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Elements from the cAMP signaling pathway are involved in the control of expression of the yeast gluconeogenic gene *FBP1*

Oscar Zaragoza, Juana M. Gancedo*

Instituto de Investigaciones Biomédicas 'Alberto Sols', CSIC-UAM, Arturo Duperier 4, E-28029 Madrid, Spain

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Abstract cAMP represses the transcription of some *Saccharomyces cerevisiae* genes sensitive to catabolite repression. The effect of cAMP on the expression of *FBP1*, encoding fructose-1,6-bisphosphatase (FbPase), has been further investigated. In yeast cells shifted to a derepressing medium, synthesis of FbPase was delayed if the strong decrease in intracellular cAMP, which occurs during the shift, was prevented. A similar delay occurred in a *RAS2*^{val19} strain, while in a *tpk1*^{w} strain, with weak protein kinase A activity, induction of FbPase occurred earlier than in a *TPK1* strain. In the *tpk1*^{w} strain, proteins which bind the UAS1 element of *FBP1* were present during growth on glucose but they were only weakly operative. Expression of *CAT8* and *SIP4*, encoding proteins which bind the UAS2 element, was blocked by a high concentration of cAMP, but catabolite repression of these genes was not much relieved in a *tpk1*^{w} strain. We conclude that in *S. cerevisiae*, as reported for *Schizosaccharomyces pombe*, control of *FBP1* requires both cAMP-dependent and independent pathways; however, the mechanisms operating in the two yeasts are different.

Key words: cAMP; Catabolite repression; Fructose-1,6-bisphosphatase; *CAT8*; *SIP4*; *Saccharomyces cerevisiae*

1. Introduction

To avoid the simultaneous functioning of enzymes catalyzing antagonistic reactions, elaborate regulatory mechanisms have evolved and have been selected in different organisms. A well-documented case is that of antagonistic enzymes involved in glycolysis and gluconeogenesis. Genes encoding gluconeogenic enzymes are usually repressed by glucose and cAMP appears implicated in different ways in the control of these genes.

In *Escherichia coli* and in mammalian cells, where cAMP concentration falls when glucose is available [1], induction of the genes which encode phosphoenolpyruvate carboxykinase requires cAMP [2,3]. However, if glucose is present the genes are not induced even at a high concentration of cAMP [2,4].

In contrast, in yeasts, which show high levels of cAMP during growth on glucose [5], the expression of the genes which encode the gluconeogenic enzyme fructose-1,6-bisphosphatase (FbPase) is negatively regulated by cAMP. In *Schizosaccharomyces pombe*, any mutation which impairs cAMP-dependent protein kinase activity allows the derepression of the *fbp1*$^{+}$ gene in the presence of glucose [6,7]. In addition, *fbp1*$^{+}$ expression depends on a pathway which includes the mitogen-activated protein kinase Sty1, the Wis1 kinase acting on Sty1 and a protein tyrosine phosphatase, Pyp1, able to dephosphorylate and inactivate Sty1. Both Wis1 and Sty1 are required for *fbp1*$^{+}$ expression while overexpression of *pyp1*$^{+}$ inhibits the transcription of *fbp1*$^{+}$ [8–10]. In *Saccharomyces cerevisiae*, although genes such as those encoding galactokinase [11] or invertase [12] are not affected by cAMP, cAMP has been shown to interfere with the transcription of a large number of genes normally expressed at the diauxic shift [13], and it has been observed that cAMP is able to block the expression of the *FBP1* gene in a derepressing medium [12,14].

The promoter of *FBP1* from *S. cerevisiae* contains two activating elements, UAS1 and UAS2, which both show carbon source-dependent regulation [15–18], and respond, to different extents, to the presence of cAMP [12]. To explore further the effect of cAMP on the control of *FBP1*, we have studied how mutations in the cAMP signaling pathway, which may cause either an increase or a decrease in protein kinase activity dependent on cAMP, affect *FBP1* expression or the formation of UAS$_{FBP1}$–DNA complexes. We have also examined how do changes in the cAMP signaling pathway affect the expression of the genes *CAT8* and *SIP4*, which encode two zinc cluster proteins, Cat8 and Sip4, both able to bind UAS2 [19,20], but which regulate differently the activating elements UAS1 and UAS2 [21].

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeasts strains used in this study are listed in Table 1. Yeasts were grown at 30°C or at 24°C (*cdc25 pde2* strains) in YPD (1% yeast extract, 2% peptone, 2% glucose), in Difco yeast nitrogen base (YNB) or in a synthetic complete medium (SC) [22] with 2% glucose, and collected at the exponential phase of growth, 2–3 mg wet weight/ml (repressed cells). To obtain derepressed cells, glucose-grown yeasts were washed twice with distilled water, suspended at 20 mg/ml in YP 2% ethanol and incubated for the time indicated in each case, at 30°C or at 24°C (*cdc25 pde2* strains). The media to which cAMP was added were sterilized by filtration. To isolate transformed *pde2* strains an enriched minimal medium (YNBS) was used [13].

2.2. Plasmids

Plasmids containing UAS1-*lacZ* and UAS2-*lacZ* fusion genes have been described previously [18] as well as *CYC1-lacZ* (pLG669-Z) [23], *CAT8-lacZ* (pDG225) [17] and pSIP4-lacZ [24]. pPDE2 contains a 5.1-kb *Bam*HI–*Hin*dIII fragment from pW34 [25] with the complete *PDE2* gene under the control of its own promoter within the centromeric plasmid YCplac111 [26]. pRAS2^{val19} contains the mutated

*Corresponding author. Fax: (34)-91-5854587.
E-mail address: jmgancedo@iib.uam.es (J.M. Gancedo).

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Table 1
S. cerevisiae strains used in this work

Strain	Relevant genotype	Source/reference
W303-1A	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	[41]
OL556	*MAT**a**/MAT*α*cdc25-5/cdc25-5 his3/his3 leu2/leu2 rca1(pde2)/rca1 TRP1/trp1 ura3/ura3*	[13]
LOZ179	*MAT**a**/MAT*α*cdc25-5/cdc25-5 his3/his3 leu2/leu2 rca1(pde2)/rca1 TRP1/trp1 ura3/ura3::GLK1-lacZ-URA3*	this study
JF907	*MAT**a** ade8 his3 leu2 trp1 ura3::GSY2-lacZ-URA3 tpk1^{w} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 snf1::ADE8*	J.M. François
JF908	*MAT**a** ade8 his3 leu2 trp1 ura::GSY2-lacZ-URA3 tpk1^{w} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	J.M. François
S7-7A	*MAT**a** ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1*	[42]
S18-1D	*MAT*α *ade8 his3 leu2 trp1 ura3 tpk1^{w} tpk2::HIS3 tpk3::TRP1*	[42]

RAS2^{val19} under the control of its own promoter (F. Portillo, unpublished) and is derived from YEp351 [27]. To construct strain LOZ179, a *GLK1-lacZ* fusion gene was integrated in the *URA3* locus from OL556 by linearizing plasmid YIp944 [28] with *Stu*I.

Cloning procedures were performed according to standard protocols [29]. Yeast cells were transformed using the lithium acetate method [30] with minor modifications.

2.3. Preparation of extracts and enzymatic tests

Yeast extracts were prepared by shaking with glass beads as described in [31]; for β-galactosidase assays the centrifugation step was omitted. β-Galactosidase was assayed as in [32], the samples being centrifuged before reading their absorbance. FbPase was tested spectrophotometrically as described in [33]. Protein was determined using the BCA protein assay (Pierce) with bovine serum albumin as a standard.

2.4. Western and Northern blot analysis

Yeast cell-free extracts were precipitated with 2 Vol of 15% trichloroacetic acid and resuspended in Laemmli buffer [34] at a final concentration of 1.5 mg/ml. Samples were heated 5 min at 95°C, fractionated in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, and the proteins transferred to a nitrocellulose membrane by semidry blotting, using 25 mM Tris base, 190 mM glycine and 20% methanol. Anti-FbPase antibodies [33] diluted 1/50 000 in blocking buffer and goat anti-rabbit peroxidase conjugate (Biosource International, Camarillo, CA, USA) diluted 1/10 000 were used as primary and secondary antibody, respectively. The ECL kit from Amersham was used to visualize the immunoprecipitates.

Total RNA was extracted as described in [35] using the Gibco Trizol reagent and the RNA samples treated as described [36]. As probe for the Northern blot, we used a 0.82-kb *Eco*RV–*Stu*I fragment of the *FBP1* gene, from position +57 to +877. The probe was labeled as in [37] using the Pharmacia labeling kit.

2.5. Determination of cAMP

Yeast cells were collected by rapid filtration, frozen in liquid nitrogen and extracted with boiling ethanol, as described in [38]. cAMP was determined in the extracts by a competition assay [5] using the Amersham kit.

2.6. Preparation of yeast nuclear extracts and band-shift assays

Nuclear extracts were obtained as described in [39]. Band-shift assays were performed as described previously, using oligonucleotides OL1 and OL2 which correspond to UAS2$_{FBP1}$ and UAS1$_{FBP1}$ respectively [16].

3. Results and discussion

3.1. Mutations in the cAMP-dependent protein kinase pathway affect the expression of FBP1 and the formation of DNA–protein complexes with UAS1

We have studied how the kinetics of FbPase derepression change in the presence of mutations which affect the cAMP signaling pathway. Since these kinetics show large variations in different genetic backgrounds (reference strains in Figs. 1 and 2 and results not shown), it is important to test always a congenic strain as a control. In a *pde2* strain, the absence of the high affinity phosphodiesterase would be expected to damp changes in intracellular cAMP after the shift of yeast cells to a medium lacking glucose. As shown in Fig. 1A, in such a *pde2* strain derepression was delayed with respect to that of the mutant yeast transformed with a plasmid containing the *PDE2* gene. Little FbPase activity was present 2 h after the shift to derepressing medium. To investigate if this

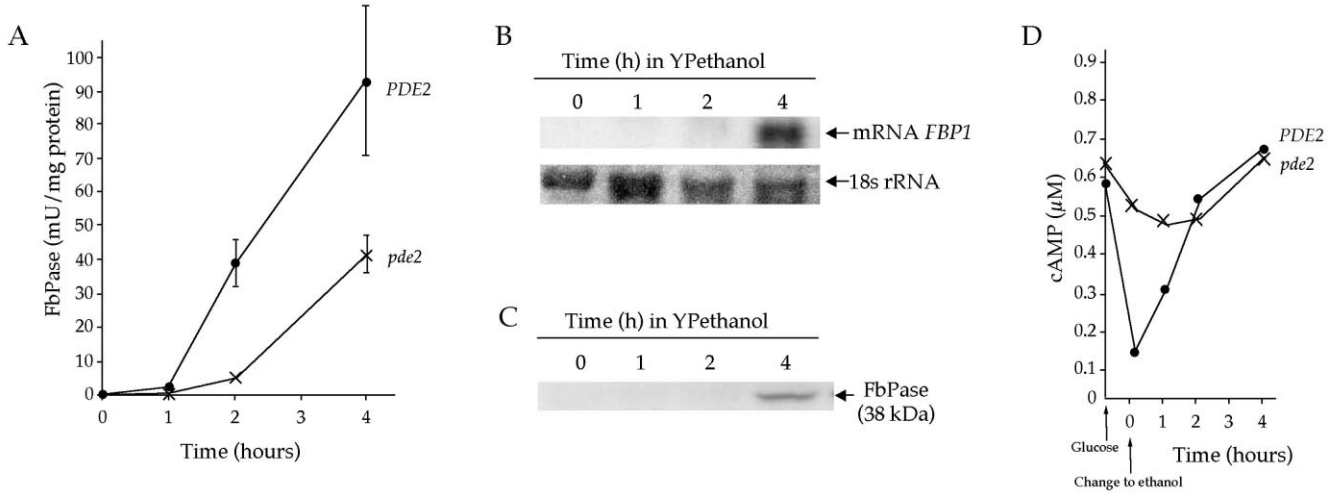

Fig. 1. Effect of the lack of Pde2 in the kinetics of expression of *FBP1*. *S. cerevisiae* OL556 (*pde2*) transformed with a control plasmid (×) or with a plasmid containing the *PDE2* gene (●) was grown in YNBS glucose and derepressed in YP ethanol. A: FbPase activity was measured as described in Section 2 in samples taken at the times indicated. D: cAMP was determined as described in Section 2 in samples taken just before transferring the cells into YP ethanol (marked as glucose with an arrow) and after the transfer, at the times indicated. B: Western blot and C: Northern blot of the different samples.

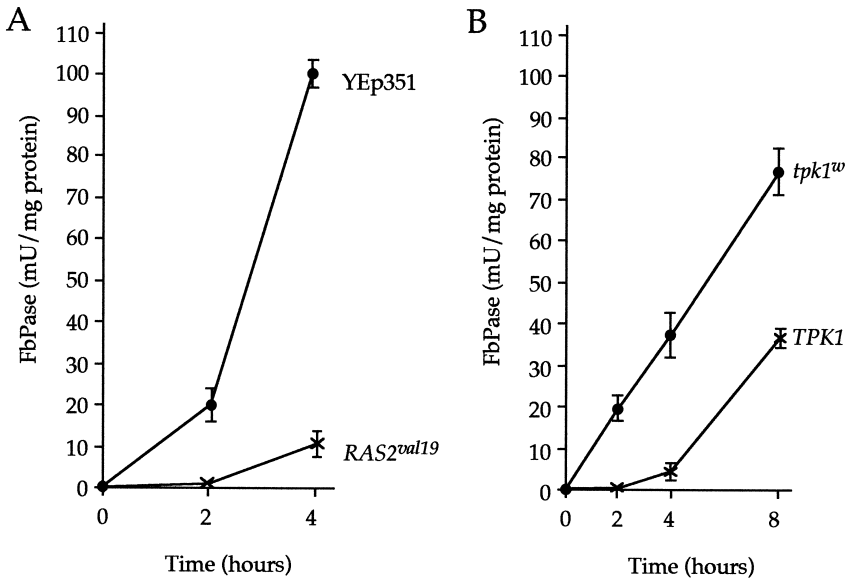

Fig. 2. Effect of different mutations in the cAMP signaling pathway on the derepression of FbPase. A: *S. cerevisiae* W303-1A transformed with a control plasmid (●) or with a plasmid containing the *RAS2*^{val19} gene (×) was grown in YNB glucose and derepressed in YP ethanol. B: Strains S7-7A (*TPK1*) and S18-1D (*tpk1*^{w}) were grown in SC glucose and derepressed in YP ethanol. At the times indicated samples were taken and FbPase activity was measured in the corresponding extracts.

low FbPase activity in the mutant was due to an effect on gene transcription or to catabolite inactivation of the enzyme, we performed Western and Northern analysis in the *pde2* strain. Neither protein reactive with specific antibodies nor mRNA corresponding to *FBP1* were detected (Fig. 1B, C). These findings suggest that the observed effect took place at the transcriptional level. When the yeast with an intact *PDE2* gene, growing exponentially in glucose, was washed and resuspended in a derepressing medium, there was a strong decrease in the intracellular cAMP concentration, which was

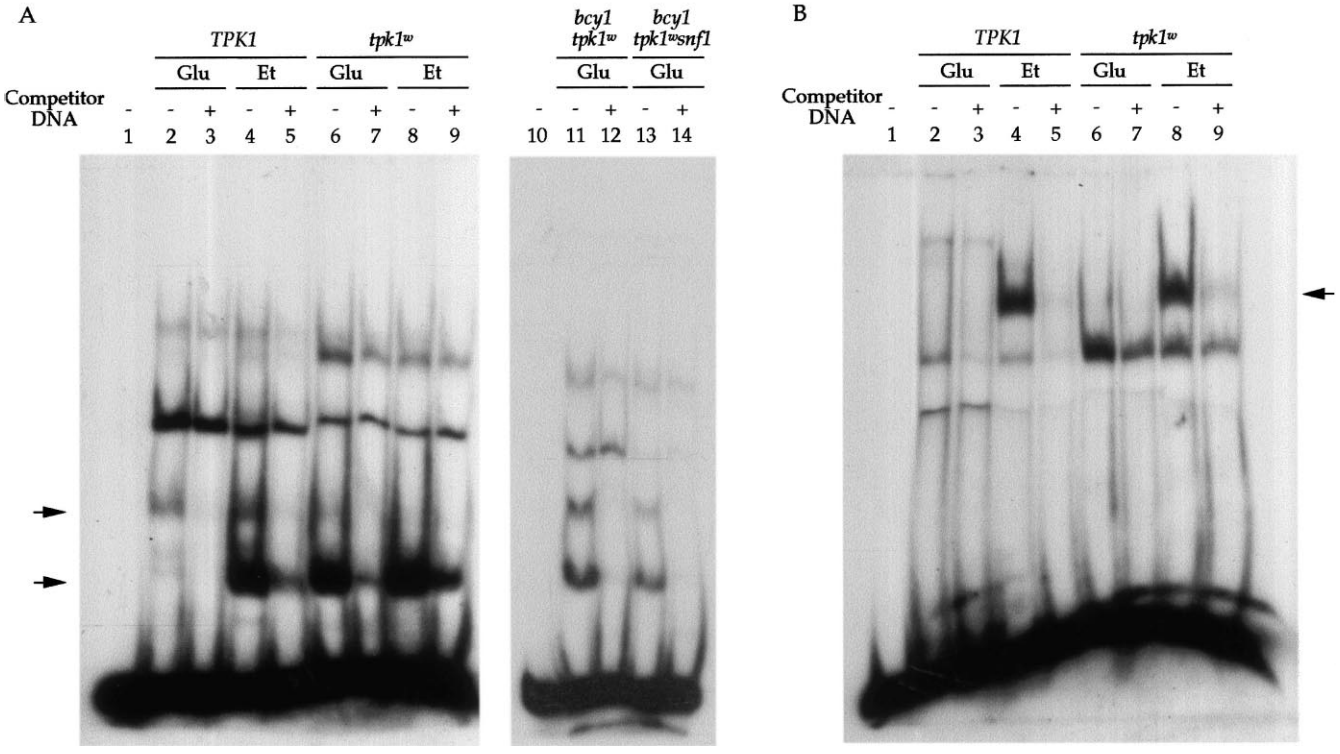

Fig. 3. Effect of a low level of cAMP-dependent protein kinase on the DNA–protein complexes formed with UAS sequences from *FBP1*. Nuclear protein extracts were prepared from strains S7-7A (*TPK1*), S18-1D (*tpk1*^{w}), JF907 (*tpk1*^{w}*bcy1snf1*) or JF908 (*tpk1*^{w}*bcy1*) grown on YPD (Glu) or derepressed in YP ethanol (Et). A: The oligonucleotide OL2 corresponding to $UAS1_{FBP1}$ was used as probe and 20 μg of nuclear proteins were added to the samples in lanes 2–9 and 11–14. B: The oligonucleotide OL1 corresponding to $UAS2_{FBP1}$ was used as probe and 40 μg of nuclear proteins were added to the samples in lanes 2–9. When indicated, a 100-fold excess of competitor DNA was used. See Section 2 for further details. Specific complexes are indicated with arrows.

followed by a gradual recovery (Fig. 1D). In the mutant the decrease was much less marked, but after 2 h in the derepressing conditions, the cAMP levels became similar to those of the wild-type yeast. We conclude that a marked decrease in the intracellular concentration of cAMP appears to facilitate the derepression of FbPase, but is not an absolute requirement for it.

In a yeast strain overexpressing the gene *RAS2*^{val19}, cAMP levels should be increased and, as expected, FbPase derepression is strongly impaired in such a strain (Fig. 2A). On the other hand, in a strain with a weak protein kinase A activity (*tpk1*^{w} strain), FbPase derepression took place much earlier than in the equivalent strain with a wild-type *TPK1* gene (Fig. 2B). In all strains, FbPase activity reached high levels after an overnight incubation in YP ethanol.

The formation of UAS1$_{FBP1}$–protein complexes in band-shift experiments is reduced when nuclear extracts from cells derepressed in the presence of cAMP are used [12]. It would be possible that, conversely, in a *tpk1*^{w} strain the formation of UAS1$_{FBP1}$–protein complexes may occur even when nuclear extracts from glucose-grown cells are used, in contrast with the situation in a wild-type strain. As shown in Fig. 3A, in *tpk1*^{w} strains (either with an intact or an interrupted *BCY1* gene) UAS1–protein complexes were formed with extracts from repressed cells. The complexes were also found in a mutant lacking Snf1, a protein kinase required for the expression of UAS1-binding proteins, in a wild-type strain, under derepressed conditions [16]. This suggests that the requirement for Snf1 observed earlier is related to the need to counteract the activity of the cAMP-dependent protein kinases. On the other hand, the UAS2–protein complex was not formed when extracts from a glucose-grown *tpk1*^{w} strain were used (Fig. 3B). Thus, a decreased activity of the cAMP-dependent protein kinase does not relieve the repression of proteins binding UAS2, but allows the formation of UAS1–protein complexes in glucose-grown cells. However, the formation of this complex is not sufficient to obtain a measurable FbPase activity in the same conditions ([12], Fig. 2B). Moreover, the UAS1–protein complexes formed with extracts from a glucose-grown *tpk1*^{w} strain operate poorly. As shown in Table 2, in such a strain the expression of UAS1-*lacZ* was still strongly repressed by glucose, as happens with that of UAS2-*lacZ* (Table 2). However derepression of FbPase occurs earlier in a *tpk1*^{w} strain than in the corresponding *TPK1* strain (Fig. 2B) and the same happens with derepression of UAS2-*lacZ* (results not shown).

Table 2
Expression of fusion genes in yeast strains with a weak protein kinase A activity (*tpk1*^{w})

Fusion gene	β-Galactosidase (mU/mg protein)	
	TPK1	*tpk1*^{w}
UAS1-*lacZ*	19 ± 1	96 ± 7
UAS2-*lacZ*	9 ± 1	47 ± 7
CAT8-lacZ	7 ± 1	160 ± 60
SIP4-lacZ	9 ± 1	23 ± 9

Yeast strains S7-7A (*TPK1*) or S18-1D (*tpk1*^{w}) transformed with plasmids pOV30n (UAS1-*lacZ*), pOV21i (UAS2-*lacZ*), pDG225 (*CAT8-lacZ*) or pSIP4-lacZ were grown in SC glucose medium and collected in the logarithmic phase of growth. The values of β-galactosidase activity represent the mean values of three independent transformants.

Table 3
Effect of cAMP on the expression of *CAT8* and *SIP4*

Fusion gene	β-Galactosidase (mU/mg protein)		
	Glucose	Ethanol	Ethanol+cAMP
CAT8-lacZ	< 2	1420 ± 190	39 ± 11
SIP4-lacZ	< 2	110 ± 27	4 ± 2
CYC1-lacZ	610 ± 110	1130 ± 120	1100 ± 115
GLK1-lacZ	31 ± 1	1535 ± 47	351 ± 20

S. cerevisiae OL556 (*pde2*) transformed with different plasmids, pDG225, pSIP4-lacz, pLG669-Z and YIp944 (see Section 2) was grown in YPD and derepressed for 12 h in YP ethanol in the absence or the presence of 5 mM cAMP. Data are the mean of at least three different transformants ± the standard deviation.

3.2. Effect of cAMP on the expression of CAT8 and SIP4

Since it has been found that cAMP affects the expression of UAS1-*lacZ* and UAS2-*lacZ* [12], we investigated whether cAMP could act by regulating the transcription of *CAT8* and *SIP4*. We tested the expression of *CAT8-lacZ* and *SIP4-lacZ* in a *tpk1*^{w} strain with the results shown in Table 2. During growth on glucose, the amount of β-galactosidase synthesized from *CAT8-lacZ* was markedly higher than in the *TPK1* strain, indicating that the expression of *CAT8* is repressed by glucose through a cAMP-dependent pathway. However, the relief of *CAT8* repression in the *tpk1*^{w} is only partial, since the β-galactosidase activity measured in glucose is 40-fold lower than the one measured in the same strain in ethanol (8100 mU/mg protein), a result which supports the idea of redundant glucose repression pathways. In the case of *SIP4-lacZ*, there was only a small difference between the two strains.

On the other hand, using a *pde2* mutant, sensitive to external cAMP, we found that derepression of *SIP4-lacZ* in the presence of ethanol was completely blocked by cAMP and that derepression of *CAT8-lacZ* was also strongly affected (Table 3). The lack of expression of the fusion genes in the presence of cAMP cannot be explained by a strong inhibition of protein synthesis by cAMP, since in these conditions the control plasmids *CYC1-lacZ* and *GLK1-lacZ* (Table 3) and UAS2-*lacZ* [12] can still direct the synthesis of β-galactosidase.

3.3. Conclusions

We can conclude that cAMP plays a role in glucose signaling towards *FBP1*, but appears to be redundant with other regulatory mechanisms [12]. A decrease in cAMP levels would facilitate the transition from growth on fermentable carbon sources to growth on non-fermentable ones, but the block of *FBP1* derepression by cAMP would only be temporary. It is interesting to remark that when yeast cells are transferred from a poor to a rich carbon source the reverse situation occurs. A sugar, such as glucose, induces a transient increase in cAMP levels which activates the cAMP-dependent protein kinases; in mutant strains, where this increase does not take place, there is an extended delay in the resumption of growth [40].

In *S. pombe* derepression of the *fbp1*$^{+}$ gene is not only controlled by cAMP, but depends on the protein kinases Wis1 and Sty1, and is negatively regulated by the protein tyrosine phosphatase, Pyp1 [8–10]. In contrast, we have observed that expression of *S. cerevisiae FBP1* is not affected by

the lack of the kinases homologous to Wis1 and Sty1 (Pbs2 and Hog1) or by overexpression of the protein tyrosine phosphatase Ptp2 (J.M. Gancedo, unpublished results). It seems therefore that, although in two widely divergent yeast species there is a tight control of the gene encoding FbPase, which involves the interplay of a cAMP-dependent pathway with other regulatory mechanisms, in each yeast the mechanisms which have been selected for are different.

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