Combinatorial control by the protein kinases PKA, PHO85 and SNF1 of transcriptional induction of the Saccharomyces cerevisiae GSY2 gene at the diauxic shift

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Abstract Genes involved in storage carbohydrate metabolism are coordinately induced when yeast cells are subjected to conditions of stress, or when they exit the exponential growth phase on glucose. We show that the STress Responsive Elements (STREs) present in the promoter of *GSY2* are essential for gene activation under conditions of stress, but dispensable for gene induction and glycogen accumulation at the diauxic shift on glucose. Using serial promoter deletion, we found that the latter induction could not be attributed to a single *cis*-regulatory sequence, and present evidence that this mechanism depends on combinatorial transcriptional control by signalling pathways involving the protein kinases Pho85, Snf1 and PKA. Two contiguous regions upstream of the *GSY2* coding region are necessary for negative control by the cyclin-dependent protein kinase Pho85, one of which is a 14-bp G/C-rich sequence. Positive control by Snf1 is mediated by Mig1p, which acts indirectly on the distal part of the *GSY2* promoter. The PKA pathway has the most pronounced effect on *GSY2*, since transcription of this gene is almost completely abolished in an *ira1ira2* mutant strain in which PKA is hyperactive. The potent negative effect of PKA is dependent upon a branched pathway involving the transcription factors Msn2/Msn4p and Sok2p. The *SOK2* branch was found to be effective only under conditions of high PKA activity, as in a *ira1ira2* mutant, and this effect was independent of Msn2/4p. The Msn2/4p branch, on the other hand, positively controls *GSY2* expression directly through the STREs, and indirectly via a factor that still remains to be discovered. In summary, this study shows that the transcription of *GSY2* is regulated by several different signalling pathways which reflect the numerous factors that influence glycogen synthesis in yeast, and suggests that the PKA pathway must be deactivated to allow gene induction at the diauxic shift.

Keywords GSY2 · cAMP · PHO85 · SNF1 · Signal transduction

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Introduction

All microorganisms are endowed with signalling systems that sense and transmit external information into the cell, enabling it to respond appropriately to changes in the extracellular environment. In yeast, changes in glycogen and trehalose levels are metabolic hallmarks that occur in response to changes in growth conditions and to various environmental stresses. The synthesis of these two reserve carbohydrates is promoted by stresses such as nutrient starvation, heat, oxidative and osmotic shocks, and also occurs at the end of the exponential growth phase on glucose (for a review, see François and Parrou 2001). A key step in glycogen biosynthesis is the elongation of linear α-1,4-linked glucosyl chains catalysed by glycogen synthase, whose major isoform is encoded by *GSY2* (Farkas et al. 1991). The activity of this enzyme is regulated by both transcriptional and post-translational controls, which appear to be tightly interconnected (for a review, see François and Parrou 2001).

Previous studies showed that glycogen induction during diauxic growth on glucose is accompanied by the coordinated transcriptional activation of all the genes involved in the biosynthesis and biodegradation of this polymer (De Risi et al. 1997; Parrou et al. 1999a; Teste et al. 2000). However, the molecular mechanism that underlies this activation is still unclear. Ni and Laporte (1995) identified two functional STREs (STress Responsive Elements) in the *GSY2* promoter, and concluded that the removal of these *cis*-acting elements prevents the

transcriptional activation of *GSY2* at the diauxic shift on glucose. We challenged their conclusions by demonstrating that absence of STREs in a *GSY2-lacZ* gene fusion does not affect transcriptional induction of this reporter gene at the diauxic shift (Parrou et al. 1999b). Our results were supported by the co-induction of *GLG1* (glycogenin), which has no STREs in its promoter, and by the STRE-independent activation of *GDB1* and *NTH1*, which code for the glycogen debranching enzyme (Teste et al. 2000) and the neutral trehalase (Zahringer et al. 2001), respectively. Thus, these data argued in favour of the existence of other *cis*-acting elements necessary for gene activation at the diauxic shift on glucose.

The Ras/cAMP (PKA) pathway is essential for nutrient-dependent cell proliferation and stress resistance (reviewed in Thevelein and de Winde 1999): cells in which the PKA pathway is hyperactive cannot undergo the diauxic shift and do not accumulate reserve carbohydrates (Boy-Marcotte et al. 1999; Parrou et al. 1999a). High PKA activity results in the sequestration of the STRE-binding transcriptional activators Msn2p and Msn4p in the cytoplasm (Görner et al. 1998) and consequently in the down-regulation of most of the genes implicated in glycogen metabolism (Smith et al. 1998). However, the PKA pathway requires additional *cis*- and *trans*-acting elements, as it is still able to regulate *GSY2* even when STREs have been deleted from its promoter (Parrou et al. 1999b). In addition, the expression of *GSY2* is negatively regulated, in a STRE-independent manner, by the cyclin-dependent protein kinase Pho85 (Timblin et al. 1996 Timblin and Bergman 1997). Control of *GSY2* also occurs at the post-translational level, because Pho85p, in association with its cyclin-partners Pcl8p and Pcl10p (Huang et al. 1998), potently inhibits Gsy2p by phosphorylating the enzyme (Wilson et al. 1999). Finally, the Snf1 protein kinase that is essential for the derepression of glucose-repressed genes (for a review, see Gancedo 1998) promotes glycogen deposition via both transcriptional and posttranslational effects on *GSY2* (Hardy et al. 1994; Huang et al. 1996; Parrou et al. 1999a).

The aim of this present work was to clarify the mechanism by which *GSY2* is induced in a STRE-independent manner at the diauxic shift, and to determine how the PKA, Pho85p and Snf1p nutrient-signalling pathways affect this transcriptional induction. Moreover, the function of STREs in enabling yeast cells to accumulate glycogen was reinvestigated. Based on our results, we propose a model that illustrates the physiological relevance of nutrient-signalling pathways in controlling levels of *GSY2* and modulating the deposition of glycogen in yeast.

Materials and methods

Plasmid constructions

Plasmids used in this study are listed in Table 1. pEB-ΔA-ΔSTRE, a YIp356 derivative bearing a modified version of the *GSY2* promoter, has been described previously (Parrou et al. 1999b) and is referred to in this work as the 'Control' gene fusion. In this construct the two STREs were replaced by unrelated sequences, and the endogenous 5′UTR was replaced by the 5′UTR of *ACT1*. All other *GSY2-lacZ* constructs (see below) are derived from this 'Control' gene fusion by standard or recombinant PCR (Innis et al. 1990). For recombinant PCR, the oligonucleotides *Bam* H1 5'+ (5′-GAATTCGAGCTCGGTACCCGGGGATCCAG-3′) and AmlacZ 3'+ (5′-AAAACGACGGCGGGATCGCAAGCTTGCATG-3′) were used as external primers, and the specific internal primers are listed in Table 1. The amplified fragments were cleaved with *Bam*HI + *Pst*I, and recloned into YIp356. All constructs were verified by sequencing prior to integration at the *URA3* locus of the recipient strains.

Strain constructions

Yeast strains used in this work are listed in Table 2 and are, unless stated otherwise, derived from JF292. Transformation of yeast strains was carried out using the lithium acetate method. Crosses, sporulation and tetrad analysis were carried out as described by Rose et al. (1990) to isolate the different single and double mutant strains. Strain JF1160 (*msn2 msn4*) was obtained after six backcrosses of W *msn2 msn4* with JF625. Mutant JF1498 (*pop2::LEU2*) was obtained after five backcrosses between strain MY1992 (gift of Dr M. Collart, Geneva, Switzerland) and JF625, and a final cross with JF1120. The deletion of *PHO85* has been described elsewhere (Enjalbert et al. 2000). The *mig1::LEU2* mutant strain was made using the 2.9 kb *Sac*I-*Sac*I fragment of plasmid pJN22 (gift of Dr H. Ronne, Uppsala, Sweden). Construction of the *snf1*Δ:: *HIS3* mutant was carried out starting from pBSK *SNF1*, which bears a 3.2-kb *Eco*RI-*Bam*HI genomic DNA fragment containing *SNF1*. In the first step, partial digestion with *Bsm*I removed a 1092-bp segment that encompasses the 3′ end of the *SNF1* promoter region and almost all the sequence encoding the catalytic domain. In the second step, a 1.2 kb *Bam*HI fragment of *HIS3*, isolated from YDp-H (Berben et al. 1991) was inserted into the *Bcl*I site located in the 3′ part of *SNF1*, to generate pBSK *snf1*Δ:: *HIS3*. The *SNF1* disruption was carried out using a 3.3-kb *Eco*RI-*Bam*HI fragment from pBSK *snf1*Δ:: *HIS3*. To construct the *sok2* mutants, the wild-type *SOK2* sequence was amplified from yeast genomic DNA with the primer pair Vsok2up (5′-CCCAAGCTTGAAAGTGGATTTGTTAAGCACAG-3′; *Hin*dIII site underlined) and Vsok2down (5′-GCGGGATCCCGCTAGGGTTTTGATTAAAGTAACA-3′; *Bam*HI underlined). The PCR product was cloned into the *Hin* dIII/*Bam*HI sites of pBSK to yield pMA12. A 1.1-kb *Sma*I fragment of *LEU2* from YDp-L (Berben et al. 1991) was inserted into the *Sna*BI/*Nsi*I sites in the *SOK2* ORF from pMA12, to generate pMA13. *SOK2*

Table 1 Plasmids carrying lacZ gene fusions with variants of the *GSY2* promoter

Name	Description or purpose/primers used in PCR-based strategies (5' to 3')	Source/refernce
pEB GSY2	Introduction of *Spe*I site into the ΔSTRE-*gsy2-lacZ* (pJL37) construct	Parrou et al. (1999b)
Control	Previously described as pEBΔA-ΔSTRE (replacement of STREs by *Nhe*I/*Bgl*II sites and 5'UTR from *ACT1*)	Parrou et al. (1999b)
Δ0	Standard PCR; truncation of the distal part of the promoter (positions –688 to –494 relative to the translation start site). Primers: Δ0 5' (ACAGGGATCCTGGGGCCTCGAGCA TGGCTCATTTTCG; *Bam*H1 site underlined) and AmlacZ nbsp;3'+ (AAAACGACGGCGGGATCGCAAGCTTGCATG)	This study
Δ1	Recombinant PCR; deletion of the promoter region from –493 to –392. Primers: Δ1 3' (CATGTGCAGATATCCCTATTCC) and Δ1 5' (AATAGGGATATCTGCACATGAATTCTGACACTGGACTGCT)	This study
Δ2	Recombinant PCR; deletion of the promoter region from –391 to –286. Primers: Δ2 3' (GGTAAGCTGCCAGAACCCC) and Δ2 5' (GGGGTTCTTGCAGCTTACCTGGGCATACAATGTTTAACCC)	This study
Δ3	Recombinant PCR; deletion of the promoter region from –285 to –185. Primers: Δ3 3' (GGCTGAACTCAGTCACATATATC) and Δ3 5' (GATATATGTGACTGAGTTCAGCCTCTTTCTTATGCAAGCTCCTCG)	This study
Δ4	Recombinant PCR; deletion of the promoter region from –182 to –93. Primers: Δ4 3' (AAGATCTTGAATTGCGTTCC; *Bgl*II site underlined) and Δ4 5' (GGAACGCAATTCAGATCTTTCCT TAAATATGTACTAGT; *Bgl*II and *Spe*I sites underlined)	This study
ΔH	Recombinant PCR; replacement of *HAP2* consensus (TAATTGGT) by TTACGTAT (*Sna*BI site underlined). Primers: ΔH 3' (TATCATCATACGTAACCATTTTCAGGGAGTCTGG) and ΔH 5' (TGGTTACGTATGATGATATATGTGACTGAGTTC)	This study
ΔHΔM	Recombinant PCR; replacement of the putative Mig1p binding sequences (gcgaCCCCGCgaCCCCGCgacc) by gcgaCTTAAGgacc (*Afl*II site underlined). Primers: ΔM 3' (CTTAAGTCGCTAA TCCGCTAGCTAGCAGTC) and ΔM 5' (GCTAGCTAGCGGATT AGCGACTTAAGGACCAGACTCCCTGAAAATGGT)	

disruption was carried out in strains JF1120, JF1263 and JF1266 (see Table 2) by transformation with the *Hin*dIII-*Bam*HI *sok2::LEU2* fragment from pMA13 (Table 1). Disruption of *MSN2* was carried out in the *ira1ira2 sok2::LEU2* strain (JF1439) using the 2.65-kb *Sal*I fragment from pJL30. This plasmid was constructed by digesting pMSN2 (4.4-kb *MSN2* fragment inserted in pRS305; a gift of F. Estruch, Valencia, Spain) with *Bgl*I and inserting a 1.1-kb *Bam*HI *Kan*^{R} fragment. Disruption of *YAK1* in JF1120 and JF1265 was carried out by the method of Wach et al. (1994) using the *LEU2* marker from Ydp-L as the template and the primers YDp-X5'_YAK1 (5′-AACTCATCCAATAA-TAACGACTCGTCCAGCTCCAATAGCAGGGTAA CGCCAGGTTTTCC-3′) and YDp-X3'_YAK1 (5′-TTCTTCGACAATGTGAAGTTTATTGAACGCGC TTGTTGGCCCGGCTCGTATGTTGTGTGG-3′) (sequences homologous to *YAK1* are underlined). All yeast constructs were checked by Southern analysis or by PCR for integration at the correct locus.

Construction of the *gsy2*-ΔSTRE allele, which is characterized by a lack of STREs in the *GSY2* promoter, was carried out as follows. pEBGSY2 was cleaved with *Spe*I to target the integration of the plasmid to the *GSY2* locus, in order to substitute the mutated promoter for the native promoter. Ura$^+$ transformants were selected on selective YNB minimal medium and further verified by PCR using GSY2_481 (5′ GTTGTGAATCGA-GATGAGCC 3′) and ΔSTRE (5′ TTCTGACACTGG-ACT 3′) as primers. The former hybridizes within the *GSY2* ORF and the latter at the *Bgl*II site that replaces the most distal STRE element in the promoter (see below). Sequencing of the PCR product confirmed the integrity of this new allele.

UV mutagenesis screen to search for *trans*-acting regulators of *GSY2* expression

UV mutagenesis was carried out to isolate *trans*-acting factors involved in the transcriptional regulation of *GSY2*. The genetic screen was based on two criteria: inability of mutant cells to accumulate glycogen, as monitored by iodine staining of plates (Enjalbert et al. 2000), and reduced expression of the *GSY2-lacZ* gene fusion, as revealed by reduced colour development after incubation of permeabilized cells with the substrate X-gal (Rose and Botstein 1983). The mutants that were defective in glycogen synthesis fell into three complementation groups. Two of them (named *gli12* and *gli15*) were cured of the reporter plasmid by two successive backcrosses with the wild type JF292, and transformed with a yeast genomic library constructed in YCp50 (ATCC No. 37415) to screen for clones that restore glycogen accumulation. The plasmids were rescued from independent colonies and found to contain overlapping

Table 2 List of strains used in this study

Strain	Genotype	Source/reference
JF292	*MATα* leu2 ura3 his3	Laboratory stock
JF625	*MATα* leu2 ura3 his3 trp1 lys2	This study
Wmsn2msn3	*MAT* **a***leu2 ura3 his3 trp1 ade2 msn2::HIS3 msn4::TRP1*	F. Estruch
JF1160	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1*	This study
JF1061	*MATα leu2 ura3 his3 ira1 ira2* (*gli12 gli15*)	This study
JF1142	*MAT* **a***leu2 ura3 his3 pho85::HIS3*	This study
JF775	*MAT* **a***leu2 ura3 his3 trp1 mig1::LEU2*	This study
JF1342	*MAT* **a***leu2 ura3 his3 snf1::HIS3*	This study
MY1999	*MAT* **a***leu2::PET56 ura3 his3::TRP1 trp1 gcn4 gal2 pop2::LEU2*	M. Collart
JF558	*MAT* **a***leu2 ura3 his3 URA3::GSY2-lacZ*	Parrou et al. (1999a)
JF1135	*MAT* **a***leu2 ura3 his3 gsy2*-ΔSTRE	This study
JF1120	*MAT* **a***leu2 ura3 his3 URA3::Control*	This study
JF1199	*MAT* **a***leu2 ura3 his3 URA3::*Δ0	This study
JF1182	*MAT* **a***leu2 ura3 his3 URA3::*Δ1	This study
JF1185	*MAT* **a***leu2 ura3 his3 URA3::*Δ2	This study
JF1183	*MAT* **a***leu2 ura3 his3 URA3::*Δ3	This study
JF1145	*MAT* **a***leu2 ura3 his3 URA3::*Δ4	This study
JF1228	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::Control*	This study
JF1217	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*Δ0	This study
JF1214	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*Δ1	This study
JF1215	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*Δ2	This study
JF1216	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*Δ3	This study
JF1325	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*Δ4	This study
JF1326	*MATα leu2 ura3 his3 URA3::*ΔH	This study
JF1259	*MATα leu2 ura3 his3 URA3::*ΔH ΔM	This study
JF1390	*MATα leu2 ura3 his3 URA3::*ΔHGC	This study
JF1327	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*ΔH	This study
JF1261	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*ΔH ΔM	This study
JF1391	*MAT* **a***leu2 ura3 his3 pho85::HIS3 URA3::*ΔHGC	This study
JF1343	*MATα leu2 ura3 his3 mig1::LEU2 URA3::Control*	This study
JF1274	*MATα leu2 ura3 his3 snf1::HIS3 URA3::Control*	This study
JF1344	*MATαleu2 ura3 his3 pho85::HIS3 mig1::LEU2 URA3::Control*	This study
JF1364	*MAT* **a***leu2 ura3 his3 snf1::HIS3 mig1::LEU2 URA3::Control*	This study
JF1275	*MAT* **a***leu2 ura3 his3 snf1::HIS3 URA3::*Δ0	This study
JF1265	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 URA3::Control*	This study
JF1270	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 URA3::*Δ0	This study
JF1271	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 URA3::*Δ1	This study
JF1272	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 URA3::*Δ2	This study
JF1273	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 URA3::*Δ3	This study
JF1324	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 URA3::*Δ4	This study
JF1263	*MATα leu2 ura3 his3 trp1 ira1 ira2 URA3::Control*	This study
JF1266	*MATα leu2 ura3 his3 trp1 ira1 ira2 URA3::*Δ0	This study
JF1267	*MATα leu2 ura3 his3 trp1 ira1 ira2 URA3::*Δ1	This study
JF1268	*MATα leu2 ura3 his3 trp1 ira1 ira2 URA3::*Δ2	This study
JF1269	*MATα leu2 ura3 his3 trp1 ira1 ira2 URA3::*Δ3	This study
JF1322	*MAT* **a***leu2 ura3 his3 trp1 ira1 ira2 URA3::*Δ4	This study
JF1438	*MATα leu2 ura3 his3 sok2::LEU2 URA3::Control*	This study
JF1439	*MATα leu2 ura3 his3 trp1 ira1 ira2 sok2::LEU2 URA3::Control*	This study
JF1563	*MAT* **a***leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 sok2::LEU2 URA3::Control*	This study
JF1538	*MATα leu2 ura3 his3 trp1 yak1::LEU2 URA3::Control*	This study
JF1550	*MATα leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 yak1::LEU2 URA3::Control*	This study
JF1575	*MAT* **a***ira1 ira2 leu2 ura3 his3 trp1 msn2::Kan*^{r} *MSN4 sok2::LEU2 URA3::Control*	This study

genomic fragments encompassing the genes *IRA1* and *IRA2*. Crosses of *gli12* and *gli15* mutants with JC673-38A (*glc1* = *ira1*) and JC757-8A (*glc3* = *ira2*) strains (Cannon et al. 1994) confirmed that the mutations were allelic to *ira1* and *ira2,* respectively. The *ira1 ira2* double mutant was isolated on the basis of its inability to accumulate glycogen on a low nitrogen/high glucose (0.01% NH_2SO_4; 8% glucose) agar medium (Yeast Nitrogen Base without amino acids, containing ammonium sulphate at 1.7 g/l).

Culture conditions

Yeast cells were grown in rich medium (YEPD) containing 10 g of Yeast Extract, 20 g of BactoPeptone and 10 g of glucose per liter, or on YNB (1.7 g Yeast Nitrogen Base without amino acids, supplemented with 5 g ammonium sulphate per liter) and supplemented with glucose (1% final concentration) and auxotrophic requirements. Cultures were performed at 30°C in 2-l shake-flasks containing 0.3 l of medium. Cell growth

was followed by measuring the $OD_{600\ nm}$ value. Samples for β-galactosidase assay were taken during the growth phase up until the end of the diauxic transition. Heat shock and other stress experiments were carried out as described in Parrou et al. (1997). All experiments were repeated twice with consistent results (standard deviations less than 15%).

Biochemical and analytical procedures

Preparation of extracts and the assay for glycogen synthase activity have been described by François et al. (1998). The assay was carried out with 0.25 mM UDP-Glucose as the substrate, in the presence of 20 mM glucose-6-phosphate. Measurement of β-galactosidase activity and determination of glycogen and trehalose were performed as described previously (Rose and Botstein 1983; Parrou et al. 1999a). A rapid qualitative assessment of glycogen was made using the iodine staining method described in Enjalbert et al. (2000).

Results

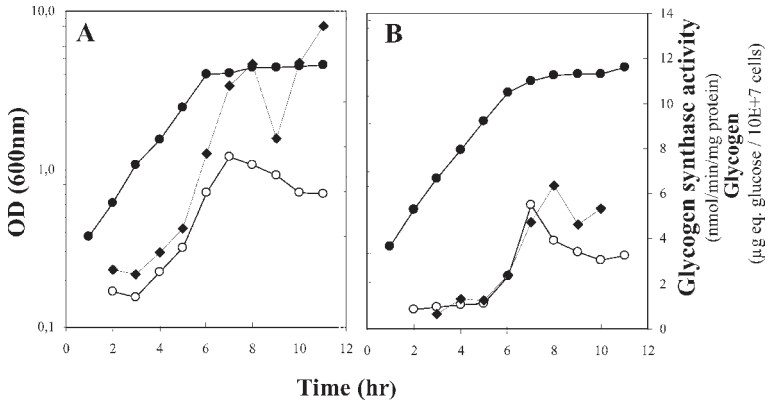
The STREs in the *GSY2* promoter are not essential for glycogen deposition during diauxic growth on glucose

We previously showed that removal of STREs from the *GSY2* promoter does not affect the growth-related induction of this gene at the diauxic shift, although these elements are indispensable for the response to other types of stress (Parrou et al. 1999b). We therefore examined the physiological relevance of STREs in glycogen accumulation using a glycogen synthase mutant (*gsy2-ΔSTRE*) in which the two STREs have been deleted from the *GSY2* promoter. As shown in Fig. 1, quite similar increases in glycogen synthase activity and glycogen accumulation occurred during diauxic growth on glucose in both the wild type cells and the *gsy2-ΔSTRE* mutant, although the magnitude of the increase was about two-fold lower in the latter strain. The glycogen accumulation seen in this mutant was not due to *GSY1*, as deletion of *GSY1* in the *gsy2-ΔSTRE* strain did not alter this response (data not shown). In contrast, no increase in glycogen levels was observed in the *gsy2-ΔSTRE* mutant following exposure of the cells to a mild heat shock (a shift from 25 to 37°C) or to 0.3 M NaCl (data not shown).

Fig. 1A, B Glycogen synthase activity and glycogen accumulation in wild type and gsy2-ΔSTRE mutant strains during growth on glucose. The control strain JF558 (*GSY2*, **A**) and the glycogen synthase mutant JF1135 (gsy2-ΔSTRE, **B**) were cultured on YEPD at 30°C. Glycogen content and glycogen synthase activity were measured during growth as described in Materials and methods. Symbols: OD_{600} (*filled circles*); glycogen levels (*open circles*); glycogen synthase activity (*filled diamonds*)

No single *cis*-acting element can account for the STRE-independent induction of *GSY2* during diauxic growth on glucose

In a previous report (Parrou et al. 1999a), we studied the STRE-independent induction of *GSY2* with a promoter-*lacZ* fusion in which the 5′UTR of the *GSY2* promoter was replaced by the 5′UTR of the constitutive *ACT1* (we refer to this construct as the 'Control'; see Fig. 2). This modification resulted in a 10-fold enhancement of β-galactosidase levels, and this modification did not affect the growth-related induction at the diauxic shift on glucose (Parrou et al. 1999b). This 'Control' *lacZ*-gene fusion was then used as a reference (Fig. 2) in our search for other *cis*-acting regulatory elements in the *GSY2* promoter. As illustrated in Fig. 3, none of the 100-bp internal deletions tested had any marked effect on the induction ratio between the exponential phase and the diauxic shift—not even removal of region 4, which led to a dramatic reduction in *GSY2* expression. This latter effect was probably due to the deletion of the two putative TATAA boxes, which reduced the overall transcriptional activity of the promoter without altering its inducibility. Conversely, this experiment revealed a putative negative regulatory element in region 1, which influenced gene expression more significantly at the diauxic shift than during the exponential phase. To summarize, this experimental design indicated that the induction of *GSY2* that occurs at the beginning of the diauxic shift is a complex event that cannot be ascribed to a single regulatory sequence.



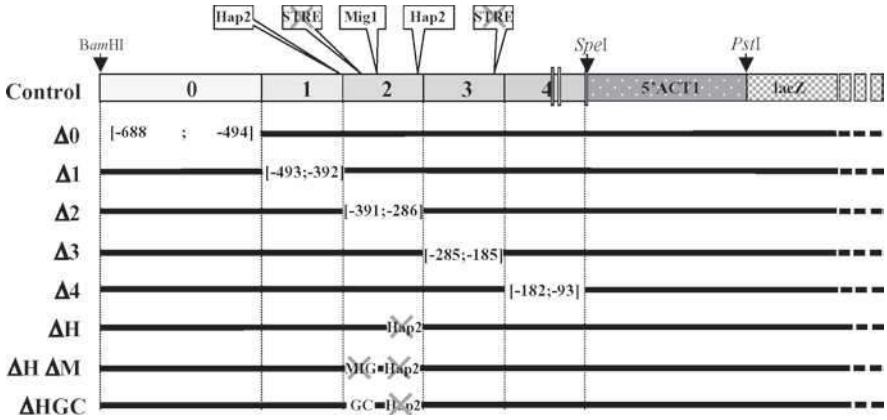


Fig. 2 Schematic representation of *lacZ* fusions to the *GSY2* promoter. The 'Control' plasmid (Table 1, previously referred to as pEBΔA-ΔSTRE) contains the Δ*STRE-gsy2-[ACT* $_{5'UTR}$*]* promoter fused to the *lacZ* reporter. This 'Control' was used to generate the different plasmids as described in Materials and methods. The *shaded boxes* numbered 0 to 4 refer to regions that were deleted. The endpoints of each the deletion relative to the translation start site in *GSY2* are indicated in *brackets*. For some constructs, consensus sequences were mutagenized as indicated by the symbol × (see Table 1 for details). The putative TATAA boxes are illustrated by the *vertical lines* in Region 4

Independent transcriptional control of *GSY2* by the protein kinases Pho85 and Snf1

Timblin and Bergman (1997) have reported that the cyclin-dependent protein kinase Pho85 represses *GSY2* in a STRE-independent manner. This repression is independent of the growth phase, as indicated by the global derepression observed in the *pho85* mutant strain during both the exponential phase and the diauxic shift (Fig. 4). Figure 4 also shows that removal of region 1 or 2 suppresses the *pho85* phenotype, which suggests that Pho85p inhibits a transcriptional activator that binds to UASs. A common feature of these two regions is the presence of a HAP consensus sequence (Fig. 2). However, deletion of this sequence from region 2 (ΔH construct) did not suppress the activating effect of the *pho85* mutation on *GSY2* (Fig. 5). Another striking feature found in region 2 of the promoter is a 14-bp G/C-rich box, which harbours a tandem repeat of two putative Mig1p binding sites (5′-CCCCGC-3′; Nehlin and Ronne 1990). Remarkably, deletion of this G/C-rich box in the ΔH construct suppressed the effect of *pho85* (Fig. 5). When we generated a scrambled 14-bp G/C-rich sequence, without altering the G/C content but eliminating the putative Mig1p consensus sequences, ~85% of the effect of *PHO85* deletion on *GSY2* expression was restored (Fig. 5). These results suggest that it is the G/C richness of this region rather than the Mig1p binding sites per se which is required for the response to the Pho85p pathway. Moreover, while deletion of the *MIG1* gene in a wild-type strain caused a moderate increase in *GSY2* expression at the diauxic shift, deletion of this gene in a *pho85* strain further increased *GSY2* derepression (Fig. 6). This synergistic effect is consistent with the idea that the Mig1p repressor does not mediate the control of *GSY2* by *PHO85*.

In agreement with Hardy et al. (1994), we found that the expression of *GSY2* at the end of growth on glucose was 2- to 3-fold lower in *snf1* mutants than in wild type, irrespective of the presence or absence of STREs (Fig. 6). Since the transcription factor Mig1p is a direct

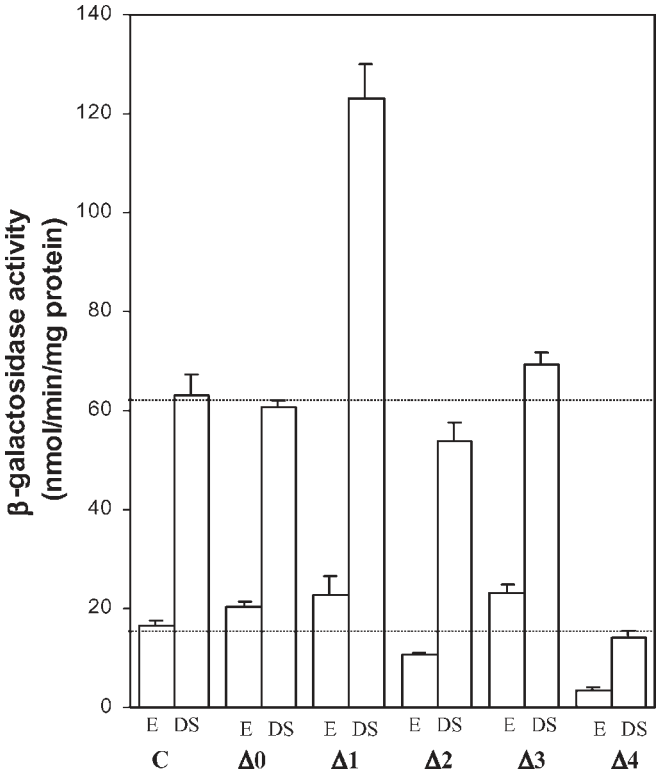


Fig. 3 Levels of *β*-galactosidase expressed from deletion derivatives of the promoter of the 'Control' gene fusion during growth on glucose. The deletion constructs described in Fig. 2 were integrated at the *URA3* locus in the wild type strain JF292 to give the strains JF1120 (C), JF1199 (Δ0), JF1182 (Δ1), JF1185 (Δ2), JF1183 (Δ3) and JF1145 (Δ4). The *bars* represent the means of three measurements of *β*-galactosidase activity, obtained from three independent samples during the early exponential phase (E, OD_{600} between 0.5 and 1.0) or after complete depletion of glucose from the medium (DS). The *horizontal lines* indicate the *β*-galactosidase levels in the reference strain JF1120 (WT strain with the 'Control' construct)

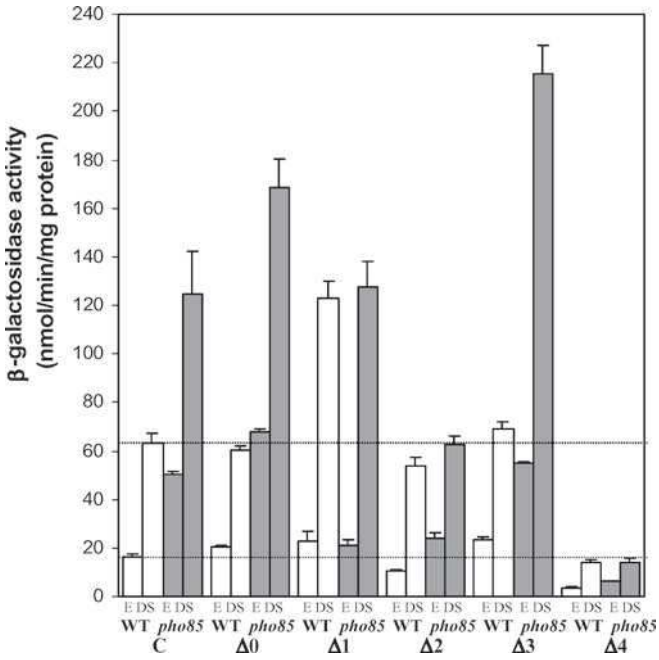


Fig. 4 Promoter regions involved in the control of *GSY2* by the cyclin-dependent Pho85 protein kinase. The 'WT' strains are those presented in Fig. 3. The constructs described in Fig. 2 were integrated in the *pho85* strain JF1142 to give the strains JF1228 (C), JF1217 (Δ0), JF1214 (Δ1), JF1215 (Δ2), JF1216 (Δ3) and JF1325 (Δ4). For other details see the legend to Fig. 3

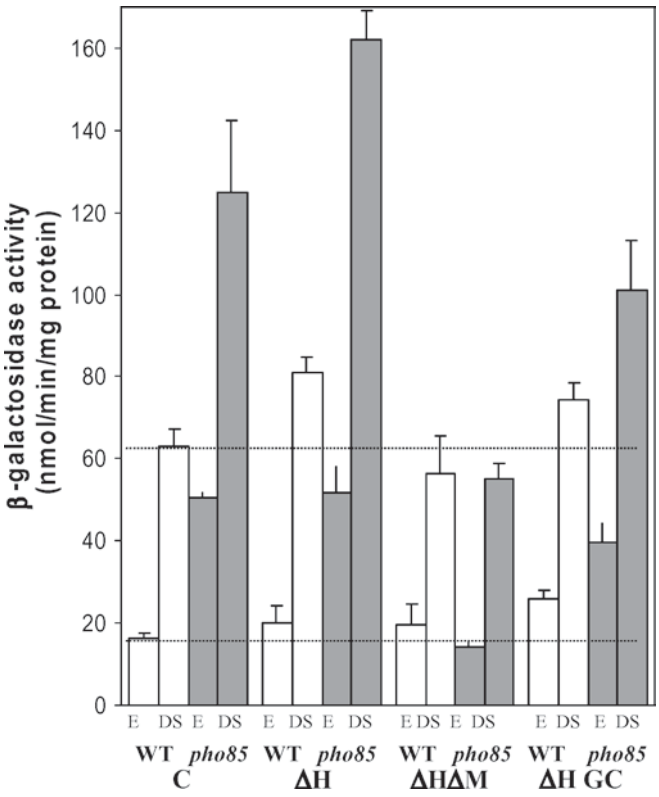


Fig. 5 Role of a G/C rich sequence in the control of *GSY2* by the cyclin-dependent protein kinase Pho85. The 'Control', ΔH, ΔHΔM and GC constructs described in Fig. 2 were integrated in the 'WT' strain JF292 to give the strains JF1120 (C), JF1326 (ΔH), JF1259 (ΔH ΔM) and JF1390 (ΔH GC), and in the *pho85* strain JF1142 to give the strains JF1228 (C), JF1327 (ΔH), JF1261 (ΔH ΔM) and JF1391 (ΔH GC). For other details see the legend to Fig. 3

target of the Snf1 protein kinase (for a review, see Gancedo 1998), we investigated whether the control of *GSY2* by Snf1p might be mediated by release from Mig1p-dependent repression. We found the expression profile of *GSY2* in a *snf1mig1* double mutant to be identical to that seen in the *mig1* mutant (Fig. 6), which is consistent with this model. Surprisingly, removal of only region 0 restored 'wild-type' *GSY2* expression in a *snf1* mutant, although this region does not include a binding site for Mig1p (Fig. 6).

Active PKA promotes repression of *GSY2* via *SOK2*

In order to look for additional *trans*-acting elements implicated in *GSY2* expression, we initiated a genetic screen (see Materials and methods) based on two criteria: inability of the mutant cells to accumulate glycogen, and reduced expression of the *GSY2-lacZ* gene fusion. This screen identified two genes, *IRA1* and *IRA2,* which encode two redundant GTPase-activating proteins. Loss of function of either protein leads to hyperactivation of the PKA pathway (Tanaka et al. 1990). In agreement with Tanaka et al. (1990), effects on cell progression through the diauxic shift and on glycogen accumulation were more pronounced in the *ira1 ira2* double mutant than in the respective single mutants (data not shown). Interestingly, levels of β-galactosidase expressed from the 'Control' construct were extremely low, and barely any induction could be observed at the diauxic shift in the double mutant (Fig. 7). A similar result was obtained with a deletion of *BCY1*, which codes for the regulatory subunit of the PKA (Hardy et al. 1994; our unpublished data). Removal of region 0 of the *GSY2* promoter resulted in a partial release from *ira1 ira2* repression, indicating the presence of a URS element in this region (Fig. 7). We then searched for the *trans*-acting element in the PKA cascade that mediates this potent repression by PKA. One potential target of the PKA pathway is *SOK2*, overexpression of which can suppress the conditional growth defect of a *tpk1*Δtpk3Δtpk2^{ts} mutant (which is temperature sensitive for PKA activity), whereas deletion of this gene exacerbates the growth defect of strains with low PKA activity (Ward et al. 1995). Remarkably, while the deletion of *SOK2* in a wild type strain had no significant effect on the expression level of the 'Control'-construct, loss of *SOK2* function in the *ira1 ira2* mutant restored the expression of this construct (Fig. 8), and glycogen accumulation occurred with almost the same kinetics as seen in wild-type cells (not shown). Since it had previously been reported that a physical interaction between Sok2p and Msn2p was apparently necessary for Sok2p to mediate transcriptional repression of *IME1* (Shenhar and Kassir 2001), we checked for a similar requirement

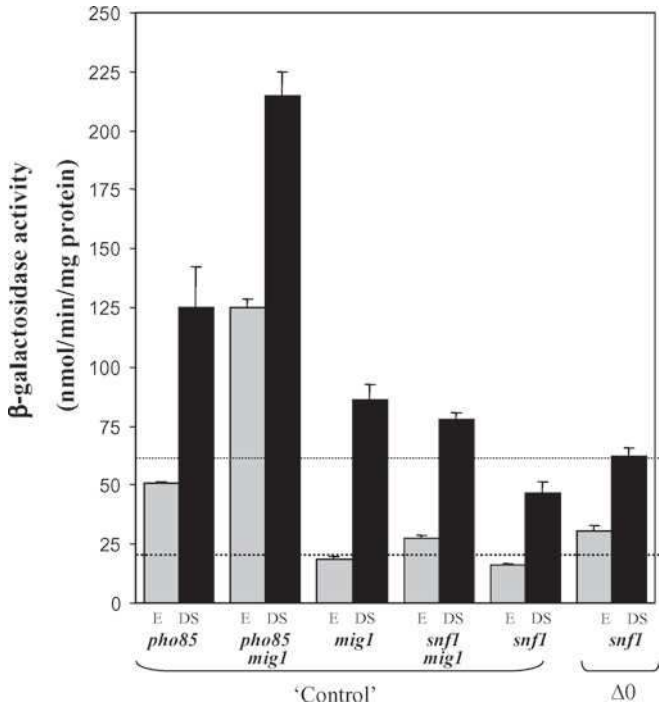


Fig. 6 Interaction of the repressor Mig1p with the Pho85p and Snf1p pathways in the control of *GSY2*. The 'Control' gene fusion was integrated in *pho85*, *mig1*, *snf1*, *pho85 mig1* and *snf1 mig1* mutant strains to give JF1228 (*pho85*), JF1343 (*mig1*), JF1274 (*snf1*), JF1344 (*pho85 mig1*) and JF1364 (*snf1 mig1*). The Δ0 construct was integrated in the *snf1* mutant strain to give JF1275 (*snf1*). For other details see the legend to Fig. 3

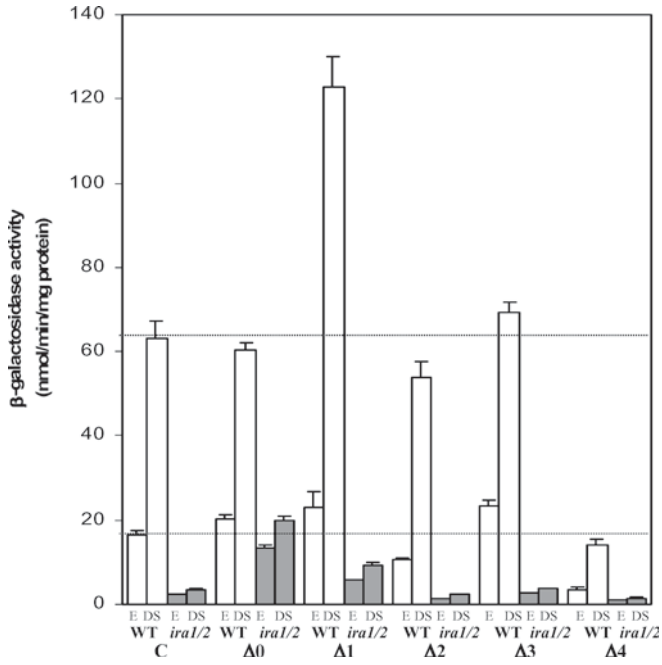


Fig. 7 STRE- and *MSN2/4*-independent control of *GSY2* by PKA. The 'WT' strains are those presented in Fig. 3. The constructs described in Fig. 2 were integrated in the *ira1ira2* strain JF1061 to give JF1228 (C), JF1217 (Δ0), JF1214 (Δ1), JF1215 (Δ2), JF1216 (Δ3) and JF1325 (Δ4). For other details, see the legend to Fig. 3

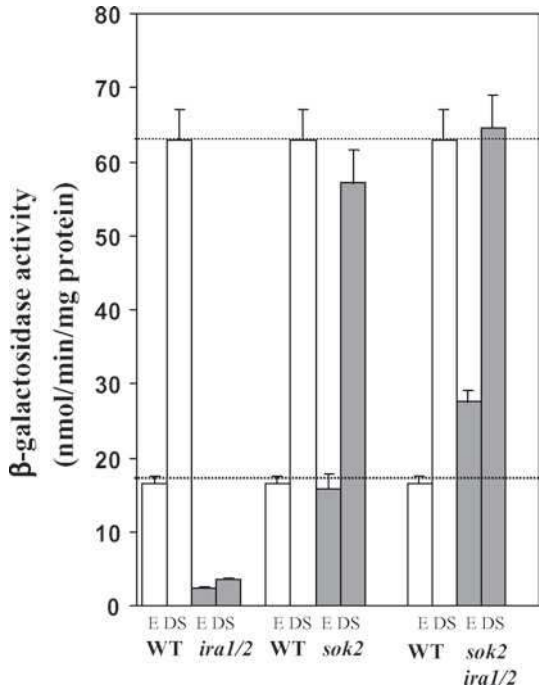


Fig. 8 Deletion of *SOK2* suppresses repression of *GSY2* in a strain with high PKA activity**.** The 'Control' construct was integrated in the wild type, *ira1ira2*, *sok2* and *ira1ira2sok2* mutants to yield the strains JF1120 (WT), JF1263 (*ira1 ira2*), JF1438 (*sok2*) and JF1439 (*sok2 ira1 ira2*). For other details, see the legend to Fig. 3

in the control of *GSY2*. In our hands, expression levels of *GSY2* in a *ira1ira2sok2*ΔMSN2 mutant were similar to those in a *ira1ira2sok2msn2*Δ mutant (data not shown), indicating that Sok2p affects *GSY2* independently of Msn2p.

The Msn2/4p-dependent activation of *GSY2* is in part independent of STREs

A major target of the PKA pathway is the STRE-binding transcriptional factor encoded by *MSN2/4* (Estruch 2000). However, in spite of the absence of STREs, the inactivation of *MSN2/4* further reduced the expression levels of our "Control" construct and caused an almost complete loss of gene activation at the diauxic shift (Fig. 9). None of the deletions in the promoter could suppress this effect, indicating that Mns2/4p might act on *GSY2* via other UAS sequences. This Msn2/4p-dependent but STRE-independent control of *GSY2* was rather puzzling, since Estruch and colleagues (Pastor-Martinez et al. 1996; Estruch 2000) concluded from detailed in vitro binding analyses that Msn2/4p only binds the consensus 5′-CCCCT-3′. Therefore, we looked for a STRE-regulated gene that could act as an intermediate in the control of *GSY2* by Msn2/4p. A possible candidate for this function is *YAK1*, since the expression of this gene depends on a functional Msn2/4p (Smith et al. 1998). Moreover, *YAK1* codes for a protein kinase

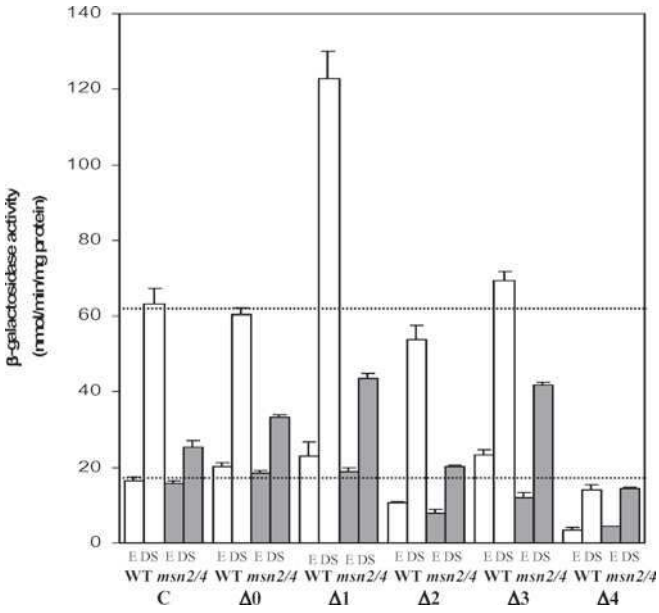


Fig. 9 STRE-minus *lacZ* fusions are sensitive to *MSN2*/*MSN4* deletion. The 'WT' strains are those presented in Fig. 3. The constructs described in Fig. 2 were integrated in strain JF1160 (*msn2::HIS3msn4::TRP1*) to give the strains JF1265 (C), JF1270 (Δ0), JF1271 (Δ1), JF1272 (Δ2), JF1273 (Δ3) and JF1324 (Δ4). For other details, see the legend to Fig. 3

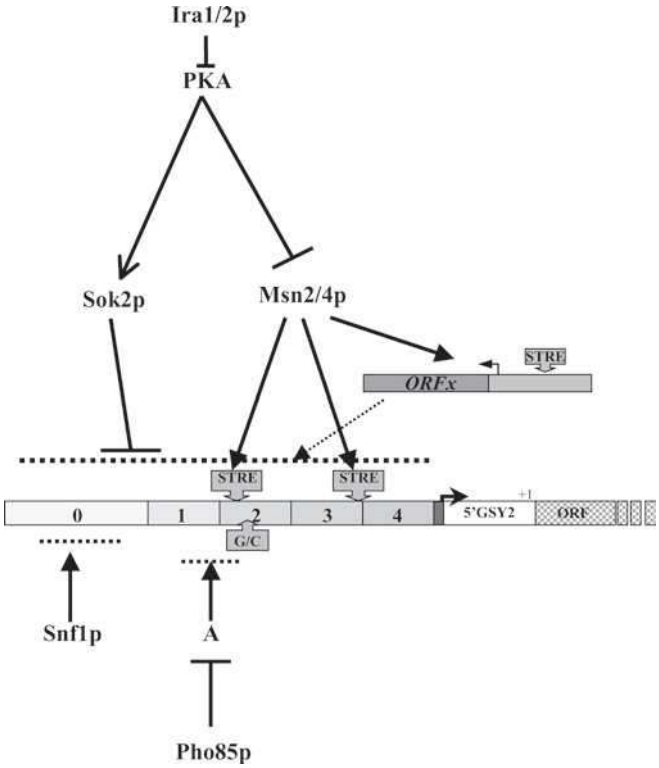


Fig. 10 A model for the transcriptional control of *GSY2* by the protein kinases PKA, Pho85p and Snf1p in *S cerevisiae*. The *dotted lines* indicate undefined *cis*-regulatory elements, and the dotted arrow is intended to suggest a hypothetical mechanism of transcriptional control. For other explanations, see the text

which plays a major role in the transient growth arrest that occurs upon glucose depletion, by phosphorylating the transcription factor Caf1p/Pop2p (Moriya et al. 2001). However, deletion of neither *YAK1* nor *POP2* altered the level of β-galactosidase expressed from our "Control" construct fusion during growth on glucose (data not shown).

It is interesting to point out that the loss of *MSN2*/*MSN4* has similar, albeit less drastic, effects on the expression of *GSY2* to those induced by hyperactivation of the PKA cascade (e.g. in the *ira1ira2* mutant). Taking this analogy into account, and based on data reported in Fig. 8, we examined whether the deletion of *SOK2* in an *msn2*/*msn4* mutant would restore wild-type expression of *GSY2*, as it does in the *ira1ira2* mutant strain. Contrary to our expectation, the expression level of the Control' construct was very similar in both *msn2msn* 4 and *msn2msn4sok2* mutants (data not shown). This result is therefore consistent with a model in which PKA regulates the transcription of *GSY2* by a branched pathway that involves Sok2p and Msn2/4p separately (Smith et al. 1998; see Fig. 10).

Discussion

In the yeast *Saccharomyces cerevisiae*, the biosynthesis of glycogen is under the control of several nutrient-signalling pathways, which act at the transcriptional and posttranslational levels (François and Parrou 2001). While the posttranslational control of Gsy2p has been studied in detail, the transcriptional control of *GSY2* is still poorly understood, and most of the work so far has focused on the function of the *cis*-regulatory STRE elements using promoter-*lacZ* gene fusion strategies (Ni and Laporte 1995; Parrou et al. 1999b). Using a mutant allele of *GSY2* that lacks STREs in its promoter, we demonstrated that STREs are dispensable for *GSY2* induction and glycogen accumulation during diauxic growth on glucose, while they are essential for the response to stress. These data also indicated that, under these growth conditions, the glycogen synthase isoform 1 encoded by *GSY1* cannot substitute for *GSY2*, despite the fact that the two genes are under similar transcriptional control (Unnikrishnan et al. 2003).

Transcription of *GSY2* is under the control of nutrient signalling pathways mediated by the protein kinases Pho85p, Snf1p and PKA (Hardy et al. 1994; Timblin et al. 1996; Smith et al. 1998; Parrou et al. 1999a; Wilson et al. 2002). However, not all *cis*- and *trans*-acting elements that are targets of these kinases have yet been identified. Pho85p has been found to repress *GSY2* in a STRE-independent manner (Timblin and Bergman 1997). We have now shown that deletion of a 14-bp G/C rich sequence (positions −358 to −345) prevents the hyperactivation of *GSY2* in a *pho85*Δ mutant. However, this 'GC' rich motif can be replaced by any other sequence that is similarly G/C rich, which suggests a structural function for this sequence. The up-

regulation of *GSY2* in the *pho85* mutant is consistent with a model in which Pho85 inhibits a transcription factor that activates *GSY2,* as in the case of the transactivator Pho4p in phosphate metabolism (Lenburg and O'Shea 1996). Timblin and Bergman (1997) provided preliminary data suggesting that Pho80p, a partner of Pho85p, could play a role in this process. In our hands, however, deletion of neither *PHO80* nor *PCL6* (which codes for another Pho85p targeting subunit; Andrews and Measday 1998) affects glycogen levels or *GSY2* expression (unpublished data). Thus, the mediator of the transcriptional effect of *PHO85* remains to be identified. From a physiological viewpoint, the control of glycogen levels by the cyclin-dependent Pho85p kinase might be restricted to the G1/G0 period of the cell cycle, in accordance with the function of this protein kinase in cell cycle progression (Andrews and Measday 1998). It has been convincingly shown that glycogen accumulates during the G1/G0 period of the cell cycle (Sillje et al. 1997, 1999; our unpublished data). One possible explanation for the stimulation of glycogen synthesis during the G1 phase is that most Pho85p monomers are recruited by other cyclins during this period, thus relieving its inhibitory action on *GSY2*.

Unlike Pho85p, Snf1p has a positive influence on glycogen metabolism. Snf1p plays a major role in the post-translational control of Gsy2p (Huang et al. 1996). However, a Snf1p-dependent control on *GSY2* at the transcriptional level cannot be excluded, since the expression of other genes involved in the metabolism of reserve carbohydrates, namely *GAC1*, *GPH1*, *GLC3*, *TPS1*, *TPS2* and *NTH1*, is reduced by 2- to 3-fold in a *snf1*Δ mutant (our unpublished data). These coordinated effects of Snf1p can therefore account for the glycogen and trehalose deficiency of *snf1* mutants. In accordance with a role of Mig1p in glucose repression (Gancedo 1998), we found that deletion of *MIG1* restored wild type expression of *GSY2* in a *snf1* mutant, suggesting that the action of Snf1p is mediated via Mig1p. However, this action is likely to be indirect, because removal of the two putative Mig1p binding sites in the *GSY2* promoter did not alter regulation of this gene by Snf1p. The finding of a synergistic effect of *PHO85* and *MIG1* disruptions on *GSY2* expression allows us to conclude that the antagonistic effects of Snf1p and Pho85p on glycogen metabolism (Huang et al. 1996) arise by independent pathways.

The cAMP/PKA pathway strongly represses gene induction at the diauxic shift. The repression involves both Msn2p/Msn4p-dependent (Smith et al 1998) and -independent pathways (Boy-Marcotte et al. 1998). The Rim15p-Gis1p cascade is a Msn2/4p-independent pathway which mediates its effects through the UAS_{PDS} element (Pedruzzi et al. 2000). However, this pathway plays no role in the transcription of *GSY2*, as the removal of the single PDS element in the *GSY2* promoter did not alter expression of the gene (Parrou et al. 1999b; unpublished data). Evidence is presented in this work that suggests that the Msn2/4p-independent branch which represses *GSY2* in hyperactivated PKA mutants involves Sok2p, a PKA-dependent repressor of gene expression (Ward et al. 1995; Shenhar and Kassir 2001). However, the mode of action of Sok2p is not clear-cut. On the one hand, overexpression of *SOK2* decreases gene expression and reduces glycogen accumulation, while loss of its function is only effective in the context of hyperactivity of PKA (Ward et al. 1995; this work). On the other hand, Shenhar and Kassir (2001) reported that the repression of *IME1* by Sok2p required its interaction with Msn2p, but this mode of transcriptional control was not found for *GSY2*. Finally, no DNA-binding activity has been identified yet for Sok2p. With respect to the Msn2/4p-dependent branch of the pathway, the control of *GSY2* is more complicated than previously anticipated, because this gene is still responsive to Msn2/4p when the STREs have been deleted from its promoter. Similar conclusions have been drawn with respect to the transcriptional control of *GSY1* by Msn2/4p (Unnikrishnan et al. 2003), and from a genome-wide analysis of *msn2msn4* mutant, which identified more than 200 genes that are subject to control by this transcription factor, although only 47 of them harbour STREs (Causton et al. 2001). Therefore, one might consider that Msn2/4p could bind to a degenerate STRE, or that the effect of the *msn2msn4* deletion on a *GSY2* gene that lacks STREs is indirect, being mediated by a STRE-regulated factor.

To summarize (Fig. 10), the cAMP/PKA pathway affects *GSY2* expression by a combination of two major routes, both of which are needed to effectively repress *GSY2*, and probably other genes that belong to the same regulon, during exponential growth on glucose. Sok2p is a transcriptional repressor of the effects of PKA (Ward et al. 1995; Smith et al. 1998). According to our data, Sok2p only plays a role in *GSY2* expression in the context of hyperactivity of PKA; e.g., in *ira1ira2* or *bcy1* mutants. Under these conditions, the Msn2/4p pathway is completely shut off, and the only way to relieve the inhibition of transcription by high PKA activity is through ablation of *SOK2*. On the other hand, gene activation at the diauxic shift requires de-activation of the PKA pathway. This in turn releases the transcription factor Msn2/4p from inhibition. In addition to the central role of PKA, the expression of *GSY2* is further enhanced because Snf1p is released from inhibition by glucose (Gancedo 1998) and Pho85p probably loses its ability to inhibit, as indicated by the activation of Gsy2p as cells reach the diauxic shift (François et al. 1988; Farkas et al. 1991; Wilson et al. 1999). A major challenge now is to understand how PKA activity is reduced as yeast cells enter the diauxic shift. This reduction could involve inactivation of the TOR pathway, which has recently been shown to control the Ras/cAMP pathway (Schmelzle et al. 2004). We therefore suggest that the dynamic consumption of glucose during growth can be sensed by the cells and that this information serves to control the activity of TOR.

Concluding remarks

The complex transcriptional control of *GSY2* illustrates the combinatorial regulation of glycogen metabolism by nutrient signalling pathways, and is consistent with the importance of the deposition of glycogen as a carbon and energy source to ensure viability and fitness, as previously suggested by competition experiments with strains containing low or high levels of glycogen (Anderson and Tatchell 2001). Because these functions are probably dispensable under standard laboratory conditions, more subtle conditions must be investigated if we are to understand the physiological function of glycogen in yeast.

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