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Combinatorial control by the protein kinases PKA, PH085 and SNF1 of transcriptional induction of the *Saccharomyces cerevisiae GSY2* gene at the diauxic shift

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 iralira2 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 *iralira2* 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

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B. Enjalbert · J. L. Parrou · M. A. Teste · J. François (⊠) Centre de Bioingenierie Gilbert Durand, UMR CNRS 5504 and INRA 792, Institut National des Sciences Appliquées, 135 Avenue de Rangueil, 31077 Toulouse, France E-mail: fran_jm@insa-tlse.fr Tel.: + 33-5-61559492 Fax: + 33-5-61559400 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 $GSY2 \cdot cAMP \cdot PHO85 \cdot SNF1 \cdot Signal transduction$

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 posttranslational 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 cyclindependent 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 glucoserepressed 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 - $\Delta STRE$, 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'-GA-ATTCGAGCTCGGTACCCGGGGGATCCAG-3') and AmlacZ 3'+ (5'-AAAACGACGGCGGGATCGCAA GCTTGCATG-3') were used as external primers, and the specific internal primers are listed in Table 1. The amplified fragments were cleaved with BamHI + PstI, 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 SacI-SacI fragment of plasmid pJN22 (gift of Dr H. Ronne, Uppsala, Sweden). Construction of the $snfl\Delta$:: HIS3 mutant was carried out starting from pBSK SNF1, which bears a 3.2-kb EcoRI-BamHI genomic DNA fragment containing SNF1. In the first step, partial digestion with BsmI 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 BamHI fragment of HIS3, isolated from YDp-H (Berben et al. 1991) was inserted into the BclI site located in the 3' part of SNF1, to generate pBSK snf1A:: HIS3. The SNF1 disruption was carried out using a 3.3-kb EcoRI-BamHI fragment from pBSK snf1A:: HIS3. To construct the sok2 mutants, the wild-type SOK2 sequence was amplified from yeast genomic DNA with the primer pair Vsok2up (5'-CCCAAGCTTGAAAGTGGATTTGTTAAGCACA G-3'; HindIII site underlined) and Vsok2down (5'-GCG GGATCCCGCTAGGGTTTTGATTAAAGTAACA-3'; BamHI underlined). The PCR product was cloned into the Hin dIII/BamHI sites of pBSK to yield pMA12. A 1.1-kb SmaI fragment of LEU2 from YDp-L (Berben et al. 1991) was inserted into the SnaBI/NsiI sites in the SOK2 ORF from pMA12, to generate pMA13. SOK2

Name	Description or purpose/primers used in PCR-based strategies (5' to 3')	Source/refernce
pEB GSY2	Introduction of SpeI site into the Δ STRE-gsy2-lacZ	Parrou et al. (1999b)
	(pJL37) construct	
Control	Previously described as pEBAA- Δ STRE (replacement of STREs	Parrou et al. (1999b)
	by <i>Nhel/Bgl</i> II sites and 5'UTR from <i>ACT1</i>)	
$\Delta 0$	Standard PCR; truncation of the distal part of the promoter	This study
	(positions -688 to -494 relative to the translation start site).	
	Primers: $\Delta 0.5^{\circ}$ (ACAGGGATCCTGGGGGCCTCGAGCA	
	TGGCTCATTTTCG; BamH1 site underlined) and AmlacZ	
	nbsp;3'+ (AAAACGACGGCGGGGATCGCAAGCTTGCATG)	
Δ1	Recombinant PCR; deletion of the promoter region from -493 to	This study
	-392. Primers: AI 3' (CAIGIGCAGAIAICCEIAIICC)	
4.2	and $\Delta I S'$ (AATAGGGATATCIGCACAIGAATICIGACACIGGACIGCI)	This star las
Δ2	Recombinant PCR; deletion of the promoter region from -391	This study
	to -280 . Primers: $\Delta 2.3^{\circ}$ (GGTAAGCTGCCAGAAACCCC)	
A 2	and $\Delta 2.5$ (GGGGTTCTTGCAGCTTACCTGGGGCATACAATGTTTAACCC)	This study
$\Delta 3$	Recombinant PCR; deletion of the promoter region from -285 to -185. Primers:	This study
	$\Delta 3 3$ (GOUTGAACTCAGTCACATATATC) and $\Delta 3 3$	
A /	(GATATATATOTOACTOACTOACTOCATOCATOCTATOCAACTOCTCO)	This study
Δ4	Recombinant PCR; detection of the promoter region from -182 to -93 .	This study
	Prilleds. D4 5 (AADATCHTGAATTGCGTTCC,	
	TAAAT ATGTACTACTACT Paul and Spal sites underlined	
лн	Pacambinant PCP: raplacement of HAP2 consensus (TAATTGGT) by	This study
ΔΠ	TTACGTAT (Such site underlined) Drimers: AU 2'	This study
	(TATCATCATCATCATCATCATCATCAGGAGTCTGG)	
	and AH 5' (TGGTTAGGTATGATGATGATGATGTGACTGAGTTC)	
ΔΗΔΜ	Recombinant PCR: replacement of the nutative Miglin binding	
	sequences (accase CCCGC accCCCGC cacc) by accase TTAAG acc	
	(<i>AfIL</i> site underlined) Primers: AM 3' (CTTAAGTCGCTAA	
	TCCGCTAGCTAGCAGTC) and AM 5' (GCTAGCTAGCGGATT	
	AGCGACTTAAGGACCAGACTCCCTGAAAATGGT)	

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

disruption was carried out in strains JF1120, JF1263 and JF1266 (see Table 2) by transformation with the HindIII-BamHI sok2::LEU2 fragment from pMA13 (Table 1). Disruption of MSN2 was carried out in the iralira2 sok2::LEU2 strain (JF1439) using the 2.65-kb SalI 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 Bg/I and inserting a 1.1-kb BamHI 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'-<u>AACTCATCCAATAA-</u> 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 $gsy2-\Delta$ STRE allele, which is characterized by a lack of STREs in the GSY2 promoter, was carried out as follows. pEBGSY2 was cleaved with *SpeI* to target the integration of the plasmid to the GSY2locus, 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 Δ STRE (5' TTCTGACACTGG-ACT 3') as primers. The former hybridizes within the *GSY2* ORF and the latter at the *Bg*/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
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Strain	Genotype	Source/reference
JF292	MATa leu2 ura3 his3	Laboratory stock
JF625	$MAT\alpha$ leu2 ura3 his3 trp1 lys2	This study
Wmsn2msn3	MAT aleu2 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 aleu2 ura3 his3 pho85::HIS3	This study
JF775	MAT aleu2 ura3 his3 trp1 mig1::LEU2	This study
JF1342	MAT aleu2 ura3 his3 snf1::HIS3	This study
MY1999	MAT aleu2::PET56 ura3 his3::TRP1 trp1 gcn4 gal2 pop2::LEU2	M. Collart
JF558	MAT aleu2 ura3 his3 URA3::GSY2-lacZ	Parrou et al. (1999a)
JF1135	MAT aleu2 ura3 his3 gsy2- Δ STRE	This study
JF1120	MAT aleu2 ura3 his3 URA3::Control	This study
JF1199	MAT aleu2 ura3 his3 URA3:: $\Delta 0$	This study
JF1182	MAT aleu2 ura3 his3 URA3:: Δ 1	This study
JF1185	MAT aleu2 ura3 his3 URA3:: $\Delta 2$	This study
JF1183	MAT aleu2 ura3 his3 URA3:: Δ 3	This study
JF1145	MAT aleu2 ura3 his3 URA3:: $\Delta 4$	This study
JF1228	MAT aleu2 ura3 his3 pho85::HIS3 URA3::Control	This study
JF1217	MAT aleu2 ura3 his3 pho85::HIS3 URA3:: $\Lambda 0$	This study
JF1214	MAT aleu2 ura3 his3 pho85::HIS3 URA3:: Λ	This study
IF1215	MAT aleu2 ura3 his3 pho85HIS3 URA3A2	This study
IF1216	MAT aleu2 ura3 his3 pho85···HIS3 URA3··A3	This study
IF1325	MAT aleu2 ura3 his3 pho85···HIS3 URA3··A4	This study
JF1326	MAT w land mas his 3 IIR 43 AH	This study
JF1250	MATA lan2 ma3 his3 UKA3AH	This study
JF1200	MATA land mad hisd URAS. AHGC	This study
JF1327	MAT algul uras his o hAss. HIS3 URAS. AH	This study
JF1327 JF1261	MAT alau und mis photo: HIS UNA: AN	This study
JF1201 JF1301	MAT aleuz uras hiss phoss-thiss URAS. AH GC	This study
JF13/3	MAT a lang mag his photo: IFU2 UPA: Control	This study
JF1345	MATE law was his mgt. LEC2 UNAS. Control	This study
JF12/4 JF13/4	MATChev 2 was his above USS ministration of the second second	This study
JE1264	MATHERZ WIDS HISS PHOOSTHISS HIGHLEU2 UKASControl MAT alay ang king mili uHS2 mia u EU2 UKASControl	This study
JF1304 JF1275	MAT aleuz und mis shit. miss migt. LEOU OKAS. Comroi	This study
JF1275 JE1265	MAT aleaz und miss shi1miss OKASΔ0	This study
JF1203 JE1270	MATA leuz uraz hisz trp1 msn2;:HISZ msn4;:TRF1 UKAS::CONIFOL MATA leuz uraz hisz trp1 msn2;:HISZ msn4;:TRP1 UKAS::CONIFOL	This study
JF1270 JE1271	$MA1\alpha$ let 2 uras hiss irps msn2;::h1ss msn4;::1 RF1 URAS:: $\Delta 0$	This study
JF12/1 JE1272	$MA13$, leuz uras miss irp1 msn2:: $H155$ msn4:: $I KF1 \cup KA5$:: ΔI	This study
JF12/2 JF1272	$MA10$, leuz uras miss trp1 msn2::HIS5 msn4::IRP1 URAS:: $\Delta 2$	This study
JF12/3	$MA1\alpha$ leuz uras miss irp1 msn2::HISS msn4::IRP1 URAS:: Δ S	This study
JF1324 JF1262	MATCheu 2 uras hiss trp1 msn2::HISS msn4::1RP1 URAs::\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\	This study
JF1203	MATCHEUZ Uras hiss trp1 tra1 tra2 URAS::Control	This study
JF1266	$MA1\alpha$ leuz uras hiss trp1 tra1 tra2 URAS:: $\Delta 0$	This study
JF126/	MATa leuz uras hiss trp1 ira1 ira2 URAS::X1	This study
JF1268	$MA1\alpha$ leu2 ura3 his3 trp1 ura1 ira2 URA3:: $\Delta 2$	This study
JF1269	MATa leu2 ura3 his3 trp1 ira1 ira2 URA3::\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\	This study
JF1322	MAT aleu2 ura3 his3 trp1 ira1 ira2 URA3:::Δ4	This study
JF1438	MATa leu2 ura3 his3 sok2::LEU2 URA3::Control	This study
JF1439	MATa leu2 ura3 his3 trp1 ira1 ira2 sok2::LEU2 URA3::Control	This study
JF1563	MAT aleu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 sok2::LEU2 URA3::Control	This study
JF1538	MATa leu2 ura3 his3 trp1 yak1::LEU2 URA3::Control	This study
JF1550	MATa leu2 ura3 his3 trp1 msn2::HIS3 msn4::TRP1 yak1::LEU2 URA3::Control	This study
JF1575	MAT aira1 ira2 leu2 ura3 his3 trp1 msn2::Kan' 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% NH₂SO₄; 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 2-1 shake-flasks containing 0.3 1 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-\DeltaSTRE*) in which the two STREs have been deleted from the *GSY2*

Fig. 1A, B Glycogen synthase activity and glycogen accumulation in wild type and gsy2- Δ STRE mutant strains during growth on glucose. The control strain JF558 (*GSY2*, **A**) and the glycogen synthase mutant JF1135 (gsy2- Δ STRE, **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: OD₆₀₀ (*filled circles*); glycogen levels (*open circles*); glycogen synthase activity (*filled diamonds*) 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-* Δ *STRE* 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-* Δ *STRE* strain did not alter this response (data not shown). In contrast, no increase in glycogen levels was observed in the *gsy2-* Δ *STRE* 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).

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' lacZgene 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 pEB ΔA - Δ STRE) 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, $\sim 85\%$ of the effect of PHO85 deletion on GSY2 expression was restored (Fig. 5). These results suggest that it is the G/Crichness 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 (Δ 0), JF1182 (Δ 1), JF1185 (Δ 2), JF1183 (Δ 3) and JF1145 (Δ 4). The bars represent the means of three measurements of β -galactosidase activity, obtained from three independent samples during the early exponential phase (E, OD₆₀₀ 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 (Δ 0), JF1214 (Δ 1), JF1215 (Δ 2), JF1216 (Δ 3) and JF1325 (Δ 4). 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



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

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 iral 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 $tpk1\Delta tpk3\Delta tpk2^{ts}$ 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 iral 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 $\Delta 0$ 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 (Δ 0), JF1214 (Δ 1), JF1215 (Δ 2), JF1216 (Δ 3) and JF1325 (Δ 4). 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, *iralira2*, *sok2* and *iralira2sok2* 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* Δ MSN2 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 STREbinding 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 Msn2/4pdependent 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, YAKI 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 (Δ 0), JF1271 (Δ 1), JF1272 (Δ 2), JF1273 (Δ 3) and JF1324 (Δ 4). For other details, see the legend to Fig. 3

which plays a major role in the transient growth arrest that occurs upon glucose depletion, by phosphorylating the transcription factor Caflp/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 *iralira2* 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 *iralira2* mutant strain. Contrary to our expectation, the expression level of the Control' construct was very similar in both msn2msn4 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



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

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*\Delta 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 upregulation 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 (Sillie 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 posttranslational 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 $snf1\Delta$ 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 DNAbinding 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 iralira2 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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