Transcriptional regulation of the protein kinase A subunits in *Saccharomyces cerevisiae*: Autoregulatory role of the kinase A activity

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ABSTRACT

Protein kinase A (PKA) is a broad specificity protein kinase that controls a physiological response following the increment of cAMP as a consequence of a particular stimulus. The specificity of cAMP-signal transduction is maintained by several levels of control acting all together. Herein we present the study of the regulation of the expression of each PKA subunit, analyzing the activity of their promoters. The promoter of each isoform of *TPK* and of *BCY1* is differentially activated during the growth phase. A negative mechanism of isoform-dependent autoregulation directs *TPKs* and *BCY1* gene expressions. *TPK1* promoter activity is positively regulated during heat shock and saline stress. The kinase Rim15, but not the kinase Yak1, positively regulates *TPK1* promoter. Msn2/4, Gis1, and Sok2 are transcription factors involved in the regulation of *TPK1* expression during stress. *TPK2*, *TPK3*, and *BCY1* promoters, unlike *TPK1*, are not activated under stress conditions, although all the promoters are activated under low or null protein kinase A activity. These results indicate that subunits share an inhibitory autoregulatory mechanism but have different mechanisms involved in response to heat shock or saline stress.

1. Introduction

Extracellular signals are sensed by living cells triggering specific transduction pathways. These processes are critical to appropriately adjust intracellular responses to environmental changes. The cAMP–protein kinase A (PKA)-pathway controls a variety of cellular processes and the specificity is determined by several factors that partially explains the differential effects of cAMP in the signaling. In mammals, it has been proposed that PKA isoenzymes with different biochemical properties, substrate specificity, and localization through association with anchoring proteins (AKAPs) may explain the specificity in the cAMP pathway [55]. Another level of control in the specificity of the cAMP–PKA signaling is the regulation of PKA subunits expression. The regulatory (R) and catalytic (C) subunits expression levels are regulated by hormones and mitogenic signals acting through G-protein coupled receptors [25,28,40] or receptors associated with protein tyrosine kinases (PTKs) [56]. Several studies in cells and tissues have shown differential expression patterns of PKA subunits at different developmental and differentiation stages. It has been reported that the Cα, Cβ, RIα, RIβ, RIIα, and RIIβ isoforms show tissue specific expression patterns [4,10,39,48]. cAMP mediates regulation of PKA subunits

expression positively modulating their transcription level [60,61]; there have also been reports on cAMP dependent stabilization of subunits mRNA [26] as well as on R and C protein stabilities after dissociation of the holoenzyme [24,60].

cAMP–PKA pathway in *Saccharomyces cerevisiae* controls a variety of essential cellular processes, which are associated with fermentative growth, the entrance into stationary phase, stress responses and developmental pathways [15,41,52]. PKA from *S. cerevisiae* is a tetrameric holoenzyme composed of two regulatory and two catalytic subunits that resemble the mammalian counterparts. The regulatory subunit is encoded by only one gene, *BCY1*, and there are three genes encoding the catalytic subunit: *TPK1*, *TPK2* and *TPK3* [65]. In yeast, it is well described that there are two major stimuli that induce cAMP synthesis: extracellular glucose or other fermentable sugars and intracellular acidification. Both stimuli rapidly increase cAMP levels and thus mediate PKA activation [64]. The local intracellular concentration of the cAMP generated as well as the endogenous substrate concentration are key factors in the activation mechanism of the PKA holoenzyme [45], and we showed that the substrate has an important role in modulating PKA activation [14].

In summary, the kinase signaling pathway specificity depends on a dynamic interrelationship of several factors: the sequence around the phosphorylation site, the substrate and kinase expression levels, and the presence or absence of kinase anchor proteins that limit kinase–substrate interaction. Two aspects of cAMP–PKA signaling in yeast remain to be elucidated: the identification and characterization

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of anchoring proteins and the mechanism involved in the regulation of PKA subunits expression. Regarding the expression of PKA subunits, some information on Tpks and Bcy1 protein levels has been reported: Bcy1 overexpression increases *TPK3* mRNA levels and its catalytic activity [32], relative expression pattern of each Tpk changes from low levels under fermentative metabolism to high levels during stationary phase, but the increase in Tpks levels is not cross-regulated with Bcy1 levels [66].

High PKA activity in yeast leads to several phenotypes and one of them is the low stress resistance due to reduced expression of genes that are controlled by STRE (stress response element). Low PKA activity leads to opposite phenotypes and derepression of STRE-controlled genes generating a high stress resistance [2,51,62,63,71]. Yeast microarray analysis of genomic expression in response to environmental changes revealed that *TPK1*, *TPK2*, *TPK3* and *BCY1* are upregulated under heat shock [7,8,18] and saline stress [8,46,50,72] conditions. Nevertheless, the results show differences in the changes that each subunit has presented. Therefore, there is an apparent paradox or contradiction: although PKA subunits transcription is stimulated during stress, a higher PKA activity leads to a lower stress resistance. These evidences suggest that a complex mechanism regulates the expression of PKA subunits. Despite these data, the molecular mechanisms, transcription factors that modulate the transcription of *TPK1*, *TPK2*, *TPK3* and *BCY1* in a specific condition, are not known. And how the resulting levels of each PKA subunit contribute to the signaling specificity is also intriguing.

In order to elucidate the molecular mechanisms involved in the transcription of PKA subunits we first assessed the role of cAMP kinase activity in the regulation of the activity of each promoter. The results indicate that PKA activity negatively regulates the promoter activity of each PKA subunit. Differential expression of the PKA subunits was evaluated during growth and stress. We demonstrate that each of the *TPKs* and *BCY1* promoters is differentially activated during the growth phases. Heat-shock and saline stress conditions were chosen to study the specific factors that influence the regulation of *TPK1*, *TPK2*, *TPK3* and *BCY1* transcription. *TPK1* promoter activity is upregulated during heat shock and saline stress. The kinase Rim15 but not the kinase Yak1 and the transcription factors Msn2/4, Gis1, and Sok2 is involved in this regulation. *TPK2*, *TPK3*, and *BCY1* promoters, unlike *TPK1*, are not activated under stress conditions but all the promoters are activated when PKA activity is low or null within the cell. Thus, PKA subunit promoters share an inhibitory autoregulatory mechanism although they are regulated by different mechanisms in response to heat shock or saline stress.

2. Experimental procedures

2.1. Strains and culture conditions

S. cerevisiae strains used in this study are indicated in Table 1. Strains were cultivated at 30 °C to late log phase in synthetic media containing 0.67% yeast nitrogen base without amino acids and 2% glucose plus the necessary additions to fulfill auxotrophic requirement medium (SD), both supplemented with 0.15 mg/ml adenine. The cultures were grown until an OD_{600} of 3.5 at 30 °C ($\approx 0.5 \times 10^7$–1×10^8 cells/ml). For heat shock experiments, cells were grown at 25 °C (OD_{600} 3.5) and instantaneous up shift was achieved by rapidly mixing a centrifugated pellet of this culture with 37 °C prewarmed medium and further incubation with shaking at 37 °C for the indicated times. Samples were taken at various time intervals.

2.2. Plasmids

The plasmids used in this study to measure the promoter activities were derived from the YEp357 plasmid [36]. The *TPK1-lacZ*, *TPK2-lacZ*, *TPK3-lacZ*, and *BCY1-lacZ* fusion genes contain the 5′ regulatory region and nucleotides of the coding region of each gene (positions −800 to +10 with respect to the ATG initiation codon in each case). Transcription factor sites were mutagenized using a QuikChange XL site-directed mutagenesis kit on the *TPK1-lacZ* promoter. Construct A has Sok2 site mutated; construct C, Gis1 site mutated, construct B–D has two STRE/PDS sites mutated and construct A–B–C–D has all the sites mentioned mutated. All mutations are point mutations: Sok2 site (A) WT sequence T**TTTTCGCGC** was replaced by TTCAGTAACGC; STRE/PDS site (B), **AGGGG**, by AAGAT; Gis1 site (C) **AGGGAA**, by AAGATA and the second STRE/PDS (D) AGGGG, by AAGATG. Transcription factor element sequence is indicated in bold.

2.3. β-Galactosidase assays

Cells were grown on SD medium up to an OD_{600} of 3.5. Aliquots (10 ml) of each culture were collected by centrifugation and resuspended in 1 ml buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). β-Galactosidase activity measured according to Miller [34] was expressed as Miller Units. In Figs. 1 and 6 the mean ± SD for duplicates made in an experiment is shown. The results shown correspond to a representative experiment which was repeated at least three times, each performed in duplicates or triplicates. In the rest of the figures the results are expressed as the mean ± SD of the total replicate samples

Table 1
Yeast strains used.

| Strain	Genotype	Reference or source
W303 1A (WT)	*Mat a, suc2, ade2, can1, his3, leu2, trp1, ura3*	[68]
S331 (*TPK1 tpk2Δ tpk3Δ*)	(W303 1A) *Mat a, tpk2::KanR, tpk3::KanR*	[1]
S332 (*TPK2 tpk1Δ tpk3Δ*)	(W303 1A) *Mat a, tpk1::KanR, tpk3::KanR*	[1]
S330 (*TPK3 tpk1Δ tpk2Δ*)	(W303 1A) *Mat a, tpk1::KanR, tpk2::KanR*	[1]
JT20454 (*msn2/4Δ tpksΔ*)	(W303 1A) *tpk1::ade8 tpk2::HIS3 tpk3::TRP1 msn2::LEU2 msn4::HIS3*	Thevelein, J.
msn2/4Δ	(W303 1A) *msn2::LEU2 msn4::HIS3*	[12]
msn2/4Δ yak1Δ	(W303 1A) *msn2::HIS3 msn4::TRP1 yak1::KanMX6*	[29]
YHN1172	(W303 1A) *MATa msn2::loxP msn4::kanMX PtetO2-HSF1, adhP-tetR-VP16, natMX4 adhP-tetR′-SSN6::LEU2*	[13]
BY4741 (WT)	*Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
rim15Δ	(BY4741) *rim15::KanMX4*	EUROSCARF
gis1Δ	(BY4741) *gis1::KanMX4*	EUROSCARF
yak1Δ	(BY4741) *yak1::KanMX4*	EUROSCARF
sok2Δ	(BY4741) *sok2::KanMX4*	EUROSCARF
SP1	*Mat α, his3, leu2, ura3, trp1, ade8*	[65]
S18-1D (*tpk1w1BCY1*)	(SP1) *tpk1w1 tpk2::HIS3, tpk3::TRP1*	[38]
133 (*TPK1bcy1Δ*)	(SP1) *tpk2::HIS3, tpk3::TRP1, bcy1::LEU2*	[5]
Msn2-TAP	(BY4741) *Msn2-TAP::HIS3MX*	Open Biosystems
Gis1-TAP	(BY4741) *Gis1-TAP::HIS3MX*	Open Biosystems
Sok2-TAP	(BY4741) *Sok2-TAP::HIS3MX*	Open Biosystems
Rpb1-TAP	(BY4741) *Rpb1-TAP::HIS3MX*	Open Biosystems
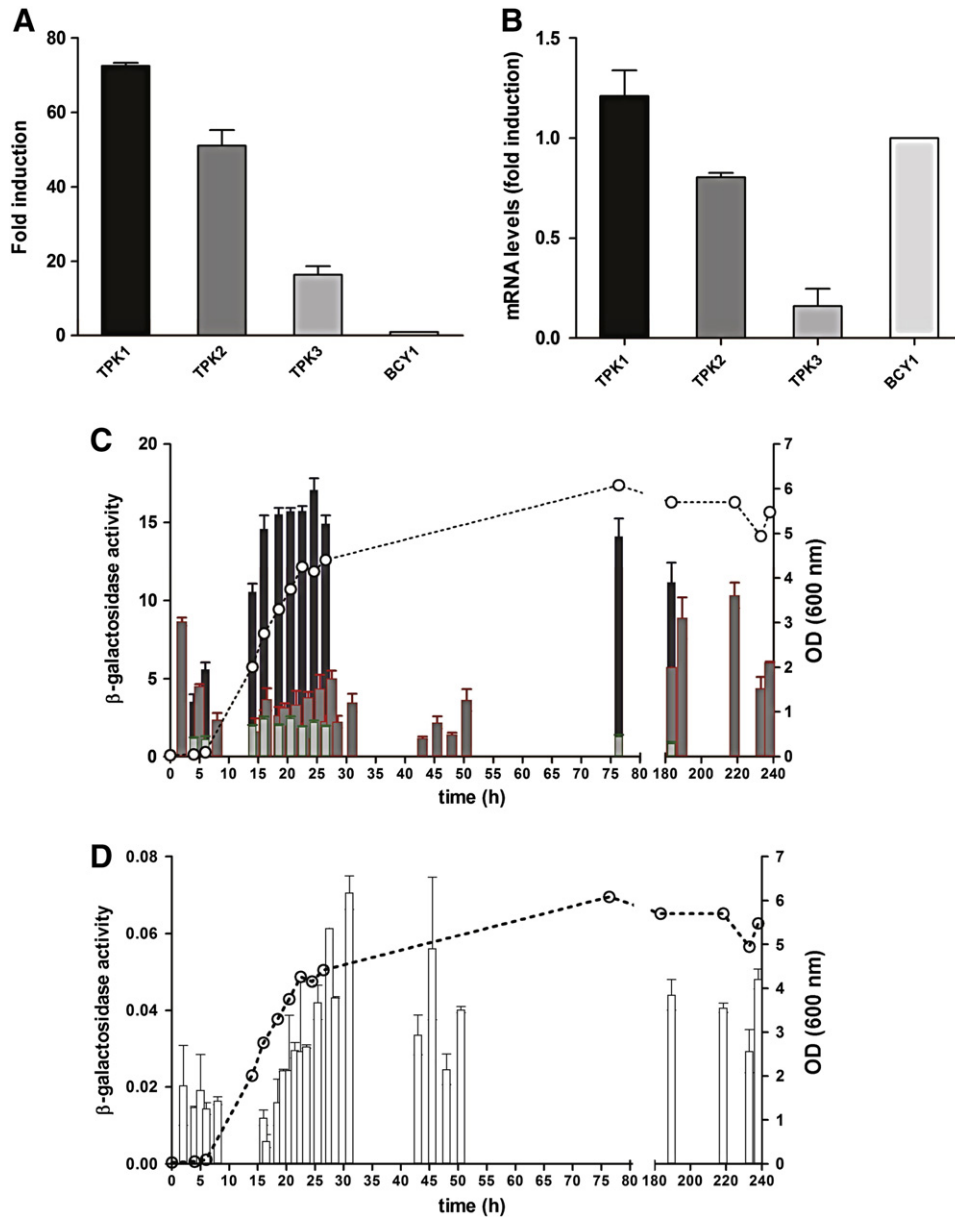
Fig. 1. Expression of *TPKs-lacZ* and *BCY1-lacZ* fusion genes in WT cells during yeast growth on glucose media. β-Galactosidase activity was determined in WT cells (W303-1A) carrying the *TPKs-lacZ* or *BCY1-lacZ* fusion gene. Cells were grown on minimal medium up to OD_{600} 3.5. (A) β-Galactosidase activity expressed in Miller Units, normalized to the *BCY1* values. Results are expressed as the mean ± SD from triplicates within a representative assay. (B) Endogenous *TPKs* and *BCY1* mRNA level quantification by qRT-PCR normalized to mRNA from *TUB1* and *BCY1* values; results are expressed as the mean ± SD from triplicates within a representative assay. Growth curve of WT cells (W303-1A) carrying the (C) *TPKs-lacZ* or (D) *BCY1-lacZ* fusion gene cultured on minimal medium for 10 days. Samples were taken for β-galactosidase activity and OD_{600} measurements at the indicated times. The graph corresponds to Miller Units showing mean ± SD from triplicate samples within a representative experiment. The gray scale colors for *TPK1*, *TPK2*, *TPK3* and *BCY1* are the same as in panels A and B but each has a colored borderline: *TPK1*, blue; *TPK2*, red; *TPK3*, green; and *BCY1*, black.

(n = 4, n = 6 or n = 8) coming from independent assays (2, 3 or 4 experiments). *tpk1^{w1}BCY1* and *TPK1bcy1Δ* strains have a SP1 genetic background and the rest of the mutants used in this study, a W303-1A or BY4741 genetic background. Promoter activities were assessed in the WT strains of the different genetic backgrounds and showed no differences (data not shown).

2.4. Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation (ChIP) was performed as described previously with modifications [27]. Cells (50 ml culture) were grown to an OD_{600} of 3.5 and after different treatments, fixed for 20 min at room temperature with 1% formaldehyde (final concentration). Glycine was then added to give a final concentration of 125 mM and incubated for 5 min. Cells were harvested, washed with ice-cold Tris buffered saline (TBS) and resuspended in 1 ml of FA lysis buffer (50 mM HEPES/KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride). An equal volume of glass beads (0.5 mm in diameter; Sigma) was added, and the cells were disrupted by vortexing for 40 min at 4 °C. The lysates were separated from glass beads, and chromatin was then pelleted by centrifugation (17,000 ×g for 15 min) and resuspended in 1 ml of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3–10 s at 15% amplitude) and clarified by centrifugation at 17,000 ×g for 15 min. 1 mg of protein was used for each immunoprecipitation. IgG-Sepharose 6 Fast Flow (GE Healthcare) was washed with PBS, 5 mg/ml BSA. The samples were incubated overnight at 4 °C in a rotator with the resin. Immune

complexes were sequentially washed four times with FA lysis buffer, four times with FA lysis buffer containing 500 mM NaCl, four times with wash buffer (10 mM Tris–HCl pH 8, 0.25 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and one time with Tris–EDTA (TE) buffer. Bound proteins were eluted from the resin by adding elution buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 1% SDS) and incubating for 1 h at 37 °C with proteinase K (0.25 mg/ml). Cross-linking was reversed incubating 5 h at 65 °C. DNA was purified using a QIAquick PCR purification kit (Qiagen). Real-time quantitative PCR (qPCR) was carried out with an Opticon Monitor 3 (Bio-Rad), using primers that amplified promoter regions of *TPK1* promoter (F/R-TPK1 Msn2/4 qPCR) corresponding to the Msn2/4, Sok2 and Gis1 sites. DNA detection was performed with SYBR Green. A pair of primers that amplify a region located outside *TPK1* promoter was used as an unbound control (F/RTPK1 control qPCR). ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over a non-TAP tagged strain control ChIP. The $\Delta\Delta C_T$ method was used to calculate the fold change of binding to the promoter of interest [31]. Propagation of error was handled using standard root mean square methods. Primers used on *TPK1* promoter: Fw: 5′ TTCAACATAGTGTTGTTGTGGTTTT 3′ and Rv: 5′ AAAAATTCTCACGTTTTTCTGG 3′. Control ORF *POL1*: Fw: 5′ CTGCACTG GCAAAACA GAAA 3′ and Rv: 5′ TCTTAAACGACGGCCAATAGA 3′.

2.5. qRT-PCR

Total RNA was prepared from different yeast strains, grown up to the same OD_{600} as for β-galactosidase assays, using standard procedures. To determine the relative levels of specific *TPK1*, *TPK2*, *TPK3* and *BCY1* mRNAs, a quantitative RT-PCR experiment was carried out. Aliquots (~10 μg) of RNA were reverse-transcribed into single-stranded complementary cDNA using an oligo-dT primer and Superscript II transcriptase reverse (Invitrogen). The single-stranded cDNA products were amplified by PCR using gene-specific sense and antisense primers (mRNA *TPK1*: Fw: 5′ CCGAAGCAGCCACATGTCAC 3′, Rv: 5′ GTACTAACGACC TCG GGTGC 3′; mRNA *TPK2*: Fw: 5′ GCTTGTGGAGCATCCGTTTC 3′, Rv: 5′ CACTAAACCA TGGGTGAGC 3′; mRNA *TPK3*: Fw: 5′ CGTTGGACAAGA CATTCCTG 3′, Rv: 5′ GTCGGT TATCTTGATATGGCC 3′; mRNA *BCY1*: Fw: 5′ CGAACAGGACACTCACCAGC 3′, Rv: 5′ GGTATCCAGTGCATCGGCAAG 3′; mRNA *TUB1*: Fw: 5′ CAAGGGTTCTTGTTTACCCAT TC 3′, Rv: 5′ GGAT AAGACTGGAGAATA TGAAAC 3′). The PCR products were visualized using SYBR Green. The relative mRNA levels of *TPK1*, *TPK2*, *TPK3* and *BCY1* were first normalized to those of *TUB1* (α-Tubulin gene) and then compared to each other. Quantitative data were obtained from three independent experiments and averaged.

2.6. Western blotting

Strains were grown in the appropriate medium up to 3.5 OD_{600}, and resuspended in buffer 5 mM Tris–HCl (pH = 7.5), 0.1 mM EGTA, 0.1 mM EDTA, 10 mM 2-mercaptoethanol and EDTA-free protease inhibitor. The cells were lysed by disruption with glass beads at 4 °C. The crude extract was resolved by SDS-PAGE and analyzed by western blotting with anti-TPK1 antibody (Santa Cruz Biotechnology). The immunoblot shown is a representative of two independent experiments.

3. Results

3.1. Gene expression of TPK1, TPK2, TPK3, and BCY1 is differentially regulated during yeast grown on glucose media

TPK1, *TPK2*, *TPK3*, and *BCY1* expressions were first assessed using promoter-lacZ-based reporter assay to measure the expression of lacZ driven by the four non-coding upstream sequences from each gene (−800 to +10 with respect to the ATG initiation codon), in WT cells grown in 2% glucose to late log phase (OD_{600} 3.5). The relative expression level of each gene was different: *TPK1* promoter presented the

highest activity followed by *TPK2* promoter (Fig. 1A). *TPK3* and *BCY1* promoters showed very low activities, corresponding the lowest to *BCY1*. mRNA levels of each subunit were analyzed by quantitative real time PCR (qRT-PCR), and were in agreement with the promoter activities, except for *BCY1* mRNA which was as abundant as *TPK1* mRNA (Fig. 1B). This discrepancy between the promoter activity, transcript abundance and protein expression could be the consequence of the mRNA stability and the extremely high stability of Bcy1 (see below and Discussion) or that *BCY1* coding sequences could be required for a proper expression. In accordance with the high mRNA levels of *BCY1*, it was demonstrated that Bcy1 protein levels, in logarithmic growth phase, are higher than the catalytic subunits [66].

To determine if the expression of the PKA subunit encoding genes was differentially regulated by yeast growth, we analyzed *TPKs-lacZ* and *BCY1-lacZ* activities along a growth curve in the presence of glucose. As shown in Fig. 1C–D, *TPK1* showed an increase in the promoter activity during the initial logarithmic phase of growth. During transition of diauxic shift and the subsequent post-diauxic period *TPK1* expression level did not show variations. Finally, when the cells entered into the stationary phase, they showed a slight decrease in the *TPK1* promoter activity. *TPK3* showed a very low expression level throughout the growth curve, with a slight increase at the beginning of the logarithmic phase. *TPK2* and *BCY1* promoter activities had a similar behavior exhibiting a decrease at the beginning of logarithmic phase. From diauxic shift to stationary phase, they showed an increment in their activities. Finally, in stationary phase, they showed expression levels nearly constant. As a control, the culture density and residual glucose concentration were measured from WT cells carrying the *TPK1-lacZ* or *TPK3-lacZ* fusion gene cultured in minimal medium. At OD_{600} of 3.5 the glucose concentration was around 60%, indicating that the cells are not at diauxic shift (data not shown). These results indicated that the genes encoding distinct PKA subunits were differentially expressed, exhibiting different promoter activities and expression patterns during the growth curve in the presence of glucose as carbon source. Cells grown up to OD_{600} 3.5 in the growth curve were chosen for the following experiments, and the OD and the hours of growth of the culture were carefully controlled to be the same in all the assays.

3.2. Activity of PKA subunit promoters is autoregulated

In order to understand the mechanisms regulating the expression of *TPKs* and *BCY1* genes, we analyzed the possibility of autoregulation by PKA activity itself. We assessed the promoter activity of each *TPK-lacZ* and *BCY1-lacZ* fusion gene in different *TPK1* yeast strains: WT (wild type), $tpk1^{w1}BCY1$ (strain carrying an attenuated form of Tpk1 and deletions of *TPK2* and *TPK3* genes resulting in a very low PKA activity strain), and $TPK1bcy1\Delta$ (strain with a deregulated PKA activity). The four promoters showed induction of their activities in the $tpk1^{w1}BCY1$ strain, completely reverted in the $TPK1bcy1\Delta$ strain (Fig. 2A). These results indicated that PKA activity generated the downregulation of PKA subunit expression. *TPK1* mRNA in $TPK1bcy1\Delta$ strain, and *BCY1* and *TPK1* mRNAs in $tpk1^{w1}BCY1$ strain were analyzed by qRT-PCR. The levels were in agreement with promoter activities (Fig. 3A). Western blot analysis of Tpk1 protein in extracts from the same mutant strains showed that Tpk1 protein amount matched with *TPK1* promoter activity and *TPK1* mRNA level (Fig. 3A inset).

To determine whether the PKA inhibition was Tpk isoform-dependent, we assessed the activity of each promoter in yeast strains carrying only one of the three *TPK* genes (S330, S331, S332 strains). As shown in Fig. 2B, the promoters exhibited different behaviors in the presence of each Tpk isoform. From these results it can be concluded that Tpk1 and Tpk3 have a similar inhibitory effect on the activity of the three subunit promoters; that Tpk2 had a stronger inhibitory effect on Tpk1 and Tpk3 promoters, and most surprisingly was the lack of inhibition toward its own promoter. *BCY1* promoter, the most inhibited, showed no significant differences between the Tpk isoforms inhibition.
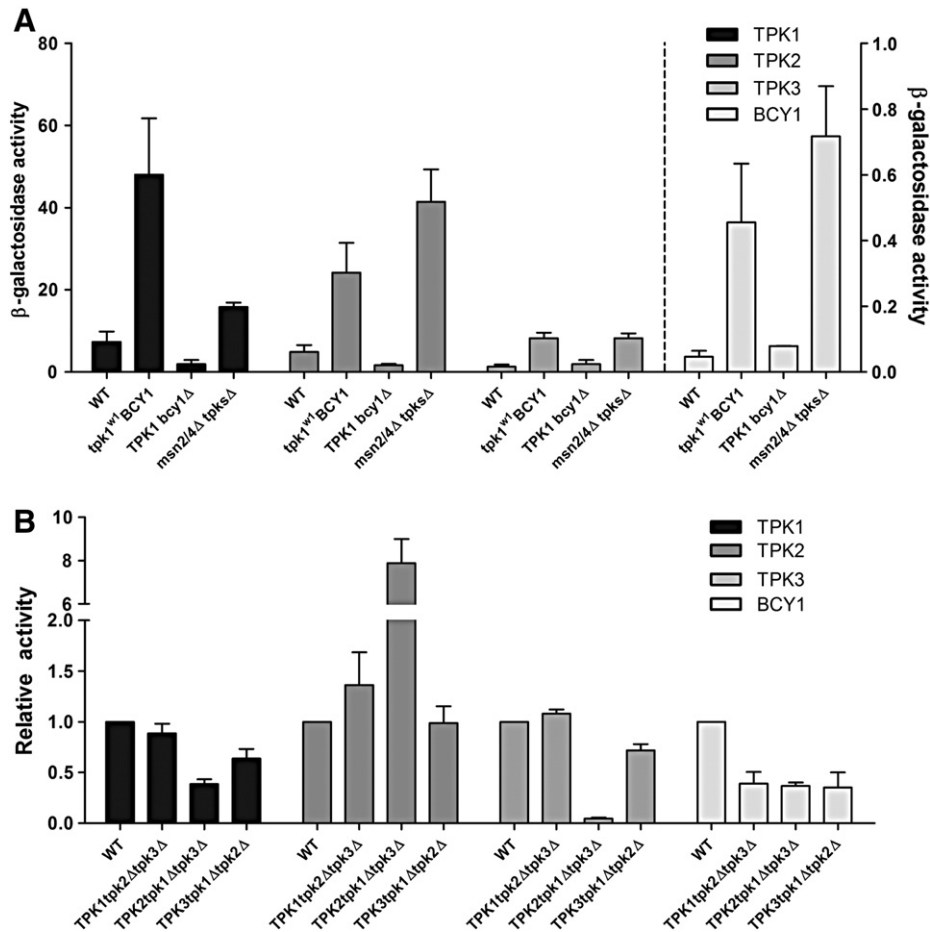
Fig. 2. Activity of PKA subunit promoters is autoregulated. (A) β-Galactosidase activity was determined in, Sp1 and W303-1A WT strains, but only the values obtained in W303-1A are shown, $tpk1^{w1}BCY1$, $TPK1bcy1\Delta$, and $msn2/4\Delta tpks\Delta$ (JT20454) cells carrying the TPKs-lacZ or BCY1-lacZ fusion gene. The results shown in Miller Units are expressed as the mean ± SD from replicate samples (n = 8) from independent experiments. (B) β-Galactosidase activity was determined for WT (W303-1A), $TPK1\ tpk2\Delta\ tpk3\Delta$ (S331), $TPK2\ tpk1\Delta\ tpk3\Delta$ (S332), and $TPK3\ tpk1\Delta\ tpk2\Delta$ (S330) cells carrying the TPKs-lacZ or BCY1-lacZ fusion gene. The values measured in each mutant strain for each reporter construct were normalized to the value obtained for each construct in the WT strain. The results are shown as fold induction means ± SD from replicate samples (n = 8) from independent experiments.

Endogenous TPK1, TPK2, TPK3, and BCY1 mRNA levels were measured by qRT-PCR in these mutant strains and the results were consistent with the promoter activities (Fig. 3A). Regardless of the difference found between the BCY1 promoter activity and its mRNA abundance (Fig. 1A and B), mRNA changes were similar to those observed for the promoter activity measured with the BCY1-lacZ construction using the same mutant strains. Thus, we considered that in spite of the low promoter activity of the BCY1-lacZ construction, it reflected the endogenous activity.

To corroborate this regulation, the TPKs-lacZ and BCY1-lacZ reporters were assayed in a strain without PKA activity and compared to a WT strain. Deletion of all three TPKs subunit genes is lethal but a concomitant deletion of MSN2/4 genes suppresses this lethality (JT20454 strain, $msn2/4\Delta tpks\Delta$) [57]. In JT20454 strain, promoter activities were higher than in the WT and the $TPK1bcy1\Delta$ strains, corroborating the inhibitory effect of PKA activity on the promoters (Fig. 2A). The results also showed that the extent of induction was different for each promoter. TPK1 promoter reached a lower level of induction in JT20454 strain than in the $tpk1^{w1}BCY1$ strain. This difference could be due to the lack of Msn2/4 in JT20454 strain which could have a role in the regulation of the promoter. The extent of induction of TPK2 promoter in the JT20454 strain was higher than in $tpk1^{w1}BCY1$ strain, suggesting a different role of Msn2/4 in the regulation of this promoter. Finally, the activities of BCY1 and TPK3 promoters induced in JT20454 strain showed almost no difference to $tpk1^{w1}BCY1$ strain, suggesting no role for Msn2/4 in their regulation.

Taken together, these results indicated that there is a negative regulation mechanism on the promoter activities of PKA subunits that involves a PKA signaling pathway that is Tpk isoform-dependent. A role for Msn2/4 transcription factors in the promoters regulation was assessed and the results of the analysis are introduced in the following section.

3.3. PKA subunit genes expression is differentially regulated during heat shock and saline stress

The preceding results led us to get further insights into the regulation of PKA subunits genes expression. Therefore, we studied whether Msn2/4 regulated the promoter activity of the genes encoding the different PKA subunits.

In S. cerevisiae, the heat shock transcription factor 1 (Hsf1) and the Msn2/4 transcription factors are responsible for the majority of the heat-shock response (HSR). However, in a few cases, these factors are not required for gene expression regulation during heat shock. Msn2 nuclear import correlates very well with the STRE expression data. In stress situations, the nuclear level of Msn2 is elevated inducing expression of stress-inducible genes [16,20]. The dephosphorylation of the Msn2 PKA target sites is induced upon stress and there is an inverse correlation between PKA activity and Msn2 nuclear localization [17,21,49].

The results shown in Fig. 2A suggested a role of Msn2/4 in the regulation of TPK1 and TPK2 promoter activities. Therefore, we analyzed the Msn2/4 involvement in the regulation of the PKA subunits expression
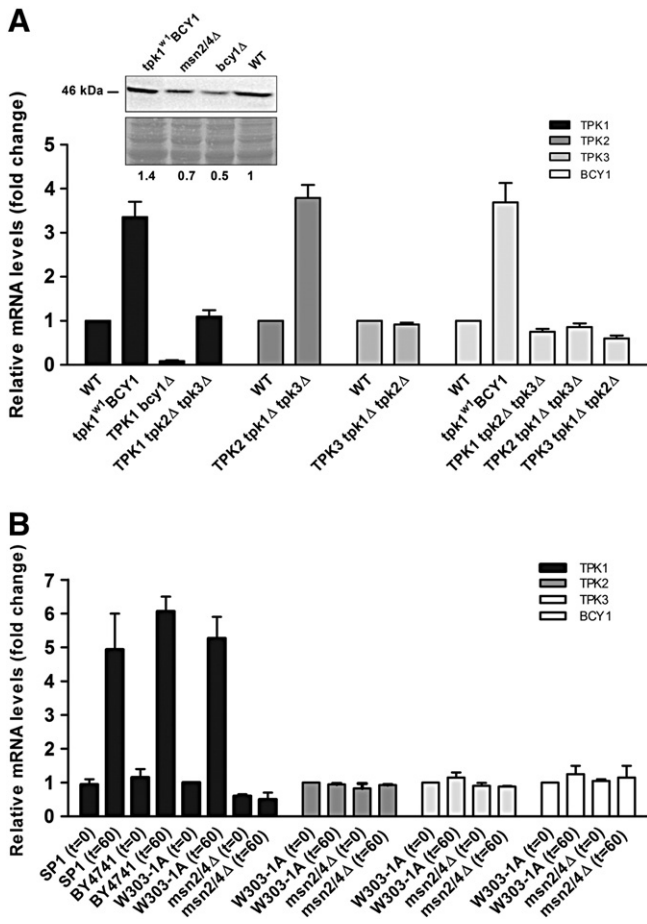
A

B

Fig. 3. Endogenous mRNA levels are autoregulated. (A) *TPKs* and *BCY1* endogenous mRNA levels were determined in WT (W303-1A), *tpk1ʷ¹BCY1*, *TPK1bcy1Δ*, *TPK1 tpk2Δ tpk3Δ* (S331), *TPK2 tpk1Δ tpk3Δ* (S332), and *TPK3 tpk1Δ tpk2Δ* (S330) by qRT-PCR and normalized to *TUB1* mRNA. The mRNA level in the WT strain was defined as 1. Inset, western blot analysis of extracts from the indicated mutant strains using anti-Tpk1 antibody; the numbers below indicate the quantification normalized to the band in the WT strain and total protein loaded. (B) *TPKs* and *BCY1* endogenous mRNA levels were determined in WT (W303-1A) and *msn2/4Δ* strains under heat-shock by qRT-PCR. *TPK1* mRNA levels were also determined in the other WT strains SP1 and BY4741 as control. Cultures of each yeast strain were grown to late log phase (OD₆₀₀ 3.5) at 25 °C, and then transferred to 37 °C for 60 min. The values were normalized to *TUB1* mRNA. The mRNA level for each subunit in the W303-1A WT strain was defined as 1.

under two stress conditions: heat shock and saline stress. *TPK1* promoter contains STRE sites experimentally documented [6]. However, although STRE sites were not predicted in *TPK2* and *BCY1* promoters by sequence inspection, Mns2/4 involvement in these promoters regulation was suggested by genomic microarray analysis [6]. As shown in Fig. 4A under heat stress there was a 1.5-fold increase of *TPK1* promoter activity. Neither *TPK2*, nor *TPK3* nor *BCY1* promoters responded to heat shock (Fig. 4B–D). Endogenous mRNA levels were measured by qRT-PCR at 60 min after heat shock and were in agreement with promoter activities (Fig. 3B). No changes in *TPK2*, *TPK3* and *BCY1* mRNA levels were detected at shorter times during heat shock (data not shown). Under saline stress conditions (1 M KCl), only *TPK1* promoter was 1.6-fold upregulated (Fig. 5).

The Msn2/4 role in *TPKs* expression regulation during heat shock and saline stress was assessed in cells lacking Msn2/4 (*msn2/4Δ*) transformed with the vectors *TPKs-lacZ* or *BCY1-lacZ*. *TPK1-lacZ* was 0.5-fold

downregulated in comparison with WT at T0, and no increment with the time of heat shock (Fig. 4A) or 1 M KCl treatment (Fig. 5) was observed. These results indicate that the *TPK1* promoter response during stress conditions (heat and saline) was regulated by the activity of Msn2/4 transcription factors. However, *TPK2*, *TPK3*, and *BCY1* promoters showed a different behavior in the absence of Msn2/4. *TPK2* showed a slight stimulation with no change over the time of heat shock treatment. *BCY1* and *TPK3* promoters exhibited no response to the lack of these transcription factors (Fig. 4B–D). Endogenous mRNA levels were analyzed in WT and *msn2/4Δ* strains by qRT-PCR, under heat shock (60 min), and the results were consistent with the promoter activities (Fig. 3B).

Msn2 and Msn4 were described as redundant transcription factors [53]. However a recent study using microarrays has reported that Msn2 and Msn4 can play nonredundant and condition-specific roles in the regulation of the expression of certain genes, arguing against a generic general-stress function [6]. Therefore, Msn2 and Msn4 redundancy on *TPK1* promoter was assessed. We found that *TPK1* promoter showed a clear downregulation defect in the double-deletion but not in the single-deletion strains, a pattern expected if the mutants were acting entirely redundantly (data not shown).

Thermal stress induces Hsf1 pathway, and the heat-inducible expression of many genes is known to be dependent on Hsf1 as well as Msn2/4. *HSF1* is essential for viability; therefore to determine whether the regulation of PKA subunit genes is dependent on Hsf1 activity, we used a conditional allele of *HSF1*. The YHN1172 strain has *HSF1* gene under a tetracycline-inducible expression system integrated into a *msn2/4Δ* strain background [13]. *HSF1* transcription is repressed in the presence of doxycycline. The activities of *TPK1* and *TPK2* promoters, differentially regulated by Msn2/4, were assayed in this strain. The growth of this mutant strain was inhibited on plates containing doxycycline indicating that Hsf1 levels had been depleted. The repression of *HSF1* for 12 h and 24 h with doxycycline produced no change in the transcriptional heat shock response of *TPK1* and *TPK2*, supporting the idea that Hsf1 is not involved in their regulation (data not shown).

Msn2/4 is under the control of the cAMP/PKA pathway, mediated in part by kinase Yak1. We analyzed the promoter activities in strains lacking Yak1 (*yak1Δ*) or lacking both Yak1 and Msn2/4 (*msn2/4Δyak1Δ*). As shown in Fig. 4 the deletion of *YAK1* had no effect on the expression of *lacZ* under the control of *TPK1*, *TPK2*, *TPK3* or *BCY1* promoters. However, the activity of *TPK1* promoter still responded to heat stress, with a response slightly higher than the control WT. The deletion of *MSN2/4* together with *YAK1* had a striking upregulatory effect, independent of heat stress on the *TPK1*, *TPK3* and *BCY1* promoters, with values similar to those obtained when PKA activity was very low (*msn2/4Δtpks Δ* (Fig. 4) or in *tpk1ʷ¹BCY1* strain (Fig. 2)). Transformation of *msn2/4 Δyak1Δ* strain with *TPK2-lacZ* reporter was unsuccessful. It has been reported that in the *msn2/4Δ* and *msn2/4Δyak1Δ* strains there is an increased nuclear localization of Bcy1 [23]. These differences in Bcy1 nuclear accumulation could explain different extent of PKA activity in the nucleus that might regulate each promoter activity (see Discussion).

The *RIM15* gene encodes a kinase protein that is negatively regulated by PKA [47]. Msn2/4 and Gis1 are Rim15-regulated stress-responsive transcription factors. We analyzed the effect of Rim15 and Gis1 on the heat shock response of *TPKs* and *BCY1* promoters. As expected neither Gis1 nor Rim15 regulated the activities of *TPK2-lacZ*, *TPK3-lacZ*, or *BCY1-lacZ*, as these promoters were not regulated during stress. However, Gis1 and Rim15 showed to be involved in heat stress regulation of *TPK1* promoter. In *gis1Δ* and *rim15Δ* strains, *TPK1* promoter showed a decreased activity compared to WT. In *gis1Δ* in spite of this decreased

Fig. 4. *TPKs* and *BCY1* promoter activities regulation during heat shock. β-Galactosidase activity was determined in WT (only the activity measured in W303-1A is shown), *msn2/4Δ*, *yak1Δ*, *msn2/4Δyak1Δ*, *msn2/4ΔtpksΔ* (JT20454), *gis1Δ*, and *rim15Δ* strains carrying the *TPKs-lacZ* or *BCY1-lacZ* fusion gene. Cultures of each yeast strain were grown to late log phase (OD₆₀₀ 3.5) at 25 °C, and then transferred to 37 °C for 15, 30, or 60 min. 60 °C corresponds to the control culture that was grown at 25 °C for 60 min. The results are expressed in Miller Units and as the mean ± SD for replicate samples (n = 6) from independent experiments. Panels A, B, C, and D correspond to *TPK1-lacZ*, *TPK2-lacZ*, *TPK3-lacZ* and *BCY1-lacZ* analyses respectively.
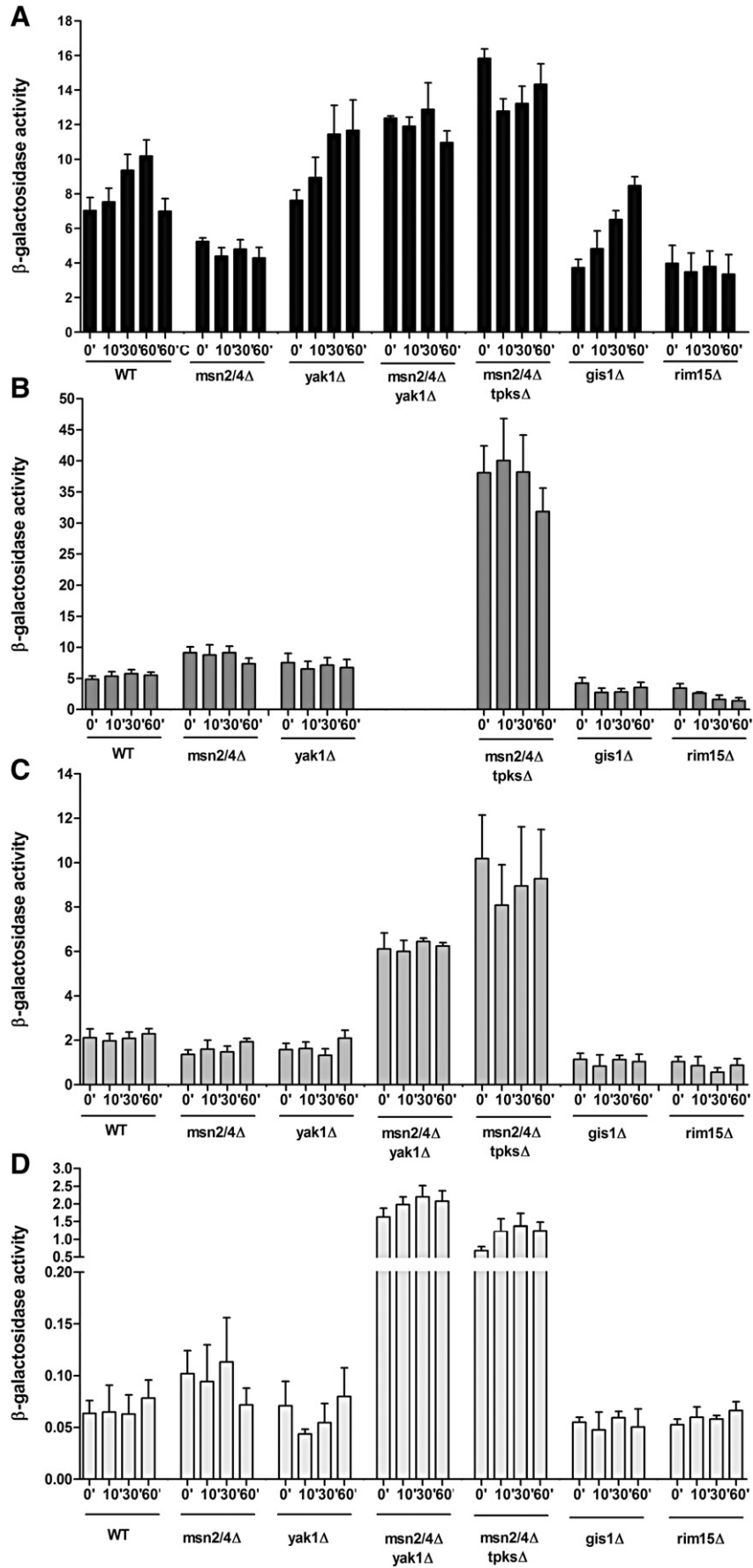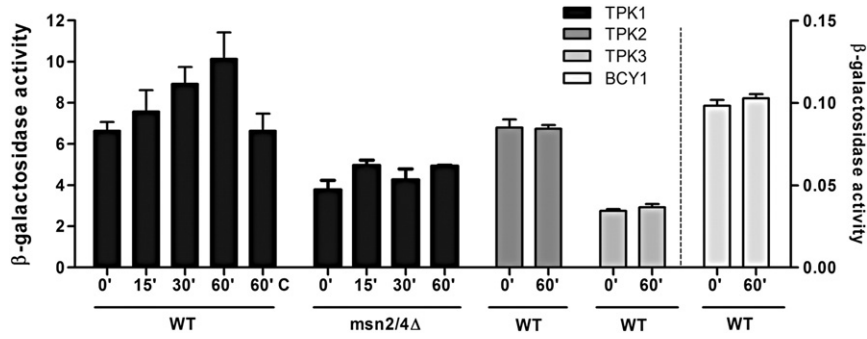
Fig. 5. *TPK1* promoter activity is regulated during saline stress. β-Galactosidase activity was determined in WT (W303-1A) and *msn2/4Δ* strains carrying *TPK1-lacZ* fusion gene. Cultures of each yeast strain were grown to late log phase (OD$_{600}$ 3.5) at 30 °C, and transferred to minimal medium with the addition of 1 M KCl for 15, 30 and 60 min. β-Galactosidase activity from *TPK2-lacZ*, *TPK3-lacZ* and *BCY1-lacZ* was assessed in WT strains at 0 and 60 min after addition of 1 M KCl. 60 °C corresponds to the control cultures that were grown for additional 60 min without KCl. The results are expressed in Miller Units and as the mean ± SD of replicate samples (n = 6) from independent assays.

activity, the promoter responded to the heat shock (Fig. 4). It was suggested that Gis1 and Msn2/4 not only functionally overlap in vivo, but also cooperatively regulate transcription of a large set of genes during the diauxic shift [73]. Thus, during heat shock and in a *gis1Δ* strain, the *TPK1* promoter response was decreased by the absence of Gis1 but upregulated by the presence of Msn2/4. In *rim15Δ* strain, however, there was no response to heat shock since Rim15 is upstream Gis1 and Msn2/4. *TPK1* promoter sequence inspection strongly supported this conclusion, since three PDS elements (Gis1) were predicted on *TPK1* promoter, being two of them overlapped with STRE elements (Msn2/4) (see Fig. 8).

3.4. Mns2/4, Sok2, and Gis1 cooperate in TPK1 promoter activity regulation

In order to corroborate the participation of Msn2/4 on the *TPK1* promoter stress-dependent regulation, we performed ChIPs (chromatin immunoprecipitation) using a yeast strain with TAP-tagged Msn2 during the heat shock time course (Fig. 6A). We found that the Msn2 level at the *TPK1* promoter declined below the background level starting at 15 and 60 min of heat shock. A decrease of Msn2 has been described to occur in the upregulation of some heat shock proteins [11], supporting our conclusion that the level of Msn2 at the *TPK1* promoter indeed decreases during heat shock (see Discussion). We also
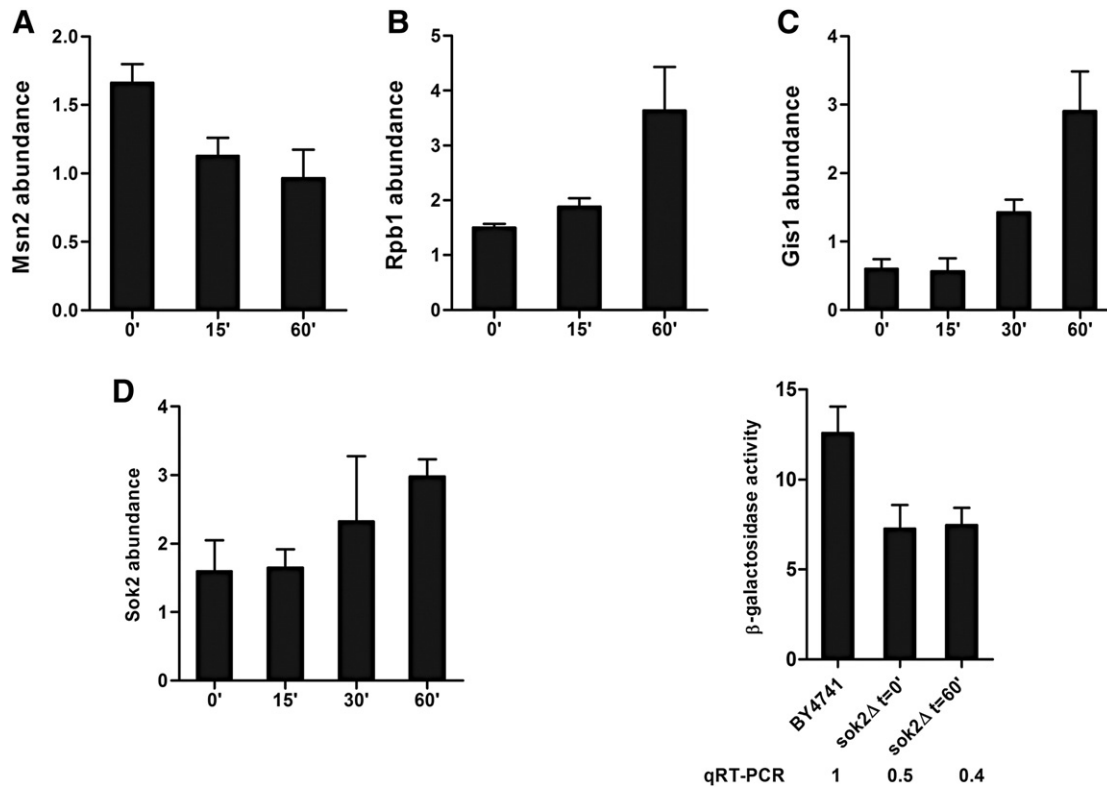

Fig. 6. Mns2/4, Sok2 and Gis1 bind *TPK1* promoter. BY4741 WT cells expressing (A) Msn2-TAP, (B) Rpb1-TAP, (C) Gis1-TAP or (D, left panel) Sok2-TAP fusion proteins were grown in minimal medium at 25 °C, and then transferred to 37 °C for 15, 30, or 60 min. ChIP assays were carried out using IgG-Sepharose. qPCR was performed with specific primers that amplify the −399 to −208 region of *TPK1* promoter. ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio to the background values of the non-tagged strain. Results are expressed as mean ± SD (n = 3). (D, right panel) β-Galactosidase activity was determined in WT (BY4741) and *sok2Δ* cells carrying the *TPK1-lacZ* fusion gene, at 0 and 60 min after heat shock at 37 °C. The results are expressed in Miller Units as the mean ± SD for duplicates within a representative assay. The numbers below the bars represent the *TPK1* mRNA amount that was quantified by qRT-PCR, normalized to WT strain. Values represent mean for duplicates within a representative experiment (n = 3).

performed a parallel ChIP experiment in a strain containing TAP-tagged Rpb1 in order to evaluate the recruitment of Pol II to the *TPK1* promoter as a measure of ongoing transcription and verify that transcription of this promoter was induced during heat shock. As shown in Fig. 6B Pol II increased its abundance at the *TPK1* promoter during heat shock response, indicating a strong correlation between Pol II loading and loss of Msn2. ChIP experiments of TAP-tagged Gis1 during heat shock were also performed (Fig. 6C) using the same primers than those for Msn2. As expected, Gis1 recruitment to the *TPK1* promoter increased during the heat shock. Gis1 recruitment and Msn2 decrease, correlated with Pol II recruitment to the *TPK1* promoter and with promoter activity during heat shock (Fig. 4A). Taken together, these results suggested that during heat shock response the *TPK1* promoter was activated by a coordinate action of Msn2 and Gis1 on the same DNA elements.

TPKs and *BCY1* promoters showed to be highly inhibited and their activities were upregulated under conditions in which PKA activity was decreased. Therefore, we looked for repressors that could contribute to the promoters downregulation. Using deletion strains and measurement of reporter β-galactosidase activity, several transcription factors described as repressors such as Rgt1, Sko1 and Adr1 were assessed. None of them showed to have a role in the regulation of the promoter activities (data not shown). However, *TPK1* promoter activity and endogenous *TPK1* mRNA levels were decreased in a *sok2Δ* mutant strain compared to a WT and showed no change over the time of heat shock treatment, indicating a role for Sok2 (Fig. 6D, right panel). Sok2 activity has been reported as an activator but also as a repressor and dependent on PKA activity [54]. Sok2 association with Msn2/4 has been demonstrated by co-immunoprecipitation assay, indicating that Sok2 could assist to the binding of Msn2/4, when their elements are close [54]. *TPK1* promoter sequence contains a possible Sok2 element close to one of the STRE sites. A ChIP assay in a TAP-tagged Sok2 strain during heat shock showed that Sok2 was present before heat shock in low levels and was recruited during the time course of heat shock (Fig. 6D, left panel). Taking into account our results and those previously reported by others, we concluded that Sok2 could assist the binding of Msn2 to the STRE sequence. Thus, under this condition of growth, Sok2 had an activator behavior on *TPK1* promoter. The three transcription factors, Msn2, Gis1, and Sok2, were necessary for *TPK1* promoter regulation during heat shock, and they bound to DNA elements that are very close.

The involvement of Msn2, Gis1, and Sok2 transcription factors in *TPK1* promoter activity was verified using mutant reporter constructs. The PKA negative effect on *TPK1* promoter beyond the regulation on Msn2/4 and Rim15 activities was also evaluated in this assay. Four β-galactosidase reporter constructs carrying different mutations in the Msn2/4, Gis1 and Sok2 elements were prepared and assessed in WT, *tpk1^w1BCY1* and JT20454 (*msn2/4Δtpks Δ*) strains without and with heat shock treatment. In construct A Sok2 site was mutated, in construct C Gis1 site was mutated, in construct B–D two Msn2/4 (STRE/PDS) sites were mutated, and in construct A–B–C–D all the mentioned sites were mutated. The constructs were transformed in WT and *tpk1^w1BCY1* strains. As shown in Fig. 7A–B, the mutant with all the sites mutated had very low β-galactosidase activity in the WT strain, before or after the heat shock. Mutant A showed a striking decrease in activity in WT and *tpk1^w1BCY1* strains, and lost the activation after heat shock treatment. Mutant C showed a small decrease in β-galactosidase activity in both strains, and only in WT strain a response during heat shock was still observed, resembling the results observed in the *gis1Δ* strain (Fig. 4A). Construct B–D showed also decreased activity in an intermediate level between A and C constructs. This result was in agreement with the β-galactosidase activity measured in the *msn2/4Δ* (Fig. 4A). The results indicated that Sok2 was involved in the regulation and that it modulated Msn2/4 and Gis1 activities. Finally, β-galactosidase activities from JT20454 (*msn2/4ΔtpksΔ*), WT and *tpk1^w1BCY1* strains transformed with the construct containing all the sites mutated (A–B–C–D) were analyzed. Although none of the mentioned transcription factors could bind to this mutated promoter, the mutant construct still
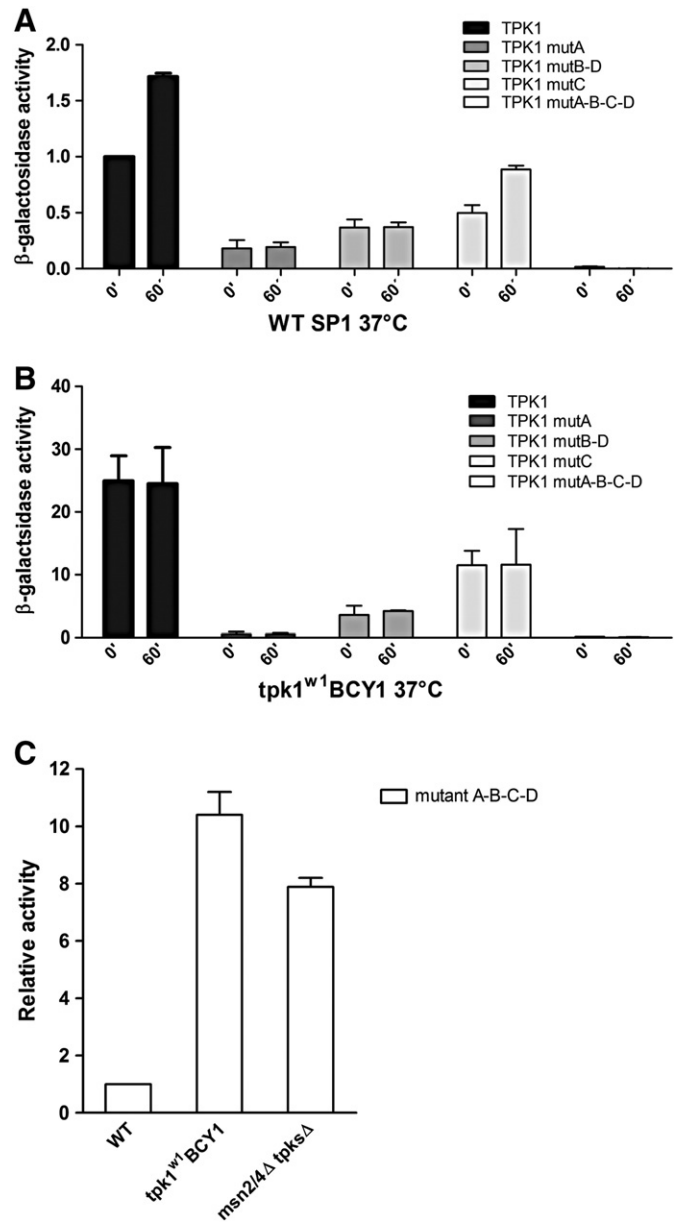

Fig. 7. Mns2/4, Sok2, and Gis1 regulate *TPK1* promoter activity. β-Galactosidase activity was determined in (A) SP1 WT strain and (B) *tpk1^w1BCY1* strain carrying *TPK1-lacZ* fusion WT gene and *TPK1-lacZ* fusion mutant constructs. Mutant A contains Sok2 site mutated; mutant B–D, two STRE/PDS sites mutated; mutant C, Gis1 site mutated and mutant A–B–C–D, all sites mutated. (C) β-Galactosidase activity was determined in WT (only SP1 is shown), *msn2/4ΔtpksΔ* (JT20454) and *tpk1^w1BCY1* strains carrying *TPK1-lacZ* fusion mutant A–B–C–D, all sites mutated construct. Cultures of each yeast strain were grown to late log phase (OD_{600} 3.5) at 25 °C and then transferred to 37 °C for 60 min. Results are expressed in Miller Units, normalized to the *TPK1-lacZ* fusion WT values from the WT strain. Results are expressed as the mean ± SD of replicate samples (n = 4) from independent assays.

showed a robust increase in β-galactosidase activity in JT20454 (*msn2/4ΔtpksΔ*) and *tpk1^w1BCY1* strains when compared with WT strain (Fig. 7C). This result indicated that there was a parallel pathway to the one of Rim15 and Msn2/4 that also involved PKA and negatively regulated the *TPK1* promoter activity (Fig. 8).

4. Discussion

In this study we investigated the regulation of PKA subunit promoters: *TPK1*, *TPK2*, *TPK3*, and *BCY1*. We demonstrated that all of them display a common feature in their regulation that is the autoregulation
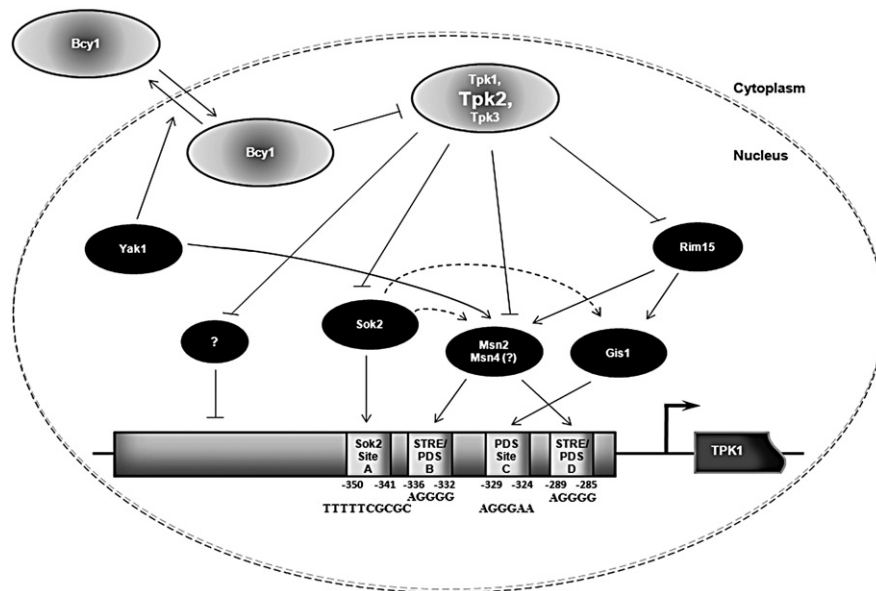
Fig. 8. Model of regulation of *TPK1* promoter by Msn2, Gis1, Sok2, and PKA activities. *TPK1* promoter is under PKA isoform-dependent auto-regulation, Tpk2 showed to be the isoform with the highest inhibitory effect. PKA inhibits Msn2/4 and Rim15. Msn2 is already bound to *TPK1* promoter in cells growing logarithmically on glucose and its abundance decreases throughout heat shock, in agreement with the "black widow" model. Gis1 shares the DNA elements on *TPK1* promoter with Msn2/4 and both of them are necessary to the upregulation of the promoter in heat shock response. Sok2 could assist the binding of Msn2/4 and Gis1 to the *TPK1* promoter (dashed line). During the heat shock Msn2 leaves the promoter and Gis1 is recruited to its sites in response to this stress. Cytoplasmic-nuclear localization of Bcy1 is regulated by Yak1 phosphorylation [23], contributing to the regulation of nuclear PKA activity. A, B, C, and D correspond to the mutated sites. The element sequences are indicated below.

by PKA activity (Fig. 2). All four promoters exhibited different degrees of upregulation in the absence of PKA activity. Moreover, the autoregulation mechanism was Tpk isoform-dependent. Tpk2 was the catalytic isoform that produced the highest inhibition on *TPK1* and *TPK3* promoters, and by contrast it was the isoform that caused the lowest inhibition on its own promoter. *TPK3* promoter activity was quite below the other two catalytic isoform promoter activities (Fig. 1). *BCY1* promoter exhibited the lowest activity of all the PKA subunit promoters; however, it was the promoter with the highest degree of upregulation in the absence of PKA activity.

Even though we clearly could observe differences in the individual behavior of each strain containing one Tpk isoform at a time, it is difficult to predict the composite behavior of the three isoforms coexisting in a wild type strain. Taking into account that *TPK2* promoter is almost not inhibited by Tpk2 catalytic activity, which is evident in the strain that only expresses Tpk2 isoform, we can hypothesize that the expression level of *TPK2* in this strain would be much higher than in the wild type strain and thus, its inhibitory effect on *TPK1*, *TPK3* and *BCY1* promoters is more strongly evidenced. It has been also reported that during exponential growth on glucose, Tpk2 localizes in the nucleus, whereas Tpk1 and Tpk3 show a mixed pattern of nucleo-cytoplasmic localization [66] suggesting that the inhibition state of the promoters could be a consequence of the localization of the subunits.

There are few examples of the transcription regulation of PKA subunits, one of them is described in the fission yeast *Schizosaccharomyces pombe* [58], the other in *Candida albicans* [19]. The results in *S. pombe* are in agreement with our results as the authors show that cgs1 and pka1 genes which encode the regulatory and the catalytic subunits of PKA respectively are negatively regulated by the PKA pathway. The authors also show in this work that nutrient starvation activates a stress-activated protein kinase (SAPK) pathway that positively regulates the transcription of both genes, providing a mechanism for cross talk between these two antagonistically acting pathways. In our work, we show that at least one component of the MAPK pathway, Sko1, is not involved in the regulation of the promoters of PKA subunits. On the other hand in *C. albicans* it was described a cross regulation event between Bcy1 and Tpk expression. These results indicate that this yeast is able to compensate increased levels of either Tpk1 or Tpk2 subunits with

an increase of Bcy1 protein levels and vice versa, suggesting a regulated mechanism, although not inhibitory by PKA activity in this case.

The mRNA levels strongly correlated with the promoter activities except for *BCY1* (Fig. 1). Published transcriptome reports under normal growth conditions in glucose indicate that *BCY1* levels are in the range of the *TPKs* levels [33,35,37,43,44]. The discrepancy between the *BCY1* promoter activity and mRNA abundance could be due to a high stability of its messenger. This difference is noteworthy and suggests that a regulation at the level of mRNA stability and translation might be involved in the expression of this PKA subunit. On the other hand, *TPK3* mRNA levels agree with the reported data that indicate that *TPK3* mRNA is the one with the lowest expression levels among the three Tpk subunits [14,32].

The activity of each PKA subunit promoter was differentially regulated during the yeast growth on glucose. *TPK1* and *BCY1* promoters showed an increase in activity during the initial logarithmic growth phase and *TPK3* promoter had a similar, although less pronounced, variation. By contrast, *TPK2* promoter activity decreased during the first part of the growth curve and then increased slowly up to stationary phase. These results indicate that the genes encoding each PKA subunit are differentially expressed, with promoters having different relative activities during the growth curve.

Each promoter shows a differential response to the absence of Msn2/4 (Figs. 4 and 5). Only *TPK1* promoter was upregulated during heat shock and saline stress conditions, and this response was lost in the absence of Msn2/4, showing a key role for these transcription factors in both stress conditions. Neither *TPK2*, nor *TPK3* nor *BCY1* was upregulated during these two stress conditions, although there are genomic reports which classified these ORFs as upregulated under stress [7,8,18,46,50,72]. One possibility for this discrepancy is that the heat shock response in yeast involves changes in both transcription rates and mRNA stabilities [7]. *BCY1*, *TPK2* and *TPK3* show more constant transcription rate values during the heat shock than *TPK1* which was increased up to 1.5-fold. However all the subunits show an increase in their mRNA abundance indicating a role of mRNA stability [7].

The analysis of the participation of Yak1 in the promoters regulation showed intriguing results. In strains lacking *YAK1*, *TPK1* promoter

activity was not downregulated in comparison to WT strain, and still exhibited the Msn2/4-mediated heat shock response. This result would indicate that the absence of Yak1 had no net effect on Msn2/4. It has been reported that under glucose starvation or heat shock, both Yak1 and Rim15 might regulate Msn2/4 by phosphorylation, but their effects seem to be different depending on the promoter context of the targets [29,30]. In the case of *TPK1* promoter, in the absence of Yak1, Rim15 would positively regulate Msn2/4 activity under heat shock. On the other hand, the lack of Yak1 would also lead to a more inhibited nuclear PKA as a consequence of the increased localization of Bcy1 in the nucleus (see below). A lower PKA activity is in agreement with an active *TPK1* promoter. *TPK2*, *TPK3*, and *BCY1* promoters showed no response to heat shock stress in *yak1Δ* strain. Surprisingly, in assays performed with strains lacking *MNS2/4* and also *YAK1*, all the promoter activities were upregulated. A possible explanation could be that Yak1 indirectly affects the activity of the promoters. One substrate of the kinase Yak1 is Bcy1 and its phosphorylation is important for its localization [3,23]. Bcy1 localization is carbon source dependent and in cells growing on glucose, it is predominantly nuclear [22]. Bcy1 has been shown to have an increased nuclear localization in *yak1Δ* and *msn2/4Δ* strains. This localization is further increased in a strain with deletions in both *MSN2/4* and *YAK1* [23]. The results show that this strain is the one that showed the highest increase in the promoter activities (Fig. 4). By contrast, an adenylate cyclase mutant strain, with low PKA activity, shows a high cytoplasmic localization of Bcy1 in glucose-grown cells [23]. Thus, PKA controls localization of its own regulatory subunit Bcy1. In *msn2/4Δ yak1Δ* strain, PKA activity may have been inhibited as a result of the increased Bcy1 nuclear localization, leading to the promoter upregulation.

The summary of the results related to *TPK1* promoter regulation is schematically shown in Fig. 8. Gis1, Msn2/4, and Rim15 are involved in the *TPK1* activity regulation. Msn2 positively regulates *TPK1* promoter. Although the decrease of Msn2 on *TPK1* promoter during heat shock is not an expected behavior for an activating transcription factor, it has been described for the upregulation of some heat shock protein promoters [11]. The Msn2/4 factors are known to be proteolytically degraded in a heat shock-dependent manner to mediate the induction of transcription. This behavior is named the "black widow" model of transcription activation and it is triggered to avoid unnecessary transcription. In this model the transcription factor is degraded at the promoter right after initiation of transcription [9,11,59].

Gis1 and Rim15 have been described to be important in the reprogramming of metabolism during the diauxic shift. It has been described that 95% of the Gis1-dependent genes are included within a set of Msn2/4-dependent genes, suggesting that Gis1 and Msn2/4 not only functionally overlap in vivo, but also cooperatively regulate transcription of a large set of genes [6,42,73]. Our results suggest that during heat shock, Gis1 and Msn2/4 transcription factors mediate a coordinate response on *TPK1* promoter (Figs. 4 and 6).

We have also demonstrated the participation of Sok2 in the regulation of the *TPK1* promoter activity. Sok2 is known as a general repressor, but also it has been described as a leaky activator [54]. Sok2 repressor activity depends on phosphorylation by PKA [54]. *TPK1* promoter contains a distribution of STRE and putative Sok2 binding sites in close proximity (Fig. 8). Here we have shown that Sok2 is involved in *TPK1* promoter regulation acting as an activator (Fig. 6). We have also evidenced that Sok2 is bound to *TPK1* promoter close to Msn2/4 binding sites. We suggest that Sok2 assists the binding of Msn2/4, but when Msn2/4 leaves the promoter in response to heat shock, Sok2 is recruited together with Gis1. Sok2 is also necessary to Gis1 binding (Fig. 7). A strain lacking Sok2 has an increased thermotolerance and also an increased glycogen accumulation, phenotypes associated with low PKA activity [69,70]. These effects are consistent with a model in which *TPK1* expression and consequently PKA activity are positively regulated by the Sok2 transcription factor.

Finally, in addition to the regulatory pathway that involves Msn2/4, Gis1, Sok2 and Rim15, there is a PKA dependent parallel pathway that negatively regulates *TPK1* promoter activity through a regulator/s that we have not yet identified (Fig. 8).

In the cAMP–PKA pathway, cAMP plays a crucial role as a second messenger with strictly controlled levels. It has been demonstrated ([67]; and references therein) that the activation of PKA triggers a feedback down-regulation process regulated at different levels by the same PKA activity. PKA also regulates its own localization through Yak1 and Mns2/4 action [23]. We now add one new level of PKA autoregulation of its cAMP–PKA pathway by demonstrating that PKA subunit expression is regulated by PKA activity. The regulation of the expression of each PKA subunit seems to be another level that controls the specificity of the cAMP–PKA signaling. Although the expression of all the subunits is autoregulated by PKA activity, the global result is that each is expressed differentially during growth and during heat shock and saline stress.

5. Conclusions

TPK1, *TPK2*, *TPK3*, and *BCY1* promoters are differentially regulated, contributing in this way to the specificity of signal transduction by cAMP–PKA pathway. All the promoters share the inhibition by PKA activity, however they have different activities during yeast growth on glucose media or stress conditions. Unlike *TPK1* promoter which is upregulated, *TPK2*, *TPK3* and *BCY1* promoters are not upregulated under stress conditions. The kinase Rim15, but not the kinase Yak1, positively regulates *TPK1* promoter. Msn2/4, Gis1, and Sok2 are transcription factors involved in the regulation of *TPK1* expression during stress. Our results also show that Bcy1 localization, regulated also by PKA activity, might be important in the regulation of the promoter activities.

References

[1] M. Bolte, P. Dieckhoff, C. Krause, G.H. Braus, S. Irniger, Synergistic inhibition of APC/C by glucose and activated Ras proteins can be mediated by each of the Tpk1–3 proteins in *Saccharomyces cerevisiae*, Microbiol. 149 (2003) 1205–1216.
[2] J.R. Broach, R.J. Deschenes, The function of Ras genes in *Saccharomyces cerevisiae*, Adv. Cancer Res. 54 (1990) 79–139.
[3] R. Budhwar, A. Lu, J.P. Hirsch, Nutrient control of yeast PKA activity involves opposing effects on phosphorylation of the Bcy1 regulatory subunit, Mol. Biol. Cell 21 (2010) 3749–3758.
[4] G. Cadd, G.S. McKnight, Distinct patterns of cAMP dependent protein kinase gene expression in mouse brain, Neuron 3 (1989) 71–79.
[5] S. Cameron, L. Levin, M. Zoller, M. Wigler, cAMP independent control of sporulation, glycogen metabolism and heat shock resistance in *S. cerevisiae*, Cell 53 (1988) 555–566.
[6] E. Cameroni, N. Hulo, J. Roosen, J. Winderickx, C. De Virgilio, The novel yeast PAS kinase Rim15 orchestrates G0-associated antioxidant defense mechanisms, Cell Cycle 3 (2004) 462–468.
[7] L. Castells-Roca, J. Garcia-Martinez, J. Moreno, E. Herrero, E. Belli, J.E. Pérez-Ortín, Heat shock response in yeast involves changes in both transcription rates and mRNA stabilities, PLoS ONE 6 (2011) e17272.
[8] H.C. Causton, B. Ren, S.S. Koh, C.T. Harbison, E. Kanin, Remodeling of yeast genome expression in response to environmental changes, Mol. Biol. Cell 12 (2001) 323–337.
[9] G.A. Collins, W.P. Tansey, The proteasome: a utility tool for transcription, Curr. Opin. Genet. Dev. 16 (2006) 197–202.

[10] D.E. Cummings, E.P. Brandon, J.V. Planas, K. Motamed, R.L. Idzerda, G.S. McKnight, Genetically lean mice result from targeted disruption of the RII b subunit of protein kinase A, Nature 382 (1996) 622–626.

[11] T.Y. Erkina, P.A. Tschetter, A.M. Erkine, Different requirements of the SWI/SNF complex for robust nucleosome displacement at promoters of heat shock factor and Msn2- and Msn4-regulated heat shock genes, Mol. Cell. Biol. 28 (2008) 1207–1217.

[12] F. Estruch, M. Carlson, Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*, Mol. Cell. Biol. 13 (1993) 3872–3881.

[13] S.B. Ferguson, E.S. Anderson, R. Harshaw, T. Thate, N.L. Craig, H.C.M. Nelson, PKA regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in *Saccharomyces cerevisiae*, Genetics 169 (2005) 1203–1214.

[14] F. Galello, P. Portela, S. Moreno, S. Rossi, Characterization of substrates that have a differential effect on *Saccharomyces cerevisiae* protein kinase A holoenzyme activation, J. Biol. Chem. 285 (2010) 29770–29779.

[15] J.M. Gancedo, The early steps of glucose signalling in yeast, FEMS Microbiol. Rev. 32 (2008) 673–704.

[16] H. Garreau, R.N. Hasan, G. Renault, F. Estruch, E. Boy-Marcotte, M. Jacquet, Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae*, Microbiology 146 (2000) 2113–2120.

[17] S. Garrett, J. Broach, Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, YAK1, whose product may act downstream of the cAMP dependent protein kinase, Genes Dev. 3 (1989) 1336–1348.

[18] A.P. Gasch, P.T. Spellman, C.M. Kao, O. Carmel-Harel, M.B. Eisen, G. Storz, D. Botstein, P.O. Brown, Genomic expression programs in the response of yeast cells to environmental changes, Mol. Biol. Cell 11 (2000) 4241–4257.

[19] R. Giacometti, F. Kronberg, R.M. Biondi, A.I. Hernández, S. Passeron, Cross regulation between *Candida albicans* catalytic and regulatory subunits of protein kinase A, Fungal Genet. Biol. 49 (2012) 74–85.

[20] W. Görner, E. Durchschlag, M.T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis, C. Schüller, Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity, Genes Dev. 12 (1998) 586–597.

[21] W. Görner, E. Durchschlag, J. Wolf, E.L. Brown, G. Ammener, H. Ruis, C. Schüller, Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor, EMBO J. 21 (2002) 135–144.

[22] G. Griffioen, P. Anghileri, E. Imre, M.D. Baroni, H. Ruis, Nutritional control of nucleocytoplasmic localization of cAMP-dependent protein kinase catalytic and regulatory subunits in *Saccharomyces cerevisiae*, J. Biol. Chem. 275 (2000) 1449–1456.

[23] G. Griffioen, P. Branduardi, A. Ballarini, P. Anghileri, J. Norbeck, M.D. Baroni, H. Ruis, Nucleocytoplasmic distribution of budding yeast protein kinase A regulatory subunit Bcy1 requires Zds1 and is regulated by Yak1-dependent phosphorylation of its targeting domain, Mol. Cell. Biol. 21 (2001) 511–523.

[24] G. Houge, O.K. Vintermyr, S.O. Døskeland, The expression of cAMP-dependent protein kinase subunits in primary rat hepatocyte cultures. Cyclic AMP downregulates its own effector system by decreasing the amount of catalytic subunit and increasing the mRNAs for the inhibitory (R) subunits of cAMP-dependent protein kinase, Mol. Endocrinol. 4 (1990) 481–488.

[25] T. Jahnsen, S.M. Lohmann, U. Walter, L. Hedin, J.S. Richards, Purification and characterization of hormone-regulated isoforms of the regulatory subunit of type II cAMP dependent protein kinase from rat ovaries, J. Biol. Chem. 260 (1985) 15980–15987.

[26] H.K. Knutsen, K.A. Taskén, W. Eskild, T. Jahnsen, V. Hansson, Adenosine 3′,5′-monophosphate-dependent stabilization of messenger ribonucleic acids (mRNAs) for protein kinase-A (PKA) subunits in rat Sertoli cells: rapid degradation of mRNAs for PKA subunits is dependent on ongoing RNA and protein synthesis, Endocrinology 129 (1991) 2496–2502.

[27] L. Kuras, K. Struhl, Binding of TBP to promoters *in vivo* is stimulated by activators and requires Pol II holoenzyme, Nature 399 (1999) 609–613.

[28] B.F. Landmark, O. Øyen, B.S. Skålhegg, B. Fauske, T. Jahnsen, V. Hansson, Cellular localization and age dependent changes of the regulatory subunits of cAMP dependent protein kinase in rat testis, J. Reprod. Fertil. 99 (1993) 323–334.

[29] P. Lee, B. Cho, H. Joo, J. Hahn, Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4, Mol. Microbiol. 70 (2008) 882–895.

[30] P. Lee, M.S. Kim, S.M. Paik, S.H. Choi, B.R. Cho, J.S. Hahn, Rim15-dependent activation of Msn2/4 and Hsf1 transcription factors by direct phosphorylation in *Saccharomyces cerevisiae*, FEBS Lett. 587 (2013) 3648–3655.

[31] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta DeltaC(T)) method, Methods 25 (2001) 402–408.

[32] M.J. Mazón, M.M. Behrens, E. Morgado, F. Portillo, Low activity of the yeast cAMP-dependent protein kinase catalytic subunit Tpk3 is due to the poor expression of the TPK3 gene, Eur. J. Biochem. 213 (1993) 501–506.

[33] C. Miller, B. Schwalb, K. Maier, D. Schulz, S. Dümcke, B. Zacher, A. Mayer, J. Sydow, L. Marcinowski, L. Dölken, D.E. Martin, A. Tresch, P. Cramer, Dynamic transcriptome analysis measures rates of mRNA synthesis and decay in yeast, Mol. Syst. Biol. 7 (2011) 458–471.

[34] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972. 23–56.

[35] F. Miura, N. Kawaguchi, M. Yoshida, C. Uematsu, K. Kito, Y. Sakaki, T. Ito, Absolute quantification of the budding yeast transcriptome by means of competitive PCR between genomic and complementary DNAs, BMC Genomics 9 (2008) 574–588.

[36] A.M. Myers, A. Tzagoloff, D.M. Kinney, C.J. Lusty, Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions, Gene 45 (1986) 299–310.

[37] U. Nagalakshmi, Z. Wang, K. Waern, C. Shou, D. Raha, M. Gerstein, M. Snyder, The transcriptional landscape of the yeast genome defined by RNA sequencing, Science 320 (2008) 1344–1349.

[38] J. Nikawa, S. Cameron, T. Toda, K.M. Ferguson, M. Wigler, Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*, Genes Dev. 1 (1987) 931–937.

[39] O. Øyen, A. Frøysa, M. Sandberg, W. Eskild, D. Joseph, V. Hansson, T. Jahnsen, Cellular localization and age dependent changes in mRNA for cyclic adenosine 3′,5′− specificity in the cAMP/PKA signaling pathway monophosphate-dependent protein kinases in rat testis, Biol. Reprod. 37 (1987) 947–956.

[40] O. Øyen, M. Sandberg, W. Eskild, F.O. Levy, G. Knutsen, S. Beebe, V. Hansson, T. Jahnsen, Differential regulation of messenger ribonucleic acids for specific subunits of cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase by cAMP in rat Sertoli cells, Endocrinology 122 (1988) 2658–2666.

[41] S.P. Palecek, A.S. Parikh, S.J. Kron, Sensing, signalling and integrating physical processes during *Saccharomyces cerevisiae* invasive and filamentous growth, Microbiol. 4 (2002) 893–907.

[42] I. Pedruzzi, N. Bürckert, P. Egger, C. De Virgilio, *Saccharomyces cerevisiae* Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1, EMBO J. 19 (2000) 2569–2597.

[43] V. Pelechano, J.E. Pérez-Ortín, There is a steady-state transcriptome in exponentially growing yeast cells, Yeast 27 (2010) 413–422.

[44] V. Pelechano, S. Chávez, J.E. Pérez-Ortín, A complete set of nascent transcription rates for yeast genes, PLoS One 5 (2010) e15442.

[45] P. Portela, S. Moreno, Glucose-dependent activation of protein kinase A activity in *Saccharomyces cerevisiae* and phosphorylation of its TPK1 catalytic subunit, Cell. Signal. 18 (2006) 1072–1086.

[46] F. Posas, J.R. Chambers, J.A. Heyman, J.P. Hoeffler, E. de Nadal, J. Arino, The transcriptional response of yeast to saline stress, J. Biol. Chem. 275 (2000) 17249–17255.

[47] A. Reinders, N. Bürckert, T. Boller, A. Wiemken, C. De Virgilio, *Saccharomyces cerevisiae* cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase, Genes Dev. 12 (1998) 2943–2955.

[48] N. Reinton, T.B. Haugen, S. Ørstavik, B.S. Skålhegg, V. Hansson, T. Jahnsen, K. Taskén, The gene encoding the C catalytic subunit of cAMP-dependent protein kinase is a transcribed retroposon, Genomics 15 (1998) 287–290.

[49] W. Reiter, E. Klopf, V. De Wever, D. Anrather, A. Petryshyn, A. Roetzer, G. Niederacher, E. Roitinger, I. Dohnal, W. Görner, K. Mechtler, C. Brocard, C. Schüller, G. Ammerer, Yeast protein phosphatase 2A-Cdc35 regulates the transcriptional response to hyperosmolarity stress by regulating Msn2 and Msn4 chromatin recruitment, Mol. Cell. Biol. 33 (2013) 1057–1072.

[50] M. Rep, M. Krantz, J.M. Thevelein, S. Hohmann, The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes, J. Biol. Chem. 275 (2000) 8290–8300.

[51] H. Ruis, C. Schuller, Stress signaling in yeast, BioEssays 17 (1995) 959–965.

[52] G.M. Santangelo, Glucose signaling in *Saccharomyces cerevisiae*, Microbiol. Mol. Biol. Rev. 70 (2006) 253–282.

[53] A.P. Schmitt, K. McEntee, Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5777–5782.

[54] G. Shenhar, Y. Kassir, A positive regulator of mitosis, Sok2, functions as a negative regulator of meiosis in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 21 (2001) 1603–1612.

[55] B. Skålhegg, K. Taskén, Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation and subcellular localization of subunits of PKA, Front. Biosci. 5 (2000) 678–693.

[56] B.S. Skålhegg, K. Taskén, A.M. Rasmussen, V. Hansson, T. Jahnsen, T. Lea, Cyclic AMP sensitive signalling by the CD28 marker requires concomitant stimulation by the T-cell antigen receptor (TCR/CD3) complex, Scand. J. Immunol. 40 (1994) 201–208.

[57] A. Smith, M.P. Ward, S. Garrett, Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation, EMBO J. 17 (1998) 3556–3564.

[58] J. Stiefel, L. Wang, D.A. Kelly, R. Janoo, J. Seitz, S.K. Whitehall, C. Hoffman, Suppressors of an adenylate cyclase deletion in the fission yeast *Schizosaccharomyces pombe*, Eukaryot. Cell 3 (2004) 610–619.

[59] W.P. Tansey, Transcriptional activation: risky business, Genes Dev. 15 (2001) 1045–1050.

[60] K. Taskén, K.B. Andersson, B.S. Skålhegg, K.A. Taskén, V. Hansson, T. Jahnsen, H.K. Blomhoff, Reciprocal regulation of mRNA and protein for subunits of cAMP dependent protein kinase (RIα and Cα) by cAMP in a neoplastic B cell line (Reh), J. Biol. Chem. 268 (1993) 23483–23489.

[61] K.A. Taskén, H.K. Knutsen, H. Attramadal, K. Taskén, T. Jahnsen, V. Hansson, W. Eskild, Different mechanisms are involved in cAMP-mediated induction of mRNAs for subunits of cAMP-dependent protein kinases, Mol. Endocrinol. 5 (1991) 21–28.

[62] J.M. Thevelein, L. Cauwenberg, S. Colombo, J.H. De Winde, M. Donation, F. Dumortier, L. Kraakman, K. Lemaire, P. Ma, D. Nauwelaers, F. Rolland, A. Teunissen, P. Van Dijck, M. Versele, S. Wera, J. Winderickx, Nutrient induced signal transduction through the protein kinase pathway and its role in the control of metabolism, stress resistance and growth in yeast, Enzyme Microb. Technol. 26 (2000) 819–825.

[63] J.M. Thevelein, Signal transduction in yeast, Yeast 10 (1994) 1753–1790.

[64] J.M. Thevelein, J.H. de Winde, Novel sensing mechanisms and targets for the cAMP–protein kinase A pathway in the yeast *Saccharomyces cerevisiae*, Mol. Microbiol. 33 (1999) 904–918.

[65] T. Toda, S. Cameron, P. Sass, M. Zoller, M. Wigler, Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase, Cell 50 (1987) 277–287.

[66] V. Tudisca, V. Recouvreux, S. Moreno, E. Boy-Marcotte, M. Jacquet, P. Portela, Differential localization to cytoplasm, nucleus or P-bodies of yeast PKA subunits under different growth conditions, Eur. J. Cell Biol. 89 (2010) 339–348.

[67] J. Vandamme, D. Castermans, J.M. Thevelein, Molecular mechanisms of feedback inhibition of protein kinase A on intracellular cAMP accumulation, Cell. Signal. 24 (2012) 1610–1618.

[68] J.W. Wallis, G. Chrebet, G. Brodsky, M. Rolfe, R. Rothstein, A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase, Cell 58 (1989) 409–419.

[69] M.P. Ward, S. Garrett, Suppression of a yeast cyclic AMP dependent protein kinase defect by overexpression of SOK1, a yeast gene exhibiting sequence similarity to a developmentally regulated mouse gene, Mol. Cell. Biol. 14 (1994) 5619–5627.

[70] M.P. Ward, C.J. Gimeno, G.R. Fink, S. Garrett, SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription, Mol. Cell. Biol. 15 (1995) 6854–6863.

[71] R.B. Wilson, G. Renault, M. Jaquet, K. Tatchell, The pde2 gene of *Saccharomyces cerevisiae* is allelic to rca1 and encodes a phosphodiesterase which protects the cell from extracellullar cAMP, FEBS 3 (1993) 191–195.

[72] J. Yale, H.J. Bohnert, Transcript expression in *Saccharomyces cerevisiae* at high salinity, J. Biol. Chem. 276 (2001) 15996–16007.

[73] N. Zhang, J. Wu, S. Oliver, Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast, Microbiology 155 (2009) 1690–1698.