means ± S.E.M of at least four independent experiments. (**B**) Wild-type (WT) W303-1A cells and their derivatives *pde1* (CCV35), *pde2* (PM942) and *pde1 pde2* (CCV36) were spotted on to YPD plates adjusted at the indicated pH values. Growth was monitored after 2 days. (**C**) The BY4741 strain (WT) and its *bcy1* derivative (MAR231) were grown on YPD plates adjusted at the indicated pH for 3 days. (**D**) Wild-type strain BY4742 (open bars) and its derivative DC90 (*tpk1 tpk2*^w3^ *tpk3*, closed bars) were grown on liquid YPD medium as described in the Materials and methods section. Growth is represented as the percentage over the same strain at initial pH 5.5. Results are the average ± S.E.M. from two independent experiments.

in the W303-1A background (results not shown). Similarly, we tested whether a decrease in PKA activity could improve growth at high pH. Since complete lack of PKA activity is not compatible with survival, for this experiment we resorted to a strain lacking *TPK1* and *TPK3* and carrying an attenuated allele of *TPK2* (*tpk2*^w3^) as the sole source of PKA activity. As shown in Figure 2(D), the relative growth of this strain in alkaline pH medium compared with non-restrictive conditions is higher than the wild-type strain. In essence, the results presented so far show that yeast cells respond to the alkalinization of their medium with a sharp decrease in the intracellular level of cAMP. The observation that when PKA activity is attenuated cells grow better at high pH, whereas any situation resulting in increased PKA activity makes cells more sensitive to this condition, supports the notion that down-regulating PKA activity contributes to adaptation to alkaline pH stress.

Alkaline pH stress promotes entry of Msn2 in the nucleus and STRE-dependent promoter activation

Nuclear localization of Msn2, a transcription factor that together with Msn4 regulates the general stress response of *S. cerevisiae*, is controlled by PKA activity [13,14,40]. We considered that alkaline pH-triggered down-regulation of the PKA activity could result in the translocation of Msn2 to the nucleus and that this transition might have a relevant effect in the pH-induced adaptive response. To test this possibility, an Msn2–GFP fusion was introduced into wild-type cells and the cultures subjected to alkaline treatment. As shown in Figures 3(A) and 3(B), exposure to high pH (8.0) triggered an almost immediate entry of Msn2 into the nucleus, which reached a maximum after 5 min (Figure 3B). Nuclear localization of Msn2 was transient and after 10–15 min the transcription factor was again mostly cytosolic. Interestingly,

when the same experiment was carried out in a strain lacking the *IRA2* gene (sensitive to alkaline pH, see Figure 1B), entry of Msn2 into the nucleus was somewhat delayed and nuclear localization was a less general effect than in the wild-type strain. Nuclear translocation of Msn2 was also evaluated in cells subjected to alkaline stress (pH 8.0) growing in the presence of ethanol as a carbon source (Figure 3B). Under these conditions, Msn2 showed both cytoplasmic and nuclear/cytoplasmic distribution in unstressed cells. Interestingly, exposure of cells to alkalinization resulted in only a modest increase in the percentage of cells showing nuclear/cytoplasmic distribution, but very few cells with only nuclear Msn2. Therefore subcellular distribution of Msn2 in response to alkaline stress is affected by the carbon source in which cells are grown.

We then investigated whether the Msn2 and Msn4 transcription factors are necessary for a normal tolerance to high pH stress. As shown in Figure 4(A), lack of these transcription factors somewhat reduces tolerance to high pH, suggesting that they are components of the adaptive response to alkalinization. Remarkably, additional deletion of all three PKA catalytic subunits (which is feasible in the absence of the transcription factors) yields cells that are even more sensitive than the *msn2 msn4* mutant. It should be noted that in this case the *msn2 msn4* strain was transformed with a centromeric plasmid which contains a *TRP1* marker. The introduction of this marker in the reference strain was necessary for accurate comparison because the *tpk msn2,4* strain was constructed using the *TRP1* gene as a marker, and it has been reported that the absence of this gene somewhat decreases tolerance to high pH [41]. These results suggested that, besides its role in Msn2,4 function, regulation of PKA activity may exert additional effects on high pH tolerance. This notion was reinforced by the results shown in Figure 4(B). We observed that expression of the hyperactive form of *RAS2* further decreased high pH tolerance even in the absence of Msn2/Msn4 transcription factors,
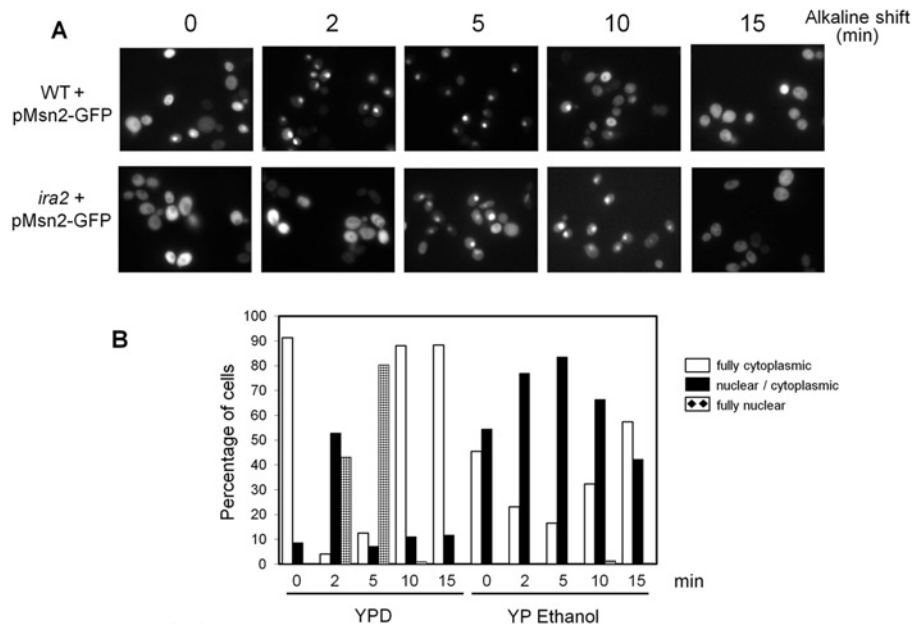
Figure 3   Alkaline pH triggers nuclear entry of the Msn2 transcription factor

Cultures of wild-type BY4741 (WT) and its *ira2::kanMX* derivative (right-hand panels) were transformed with plasmid pMsn2-GFP and subjected to pH stress (8.0) on YPD medium. Samples were collected at the specified times and fixed for fluorescence microscopy. (**B**) BY4741 cells carrying the pMsn2-GFP construct were grown to exponential phase (A_{660} of 0.8) on YPD medium or YP medium containing ethanol (2 %) as the carbon source, shifted to pH 8.0. The subcellular distribution of the Msn2–GFP fusion was monitored for at least 200 cells per time point. Open bars denote full cytoplasmic, closed bars denote nuclear/cytoplasmic and crossed bars denote full nuclear localization. The results shown correspond to a representative experiment. Three independent experiments were performed with similar results.

suggesting again that not all of the effects relevant for alkaline tolerance mediated by PKA are based necessarily on the activation of Msn2/Msn4. On the other hand, cells lacking the Snf1 kinase are sensitive to alkaline pH and Snf1 has been reported to repress Msn2 function. Therefore we considered whether the sensitive phenotype of *snf1* cells could be due to deregulation of Msn2 function. As shown in Figure 4(C), *snf1* cells are more sensitive than the *msn2 msn4* mutant to alkaline pH. In addition, deletion of *msn2 msn4* further increases sensitivity to alkaline pH of the *snf1* mutant. Therefore deregulation of Msn2/Msn4 is not the basis of the *snf1* alkali-sensitive phenotype.
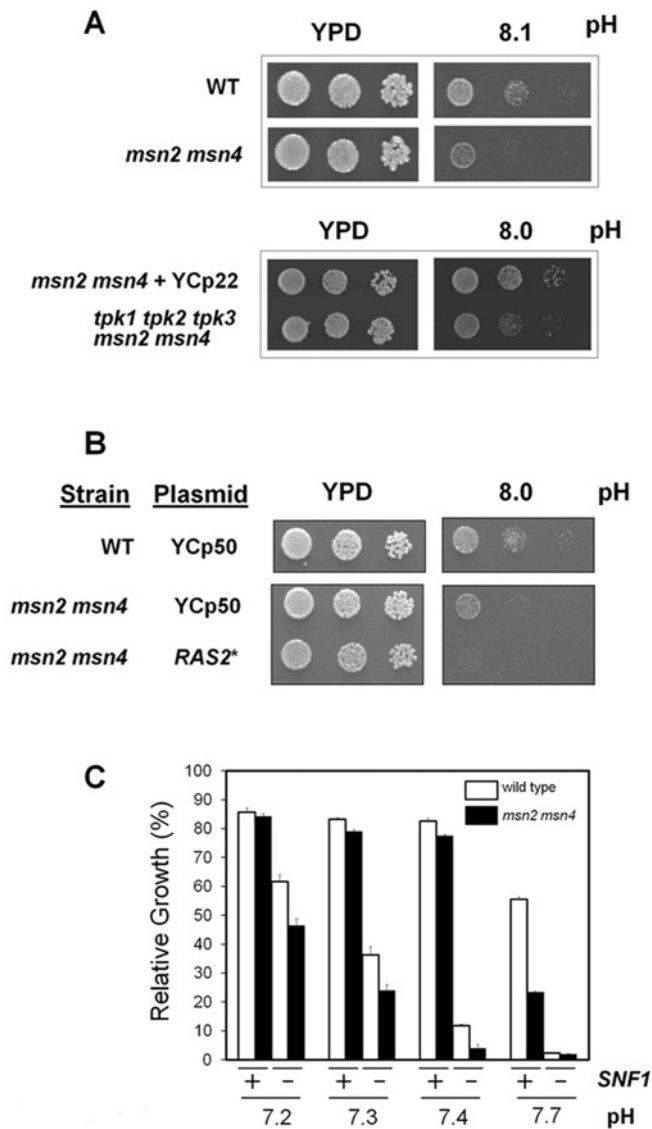
Because Msn2 is known to bind with STRE elements in response to glucose shortage and other forms of stress, we tested the activation of a synthetic promoter containing a tandem of seven STRE elements fused to the *LacZ* reporter (the AGS66 strain). As shown in Figure 5(A), this promoter exhibited a very fast activation with a peak of activity 15 min after moving the cells to a pH 8.0 environment. This activation was much faster than that observed when the *LacZ* gene is expressed from a *PHO84* promoter, which is considered a gene with a relatively late response to high pH stress [19] and it was completely abolished in a *msn2 msn4* strain (results not shown). Remarkably, the response was also faster than that obtained from a synthetic promoter based in a cluster of four copies of the CDRE motif from the calcineurin-responsive *GSC2/FKS2* gene. It must be noted that the calcineurin/Crz1-mediated response is considered to be an early response to alkaline pH stress [19,20]. We also tested the response to alkaline pH of the *HXK1* gene which encodes hexokinase, a gene with predicted STRE elements in its promoter [10] and known to be induced by high temperature (37°C) and oxidative stress in a *msn2 msn4*-dependent way [12]. As shown in Figure 5(B), expression of the *HXK1* promoter induced by high pH stress was as fast as the one induced by exposure to 37°C, although

the level of β-galactosidase activity declined faster (probably due to yeast-promoted acidification of the medium). Remarkably, in the *msn2 msn4* strain the basal activity of the promoter, as well as the response to both stress conditions, is dramatically reduced. However, a small increase can still be detected after 30 min of exposure to high pH stress, suggesting an Msn2/Msn4-independent input to the promoter, which could be attributed to activation of calcineurin as this gene was demonstrated to be induced by calcium as well as by high pH [21].

Relevance of Msn2/Msn4 transcription factors in the transcriptional response to high pH stress

Since these results indicated that Msn2/Msn4 could be responsible for the response of certain genes to alkaline pH stress, we considered it necessary to evaluate to what extent this pathway is responsible for the remodelling of gene expression observed upon exposure to high pH. To this end, DNA microarray analysis was conducted using wild-type and *msn2 msn4* strains subjected to pH 8.0 for 10 and 30 min. After 10 min of exposure to high pH, in the wild-type cells 331 genes had a magnitude of induced expression at least 2-fold greater, whereas only 186 genes found in the *msn2 msn4* cells strain (from a total number of 3463 genes with valid data; Figure 6 and Supplementary Table S1 at http://www.BiochemJ.org/bj/438/bj4380523add.htm).

GO (Gene Ontology) analysis of the genes induced in the wild-type strain yielded, as expected, an excess of genes related to carbohydrate metabolism ($P < 7.74 \times 10^{-9}$), in particular to trehalose ($P < 5.05 \times 10^{-8}$) and glucose ($P < 1.42 \times 10^{-6}$) and glycogen ($P < 1.61 \times 10^{-3}$) metabolism. Analysis of the *msn2 msn4* dependence for induction revealed that 157 genes required the presence of the transcription factors for full induction (Figure 6). Although dependence was limited in some cases (62

expression at least 2-fold in the *msn2 msn4* mutant (Figure 6). In this case, the level of dependence of the transcription factors was much lower (only 50 genes). In addition, the vast majority (40) were weakly dependent; nine were ranked as strongly dependent and only one as totally dependent. GO analysis of the set of strongly dependent plus totally dependent genes did not produce any specific profile. Therefore Msn2 and Msn4 transcription factors are responsible for the induction of a substantial subset of the early alkali-responsive genes. Analysis of repressed genes showed 146 and 279 genes repressed at least 0.5-fold in the wild-type strain 10 and 30 min after a shift to high pH respectively. The absence of *msn2 msn4* did not result in activation of any of these genes.

The *ENA1* gene encodes a Na^+-ATPase that has been reported repeatedly to be strongly induced upon alkaline pH stimulation. Interestingly, our microarray results indicate that deletion of *MSN2/MSN4* does not affect the induction of this ATPase gene in the short-term (10 min), but results in higher-than-normal expression after 30 min of stress (Figure 7A). In an attempt to identify a possible cause for this behaviour we searched our microarray raw data for changes in the expression of genes encoding known regulatory components of *ENA1*. We observed that after 10 min after alkalinization of the medium, the expression of *NRG1*, a known repressor of *ENA1* expression, was essentially identical in the wild-type and *msn2 msn4* cells. However, after 30 min the expression of *NRG1* was decreased by 37% in the *msn2 msn4* strain compared with the wild-type. To explore a possible influence of this change on the response of *ENA1*, we evaluated the expression from the ATPase gene promoter in wild-type and *msn2 msn4* cells deleted for *NRG1* by means of a *LacZ* reporter. As it can be observed (Figure 7B), high-pH-dependent expression from the *ENA1* promoter is stronger in the *msn2 msn4* mutant than in the wild-type strain, thus confirming the microarray results. Moreover, the induction observed upon alkaline pH stimulation in the *nrg1* strain is virtually identical with that observed in the *msn2 msn4 nrg1* triple mutant, suggesting that changes in the Nrg1 levels could be at the basis of the abnormal response of *ENA1* in the *msn2 msn4* mutant.

DISCUSSION

Exposure to alkaline stress triggers a set of responses that are known to be mediated by different signalling pathways, such as the calcineurin/Crz1, the Rim101 and the Slt2 pathways [16]. PKA mediates a major signalling pathway that is crucial for linking stress responses and cell proliferation. Therefore the major goal of the present study was to evaluate the relevance of the PKA pathway in the adaptive response to high pH stress. We observed that mutations leading to activation of PKA resulted in decreased alkaline pH tolerance, whereas those that lead to inhibition of the pathway yield cells with increased tolerance. Our results also show that exposure to high pH provokes a sharp and transient decrease in the cAMP levels, which may lead to down-regulation of PKA activity. In this regard, a previous study reported a transitory increase of cAMP levels as a result of sudden acidification of the cytosol [42]. It is worth noting that these authors noticed that Gpa2 was not required for the stimulation of cAMP accumulation in response to intracellular acidification. Remarkably, we observe that only alterations in the small G-proteins Ras1 and Ras2 branch of the pathway do result in changes in tolerance to alkaline pH, whereas mutations in diverse genes involved in the Gpr1/Gpa2 glucose sensor system do not produce phenotypic effects. This suggests that the observed changes are due to alteration of intracellular glucose metabolism and not to

Figure 4   Effect of the lack of Msn2 and Msn4 transcription factors on high pH tolerance

(**A**) Upper panels: wild-type W303-1A alone (WT) was plated along with strain MCY5278, a W303-1A-derived strain lacking both *MSN2* and *MSN4* genes. Lower panels: the MCY5278 strain was transformed with centromeric plasmid YCp22 (which carries a *TRP1* gene marker, see the main text for an explanation) and plated with the equivalent strain but lacking all three *TPK* genes [14]. Dilutions of the cultures were grown for 2 days at the indicated pH. (**B**) The W303-1A strain (WT) and the *msn2 msn4* derivative transformed with the empty plasmid YCp50 or the same plasmid carrying a hyperactive *RAS2Ala18Val19* allele (*RAS2**). Dilutions of the cultures were spotted on to YPD plates at the indicated pH values and growth was monitored after 2 days. (**C**) The W303-1A strain (open bars) and its *msn2 msn4* derivative (closed bars) carrying a wild-type allele of *SNF1* (+) or a *snf1* deletion (−) were grown at the indicated pH values. Growth is denoted as the percentage compared with the same strains cultured at pH 5.5. Results are means ± S.E.M. from two independent experiments performed in triplicate.

genes were rated as weakly dependent), the majority of the short-term induced genes were strongly (57) or totally (38) dependent of the presence of Msn2 and Msn4. GO analysis of the 95 genes defined as strongly or totally dependent revealed a strong excess of genes encoding proteins involved in trehalose ($P < 9.40 \times 10^{-9}$) and glycogen ($P < 1.46 \times 10^{-7}$) metabolism. A similar analysis of genes showing weak dependence provided no distinctive profile.

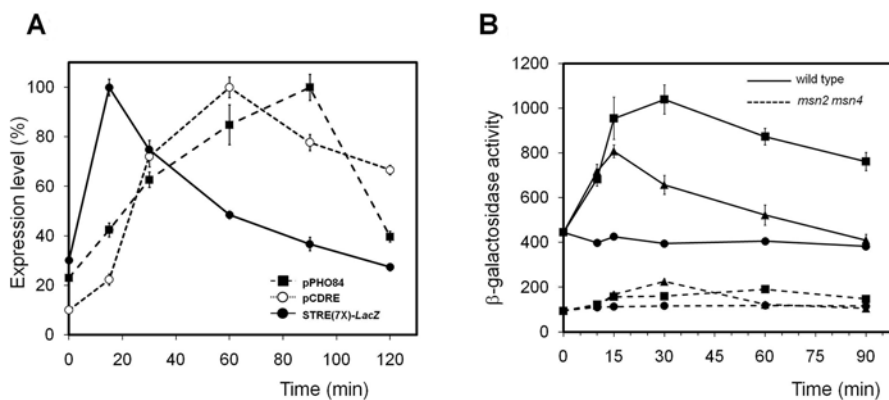Exposure of cells to pH 8.0 for 30 min resulted in 241 genes induced in the wild-type strain, whereas 296 genes increased

Figure 5   Alkaline pH stress induces STRE-mediated transcriptional activation

(**A**) The wild-type strain DBY746 was transformed with episomal plasmids pPHO84-LacZ (pPHO84) and pAMS366 (pCDRE). These strains, together with strain AGS66 [carrying a STRE(7×)–LacZ reporter system integrated at the *URA3* locus] were grown and subjected to high pH stress (pH 8.0). β-Galactosidase activity was measured as described and it is represented as the percentage over the maximum value for each strain. (**B**) The wild-type strain W303-1A (continuous lines) and its *msn2 msn4* derivative (broken lines) were transformed with the *HXK1*–LacZ reporter. Cultures grown on YP medium (plus 4 % glucose) were subjected to high pH (8.0, ▲) or high temperature (37°C, ■) and samples taken at different times for β-galactosidase activity measurements. Non-induced cultures are denoted by circles. Results are means ± S.E.M. from six independent experiments.
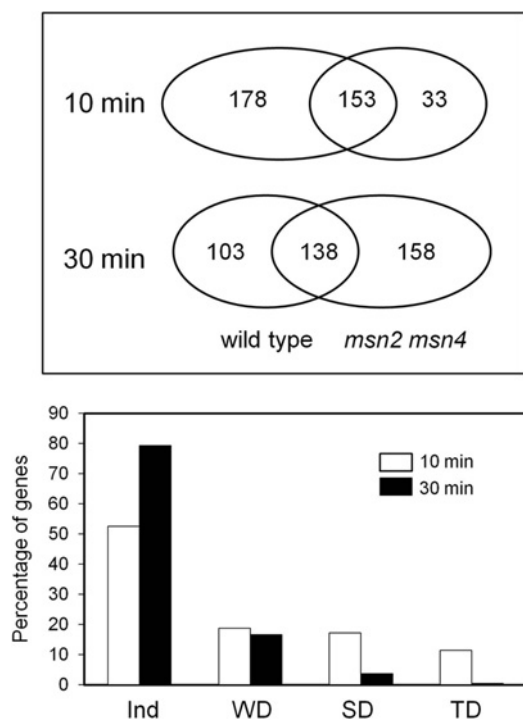
Figure 6   Msn2/Msn4 dependence of the transcriptional response to alkaline stress

Upper panel: number of genes induced in the wild-type and the *msn2 msn4* strains after 10 or 30 min of alkaline stress deduced from the DNA microarray analysis. Lower panel: evaluation of the Msn2/Msn4 level of dependence of the transcriptional response to high pH after 10 min (open bars) and 30 min (closed bars). Ind, Independent; WD, weakly dependent; SD, strongly dependent; TD, totally dependent.

defects in the extracellular glucose detection system, and reinforce the notion that changes in cytosolic pH may result in fluctuations in cAMP levels and consequent regulation of PKA activity. In the case of sudden alkalinization of the medium, the sharp decrease in cAMP levels would inhibit PKA activity and allow cells to adapt to the stress.

The results of the present study show that alkalinization of the medium triggers a very fast entry of the Msn2 transcription factor to the nucleus (2–5 min). This time frame is very similar to that observed when cells are shifted to a low-glucose medium [13,14]. Interestingly, the fact that we observe only a very modest response in ethanol-grown cells suggested that nuclear transition of Msn2 is related to alkaline stress-induced alterations in glucose signalling. We also show that Msn2 and Msn4 are important for mediating a substantial part of the transcriptional response induced by alkalinization of the medium. It is conceivable that this response constitutes one of the factors that allow normal tolerance to the stress, since the *msn2 msn4* mutant is sensitive to high pH (Figure 4A). Moreover, our results suggest that regulation of Msn2 function in response to high pH is the result of inhibition of PKA. It is known that intracellular localization of Msn2 can be regulated not only by PKA, but also by the Snf1 kinase [43,44] and the TOR (target of rapamycin) pathway [44,45], thus raising the possibility that these two pathways could contribute to the alkaline pH-triggered entry of Msn2 into the nucleus. In fact, activation of the Snf1 pathway has been previously linked to high pH stress [21,28,46]. However, we observe that mutation of *SNF1* yields cells even more sensitive to alkaline pH than those lacking *msn2 msn4*, and that deletion of both factors in a *snf1* background further decreases tolerance. Furthermore, we have observed that, within the time-span shown in Figure 3(A), entry into the nucleus of Msn2 is not affected by the lack of the protein kinase Snf1 after alkaline treatment (results not shown). Finally, comparison of the dependence level for Msn2,4 of the short-term transcriptional response to high pH described in the present paper with the dependence on Snf1, evaluated in a parallel project (A. Casamayor, A. Ruiz, R. Serrano, M. Platara, J. Ferrer-Dalmau and J. Ariño, unpublished work), indicates low overlap (only 34 of the 157 genes reported as Msn2,4-dependent in the present study are found to exhibit some degree of dependence for the Snf1 kinase). As an example, most of the genes encoding enzymes involved in trehalose metabolism whose expression is increased by alkalinization are Msn2,4-dependent, but not Snf1-dependent. Therefore the results of the present study do not support the notion that activation of Msn2/4 by high pH is mediated by the Snf1 kinase.

The PKA and TOR pathways illustrate diverse ways of functional interaction in the regulation of cell growth, showing some functional overlap [47]. However, a role of the TOR
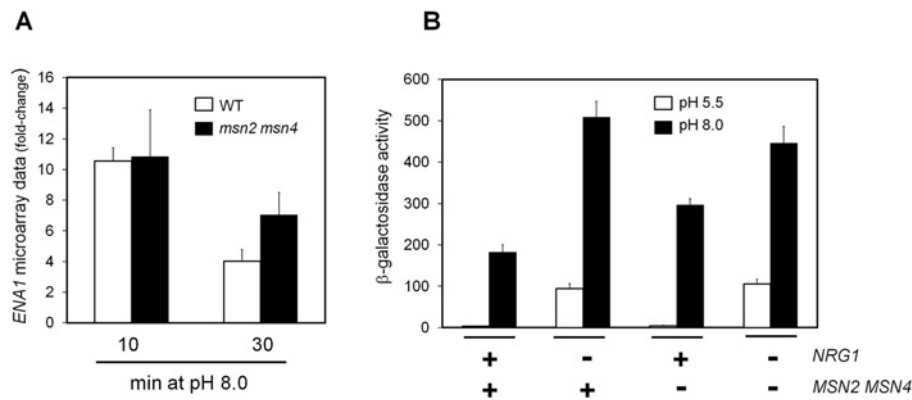
Figure 7   Dependence of *ENA1* expression on the presence of Msn2/Msn4

(**A**) Fold-change of *ENA1* expression deduced from microarray data for W303-1A wild-type cells (WT, empty bars) and MCY5278 (*msn2 msn4*, filled bars) exposed to pH 8.0 for the indicated times. Results are means ± S.E.M. from four microarray experiments. (**B**) Strains with the indicated genotype (+ , wild type allele; -, deletion mutant) were transformed with plasmid pKC201, which bears a *LacZ* fusion of the *ENA1* promoter. Exponential cultures were resuspended in medium buffered at pH 5.5 (empty bars) or pH 8.0 (filled bars) for 1 h and β-galactosidase activity was measured. Results are means ± S.E.M. from six to nine independent experiments.

pathway in regulation of Msn2 function in response to high pH is questionable because, with the exception of the *ure2* strain (a very pleiotropic strain), mutants in genes of the pathway do not show altered tolerance to high pH nor does alkalinization trigger increased expression of well-established readouts for inhibitors of the TOR pathway, such as *GAP1*, *GLN1* or *GDH1* (results not shown). Two protein kinases, Yak1 and Rim15, have been reported to modulate Msn2 function in response to PKA activation (see [5] for a review). However, *yak1* and *rim15* mutant strains are not sensitive to high pH (results not shown) and furthermore it has been proposed that Yak1 regulates Msn2 function by an unknown mechanism that does not implicate the control of Msn2 subcellular localization. Therefore the most probable scenario is that Msn2 entry into the nucleus after high pH stress is caused by inhibition of PKA activity.

Comparison of the time sequence of the events described in our work is rather coherent, with a very fast decrease in cAMP levels and subsequent entry of Msn2 in the nucleus, followed by a massive, fast (10 min) and transient activation of Msn2/Msn4-dependent genes, as deduced by DNA microarray transcriptomic analysis. It is remarkable that, after 30 min of high pH stress, the transcriptional response becomes largely *msn2 msn4* independent. In fact, our results (Figure 5A) suggest that Msn2/Msn4 may be responsible for the first chronological set of transcriptional responses to alkaline stress, even a faster response than that mediated by the calcineurin/Crz1 pathway, which were currently defined as early responses [19,20].

It has been widely documented that expression of the *ENA1* Na$^+$-ATPase is potently induced by alkaline pH (see [48] and references therein). An interesting observation derived from our microarray results is that the increase in *ENA1* expression is virtually identical in wild-type and *msn2 msn4* cells at short time frames (10 min), but is further enhanced by the lack of Msn2/Msn4 at 30 min. Interestingly, we observed previously a similar effect by using a *LacZ* translational fusion of the *ENA1* promoter and a different *msn2 msn4* mutant strain [19], although at that moment it was not further characterized. Our observations suggest that lack of Msn2/4 results in either activation of a positive regulatory element for *ENA1* expression or removal of a negative regulator. It is well known that Nrg1 acts as a repressor of *ENA1* expression by directly binding to the gene promoter, thus participating in alkaline pH signalling [18,49,50]. We observe in the present paper that *NRG1* levels decrease after 30 min of high pH stress

and that alkaline pH induction of *ENA1* is not further increased by mutation of *msn2 msn4* in a *nrg1* deletion background. This suggests that the enhanced expression of the ATPase gene in the *msn2 msn4* mutant can be due to a decrease in the amount of the Nrg1 repressor. In this regard, it must be noted that the remarkable effect of lack of *RIM101* in the expression of the ATPase gene [18,19,28], is mediated fully by a rather small change (2.8-fold) in *NRG1* mRNA levels [18].

The results of the present study also suggest involvement of the PKA pathway in high pH tolerance in way that would be independent of the Msn2/Msn4 transcription factors. This is based in the combination of *tpk* and *msn2,4* mutations and in the observation that hyperactivation of *RAS2* in the *msn2 msn4* mutant still increases high pH sensitivity (Figure 4B). A possible explanation could be based in a co-operative role for the PKA and calcineurin pathways in the adaptive response to high pH stress. In this regard, it was reported that PKA can phosphorylate Crz1, promoting its exit from the nucleus and thus opposing the action of calcineurin [26]. Therefore inhibition of PKA may result in further enhancement of calcineurin/Crz1-mediated responses. A further step of complexity may exist in the interaction between the calcineurin and the PKA pathways, as it was recently reported that Crz1 may exert a destabilization effect on Msn2 levels upon calcium treatment [51]. In this context, it is conceivable that entry of Crz1 in the nucleus, following activation of calcineurin by high pH may serve, in addition to activate calcineurin/Crz1-responsive promoters, as a negative-feedback system to avoid persistent activation of Msn2/Msn4-responsive genes. This regulatory system may contribute to the transient Msn2/4-dependent transcriptional effect observed in the present study.

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