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Jean-Luc Parrou, Brice Enjalbert, Jean Francois. STRE-and cAMP-independent Transcriptional Induction of *Saccharomyces cerevisiae* GSY2 Encoding Glycogen Synthase during Diauxic Growth on Glucose. *Yeast*, 1999, 15 (14), pp.1471-1484. hal-02146827

**HAL Id: hal-02146827**

**<https://hal.science/hal-02146827>**

Submitted on 7 Jun 2019

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# STRE- and cAMP-independent Transcriptional Induction of *Saccharomyces cerevisiae* *GSY2* Encoding Glycogen Synthase during Diauxic Growth on Glucose

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It has been shown that the so-called stationary phase *GSY2* gene encoding glycogen synthase was induced as the cells left the exponential phase of growth, while glucose and all other nutrients were still plentiful in the medium (Parrou *et al.*, 1999). Since this effect was essentially controlled at the transcriptional level, we looked for the *cis*- and *trans*-acting elements required for this specific growth-related genetic event. We demonstrated that mutations of the HAP2/3/4 binding site and of the two STress-Responsive *cis*-Elements (STRE) did not abolish the early induction of *GSY2*, although the latter mutation led to a 20-fold drop in the transcriptional activity of the promoter, as determined from *lacZ* gene fusions. Insertion of a DNA fragment (from –390 to –167 bp, relative to the ATG) of the promoter lacking the two STREs, upstream to the TATA box of a *CYCI-lacZ* fusion gene, allowed this reporter gene to be induced with a kinetic similar to that of *GSY2-lacZ*. Mutations in *BCY1*, which results in a hyperactive protein kinase A, did not alleviate the early induction, while causing a five- to 10-fold reduction in the transcriptional activity of *GSY2*. In addition, the repressive effect of protein kinase A was quantitatively conserved when both STREs were mutated in *GSY2* promoter, indicating that the negative control of gene expression by the RAS–cAMP signalling pathway does not act solely through STREs. Taken together, these results are indicative of an active process that couples growth control to dynamic glucose consumption. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — growth phases; stress; STRE; Ras–cAMP; gene expression; *S. cerevisiae*

## INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, the availability of nutrients determines metabolic regimes as well as decisions about cell division, growth arrest or sporulation. However, a system that can couple growth rate to dynamic changes in nutrient levels would be more suited to maintain a homeostatic balance of the cell than an on/off system for the decision to halt growth. An interesting example is the genetic and metabolic adaptation of yeast cells to the diauxic transition of growth on a glucose-limited medium. These changes have been characterized by metabolite patterns and enzymes

activities (François *et al.*, 1987), protein patterns using two-dimensional gel electrophoresis (Boucherie, 1985; Boy-Marcotte *et al.*, 1996; Werner-Washburne *et al.*, 1993, 1996), and gene expression using DNA microarrays (DeRisi *et al.*, 1997). To determine the decisive moment when these changes are triggered, accurate kinetic studies of gene expression have been carried out under well-defined growth media using controlled bioreactors. These studies revealed that genes involved in reserve carbohydrate metabolism (for review, see François *et al.*, 1997) and the general stress response *CTT1* gene (Marchler *et al.*, 1993) were coordinately induced while glucose and all other nutrients were still abundant in the medium (Parrou *et al.*, 1999). These genetic changes were actually triggered far in advance of other so-called diauxic and stationary-phase events (Werner-Washburne *et al.*, 1995, 1993). This prompted us to investigate the mechanism underlying this early

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Contract/grant sponsor: European Union Cell Factories Program; Contract/grant number: BIO4.CT95.132.

induction, especially with respect to existing models, which suggest the requirement of the specific STress-Responsive *cis*-Element (STRE) and a control by cAMP of STRE-regulated genes (Siderius and Mager, 1997).

Most of the genes showing an early induction indeed possess one to several copies of a stress-responsive element in their promoter (STRE, core consensus CCCCT), which confers transcriptional activation in response to a wide range of stresses, including heat, oxidative and osmotic shocks, and nutrient starvation (Ruis and Schüller, 1995; Siderius and Mager, 1997). As entry of yeast into the diauxic transition of growth on glucose—incorrectly defined as entry of yeast in the stationary phase—is assimilated to a ‘stress’ condition (Siderius and Mager, 1997; Ni and Laporte, 1995), it was suggested that STREs are the primary determinants for this specific growth-related gene induction. This assertion was further supported by the observation that activation of *GSY2* encoding glycogen synthase 2 (Farkas *et al.*, 1991) and of *HSP12* encoding a small heat shock protein (Preakelt and Meacock, 1990) was no longer detectable after mutation of STREs in their promoters (Ni and Laporte, 1995; Varela *et al.*, 1995). Against this idea, deletion of STREs in *SOD2* encoding manganese superoxide dismutase (Gralla and Kosman, 1992) did not alter its transcriptional induction (Flattery-O’Brien *et al.*, 1997), although it caused a two-fold reduction of expression at the diauxic transition. In addition, we showed that *GLG1*, which is also involved in glycogen metabolism, was co-induced with these so-called ‘STRE-controlled genes’ during diauxic growth, although there is no STRE in the promoter of this gene (Parrou *et al.*, 1999).

In *S. cerevisiae*, Ras–cAMP exerts a negative effect on the reprogramming of gene expression at the diauxic transition of growth (Boy-Marcotte *et al.*, 1996, 1998; Werner-Washburne *et al.*, 1996). With respect to stress-responsive genes such as *CTT1*, *HSP12* and *SOD2*, it has been shown that this repression is mediated by STREs (Marchler *et al.*, 1993; Varela *et al.*, 1995; Flattery-O’Brien *et al.*, 1997). STREs are binding sites for Msn2p/Msn4p transactivators (Schmitt and McEntee, 1996; Martinez-Pastor *et al.*, 1996) and Görner *et al.* (1998) provided further evidence that the nuclear localization of these transactivators is inversely correlated to cAMP levels and PKA activity, suggesting that the STRE-mediated cAMP repression of gene expression probably

occurs through Msn2p/Msn4p. Supporting this model, Boy-Marcotte *et al.* (1998) showed that the repressive effect of cAMP applied to all Msn2p/Msn4p-dependent genes, but these authors also reported a cAMP-repressive effect on Msn2p/Msn4p-independent gene targets. This result argues that the cAMP signalling pathway repression might occur through other *cis*-elements and transcriptional regulators.

Using the kinetic approach that has been reported in a previous paper (Parrou *et al.*, 1999), our aim was to gain more insight on the *cis*- and *trans*-acting elements required for the growth-related induction of *GSY2*, taken as the representative of this early genetic event. We provided evidence that this event is not signalled through the STRE-binding elements present in *GSY2* promoter, and identified a short region in this promoter that may be important for gene induction. In addition, we found that the repressing effect of the cAMP-signalling pathway on *GSY2* expression is not solely mediated through STREs.

## MATERIAL AND METHODS

### *Construction of yeast strains*

The strains used in this work are listed in Table 1. Integration at the *ura3* locus of the *GSY2*–*lacZ* fusion gene or its variants was as described in Parrou *et al.* (1997). Standard yeast genetic procedures for diploid construction, sporulation and tetrad analysis (Rose *et al.*, 1990) were used to select strains carrying both *lacZ* fusion genes and mutations in the Ras–cAMP pathway.

### *Growth conditions and analytical procedures*

Yeast strains were grown in rich medium (YEP) containing 10 g/l Yeast Extract, 20 g/l Bacto-peptone with 10 or 20 g/l glucose, or in synthetic minimal medium (YNB) containing 1.7 g/l Yeast Nitrogen Base without amino acids and ammonium sulphate, 1 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 g/l glucose and auxotrophic requirements (100 mg/l each). YNB was buffered at an initial pH of 5.8 by addition of 10 g/l of succinic acid and 6 g/l of NaOH. Cultures were performed at 30°C in a 21 fermentor (Inceltech-SGI, France). Growth curves (see Figures 3, 6) were fitted using an exponential regression analysis (Excel 5.0 software), taking into account for calculation the experimental points within the time windows indicated by arrows on the axis, i.e. during the apparent

Table 1. List of strains used.

Yeast strain	Genotype	Source
KT1112	<b>a</b> <i>leu2 ura3 his3</i>	K. Tatchell
JF326	Diploid from KT1112	This study
KT1126	<b>a</b> <i>leu2 ura3 his3 sra1-14 (SRA1=BCY1)</i>	K. Tatchell
JF415	<b>a</b> <i>leu2 ura3 his3 ras2::HIS3</i>	This study
JLP5-1A	<b>a</b> <i>leu2 ura3 his3 URA3::GSY2-lacZ</i>	This study
JLP25-15C	<b>a</b> <i>leu2 ura3 his3 URA3::GSY2-lacZ sra1-14</i>	This study
JLP36-7B	<b>a</b> <i>leu2 ura3 his3 URA3::GSY2-lacZ ras2::HIS3</i>	This study
JLP48-3B	<b>a</b> <i>LEU2 HIS3 URA3::GSY2-lacZ</i>	Parrou <i>et al.</i> (1999)
JLP86-3B	<b>a</b> <i>LEU2 HIS3 URA3::GSY2-lacZ sra1-14</i>	This study
JLP86-3A	<b>a</b> <i>LEU HIS3 URA3::GSY2-lacZ ras2::HIS3</i>	This study
JLP94-4B	<b>a</b> <i>leu2 ura3 his3 URA3::A-STRE-gsy2-lacZ</i>	This study
JLP95-8C	<b>a</b> <i>leu2 ura3 his3 URA3::A-STRE-gsy2-lacZ sra1-14</i>	This study
JLP95-8D	<b>a</b> <i>leu2 ura3 his3 URA3::A-STRE-gsy2-lacZ SRA1</i>	This study
JLP96-1A	<b>a</b> <i>leu2 ura3 his3 URA3::A-STRE-gsy2-lacZ RAS2</i>	This study
JLP96-1C	<b>a</b> <i>leu2 ura3 his3 URA3::A-STRE-gsy2-lacZ ras2::HIS3</i>	This study
JLP44-8A	<b>a</b> <i>leu2 ura3 his3 URA3::GPH1-lacZ</i>	This study
JLP80-18D	<b>a</b> <i>leu2 ura3 his3 URA3::GPH1-lacZ sra1-14</i>	This study
JLP79-1C	<b>a</b> <i>leu2 ura3 his3 URA3::GPH1-lacZ ras2::HIS3</i>	This study
JLP51-3D	<b>a</b> <i>leu2 ura3 his3 URA3::GAC1-lacZ</i>	This study
JLP53-4B	<b>a</b> <i>leu2 ura3 his3 URA3::GAC1-lacZ sra1-14</i>	This study
JLP78-2B	<b>a</b> <i>leu2 ura3 his3 URA3::GAC1-lacZ ras2::HIS3</i>	This study
JF1087	<b>a</b> <i>leu2 ura3 his3 URA3::ACT1-lacZ</i>	This study
JF1088	<b>a</b> <i>leu2 ura3 his3 URA3::GSY2[ACT1<sub>5'UTR</sub>]-lacZ</i>	This study
JF1120	<b>a</b> <i>leu2 ura3 his3 URA3::A-STRE-gsy2[ACT1<sub>5'UTR</sub>]-lacZ</i>	This study
JF1121	<b>a</b> <i>leu2 ura3 his3 URA3::A-STRE-gsy2-lacZ</i>	This study

exponential phase. The prerequisite of these time windows was to reach a correlation coefficient ( $r^2$ ) higher than 0.998. The  $\mu$  constant from the equation  $OD = b \exp(\mu \cdot t)$  of the fitting curve corresponds to the specific growth rate ( $\mu = \ln 2 / t_g$  with  $t_g$ , the doubling time). Heat shock, osmotic shocks and nutrient starvation procedures were carried out essentially as described in Martinez-Pastor *et al.* (1996) and Parrou *et al.* (1997). Glucose at 0.05% (w/v) was added in the carbon starvation medium to avoid translation deficiencies encountered under this growth condition (Martinez-Pastor and Estruch, 1996). Preparation of extracts, measurement of  $\beta$ -galactosidase activity and quantitative determination of glycogen and trehalose were performed as described previously (Parrou and François, 1997; Parrou *et al.*, 1997).

#### Plasmids and site-directed mutagenesis

Plasmid pJL1 (*GSY2-lacZ* translational fusion gene, Parrou *et al.*, 1997), carrying a *Bam*H1–*Pst*I PCR fragment containing *GSY2* promoter and its

5' UTR (from –693 to +19 relative to the translational start site) fused in frame to *lacZ* in Yep356 (Myers *et al.*, 1986), was used as a template for site-directed mutagenesis by inverse PCR (Weiner *et al.*, 1994). The mutagenic oligonucleotides were *STRE/A\_dir* 5'-CTAGCTAGCGGATTAGCGA CCCTGCGACC-3' (–351 bp) and *STRE/A\_rev* 5'-CTAGCTAGCAGCCAGTGTCAGAATTGG TAAGC-3' (–400 bp) for replacement of *STRE/A* with a *Nhe*I site (underlined); *STRE/B\_dir* 5'-GGGGAGATCTTCTTTCTTATGCAAG CTCCTCG-3' (–168 bp) and *STRE/B\_rev* 5'-GGCGAGATCTTGAATTGCGTTCTGGAG AAAAAC-3' (–219 bp) for replacement of *STRE/B* with a *Bg*/II site (underlined); *HAP\_dir* 5'-TCCCCGCGGGATGATATATGTG-ACTG AGTTCAGC-3' (–294 bp) and *HAP\_rev* 5'-TCCCCGCGGATCCATTTTCA-GGAGTCTA GTCG-3' (–347 bp) for replacement of *HAP* with a *Sac*II site (underlined) and two additional mutagenic bases (**bold**). The PCR was performed with the high-fidelity *Pfu* polymerase (Stratagene) using

the following parameters: denaturation, 94°C, 30 s; hybridation, 58°C, 30 s for five cycles and 63°C, 30 s for 20 cycles; elongation, 72°C, 18 min. After amplification, the DNA template was digested with *Dpn1*, and the amplified fragments were subsequently digested using the mutagenic site they harbour at their ends and religated. The *Bam*HI–*Pst*I promoter fragments were subcloned in YEp356, and sequences were determined (Genome Express SA, Grenoble, France) to confirm the fidelity of the amplification and the expected replacement of the consensus. Plasmids pJL35, pJL33 and pJL34 contained, respectively, *Nhe*I, *Bgl*III and *Sac*II sites, in replacement of *STRE*/A, *STRE*/B and HAP consensus. Using the procedure described above, subsequent replacement of *STRE*/B in the *STRE*/A mutated promoter yielded pJL37, carrying mutations in both *STRE*s ( $\Delta$ -*STRE*-*gsy2*-*lacZ* construct). The *Bam*HI–*Pst*I promoter fragments of all these *GSY2*-*lacZ* variants were also subcloned in YIp356 for integration at the *ura3* locus of strains JF326 or W303-1A.

The *ACT1*-*lacZ* translational fusion gene was constructed by amplification of a *Bam*HI–*Pst*I fragment of *ACT1* (–465 to +20) using the following primers: *act1*-*Bam*HI 5'-CCTAAGAATGTCCTTATCGGATCCTCAAAC-3' and *act1*-*Pst*I 5'-CAATGGATTCTGGTATGTTCTCTGCAGTG CA-3' (*Bam*HI and *Pst*I). This PCR fragment was subcloned into YIp356 to yield pEBACT1. This plasmid was used as template for introduction of a *Spe*I restriction site at position –196 (pEB2ACT1) using the following primers: *act1*-5'-*Spe*I (5'-TCTGTGACTAGTATCACGCTCTCTTTTATCTTCCTTTT-3') and *act1*-3'-*Spe*I (5'-TCATTTACTAGTGAATATATATTAAC AAGAGAGATTGGG-3'), and it yielded pEBACT2. Following the same strategy, a *Spe*I site was introduced in pJL1 and in pJL37 at position –86 using the following primers: *gsy2*-5'-*Spe*I (5'-TCTGTGACTAGTTCTCTAAAGCATCAC TGAGAAG-3') and *gsy2*-3'-*Spe*I (5'-TCATTTACTAGTACATATTTAAGGAAAGGCAAGG AAC-3'), yielding pEBGSY2. The plasmid pEB $\Delta$ - $\Delta$ STRE ( $\Delta$ -*STRE*-*gsy2*[*ACT1*<sub>5'UTR</sub>]-*lacZ* construct) was obtained by replacement of the *Spe*I–*Pvu*II fragment from pEBGSY2 by the *Spe*I–*Pvu*II fragment from pEBACT1. All these constructs were verified by sequencing (Genome Express SA, Grenoble, France). Plasmids were linearized at the *Nco*I site of the *URA3* marker to target the integration to the *ura3* locus of the strain KT1112, as described in Parrou *et al.* (1997).

The plasmid pLG669-ZX (Guarente and Ptashne, 1983; Vincent and Gancedo, 1997), bearing the *CYC1* promoter fused to *LacZ*, was used for heterologous expression of this gene with DNA fragments from *GSY2* or  $\Delta$ -*STRE*-*gsy2* promoter spanning from –390 to –167 (relative to the translation initiation codon). These constructs were generated by PCR using the following primers: *Xho*I–*GSY2* (5'-AGTAATCTCGAGCTTGCCATAAGAAAGAAAGGGGTG-3') or *Xho*I– $\Delta$ STRE *gsy2* (5'-AGTAATCTCGAGCTTGCCATAAGAAAGAAAGATCTTG-3') and *Sma*I–*GSY2* (5'-ATATGACCCGGGTGGGGCCTCGAGCATGGCT-3') (restriction site underlined) to amplify the DNA fragment using pJL1 (bearing wild-type *GSY2* promoter) and pJL37 (bearing  $\Delta$ STRE-*gsy2* promoter), respectively. The PCR fragment were inserted into the *Xho*I–*Sma*I site of pLG669-ZX to yield pJF737 and pJF738.

#### *RNA quantification, and correlation with $\beta$ -galactosidase activity from lacZ gene fusions*

Total RNA was extracted from cells (about  $2 \times 10^9$  cells) harvested at different times during growth by centrifugation. The RNA was separated in formaldehyde agarose gels, as described previously (Guthrie and Fink, 1991) and transferred to Hybond N<sup>+</sup> filters (Amersham, UK). Hybridization and washings were performed as recommended by the manufacturer. Blots were probed with <sup>32</sup>P-radiolabelled fragments of *ACT1*, *GPH1* and *GSY2* using the Megaprime<sup>™</sup> DNA labelling system (Amersham, UK). Signal quantification of the autoradiographic films from Northern analysis was performed using the Tina 2.0.PC Bas software. Unless otherwise stated,  $\beta$ -galactosidase activity has been determined instead of mRNAs for quantitative analysis of *GSY2* expression in different genetic backgrounds and under various growth conditions, because we demonstrated in a previous paper (Parrou *et al.*, 1999) a close quantitative correlation between  $\beta$ -galactosidase activity from *GSY2*-*lacZ* translational fusion gene and levels of *GSY2* mRNAs.

#### *Reproducibility of the data*

All experiments have been repeated at least three times with consistent results (standard error of ca. 10%). Only a typical representative experiment is reported for each figure.

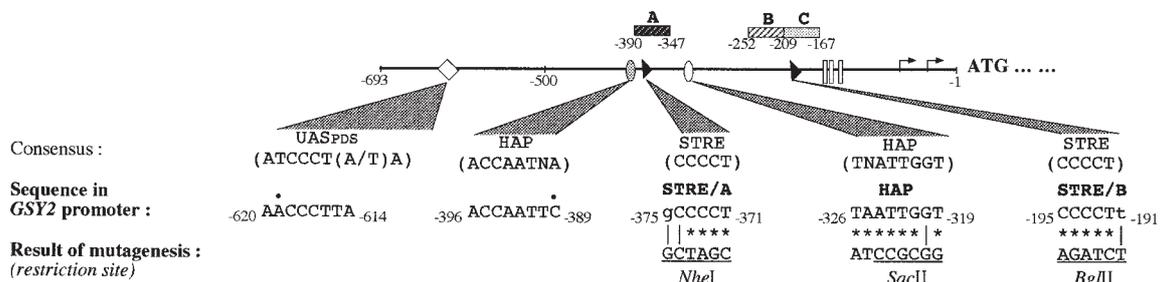


Figure 1. Putative regulatory elements in *GSY2* promoter region and result of site-directed mutagenesis. Coordinates are relative to the translation start site. Boxes A, B and C are three enhancer regions (Ni and Laporte, 1995). →, Transcriptional start sites (Laporte, personal communication); □, putative TATA boxes; ●, mismatches between the promoter sequence and the consensus sequence. The choice of these restriction sites allowed an exclusive and almost full-length replacement of the consensus by unrelated sequences (| and \* respectively, indicate conserved and replaced bases after site-directed mutagenesis).

## RESULTS

### *Mutations of STREs and other cis-elements do not alleviate the growth-related induction of GSY2*

A detailed analysis of the *GSY2* promoter revealed a number of putative regulatory elements which may be involved in transcriptional activation of this gene in response to stress and changing growth conditions (Figure 1). In addition to the two STREs previously studied by Ni and Laporte (1995) (positions -375 and -195 bp, relative to the translation initiation site), we identified one UAS<sub>PDS</sub> element at -620 bp and two putative HAP2/3/4/5 complex binding sites at -396 and -326 bp, respectively. Deletion up to -390 bp relative to the ATG did not affect *GSY2* expression during growth on glucose (Ni and Laporte, 1995; unpublished results), indicating that UAS<sub>PDS</sub> and the more distal HAP2-binding site have no regulatory function under this growth condition. We therefore investigated the role of each of the two STREs (named STRE/A and STRE/B; see Figure 1) and of the proximal HAP2-binding site by replacing most of the consensus sequences with unrelated sequences, instead of deleting them, to avoid possible alteration of the promoter structure. As shown in Figure 2, mutation of the proximal HAP2-binding site and STRE/B caused respectively a 40% and 60% reduction of *GSY2* promoter activity at the diauxic shift (after 20 h of growth). By contrast, the replacement of STRE/A reduced *GSY2* expression by about 20-fold, and this effect was only slightly reinforced after additional mutation of STRE/B ( $\Delta$ -STRE-*gsy2-lacZ*, not illustrated). These results were at variance to those of Ni and Laporte (1995), who showed that mutation in either

STRE/A or STRE/B gave a similar reduction of *GSY2* expression. Therefore, our mutational strategy probably supported the presumption of these authors that their site-directed mutagenesis on STRE/A may not have completely inactivated this element.

To determine whether STREs were either the determinants for the transcriptional induction or solely powerful enhancers of transcription,  $\beta$ -galactosidase activity from a  $\Delta$ -STRE-*gsy2-lacZ*

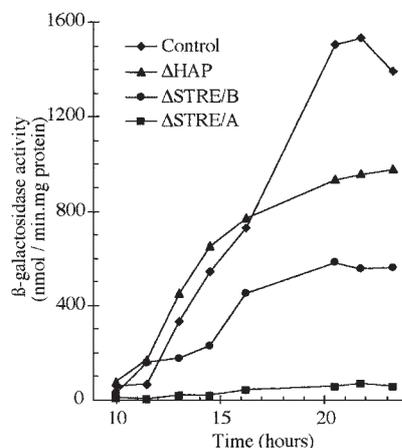


Figure 2. Individual involvement of STREs and HAP2 consensus in the expression of *GSY2-lacZ* constructs during diauxic growth on glucose. Strain KT1112 was transformed with YEp356 derivative plasmids carrying either the wild-type *GSY2-lacZ* construct or mutated constructs at STRE/A, STRE/B and the proximal HAP2-consensus (respective symbols reported on the graph). These transformants were grown in shake flasks, with YNB containing 10 g/l glucose and auxotrophic requirements. The three latest points of  $\beta$ -galactosidase activity curves were taken during the diauxic shift (growth curves not plotted).

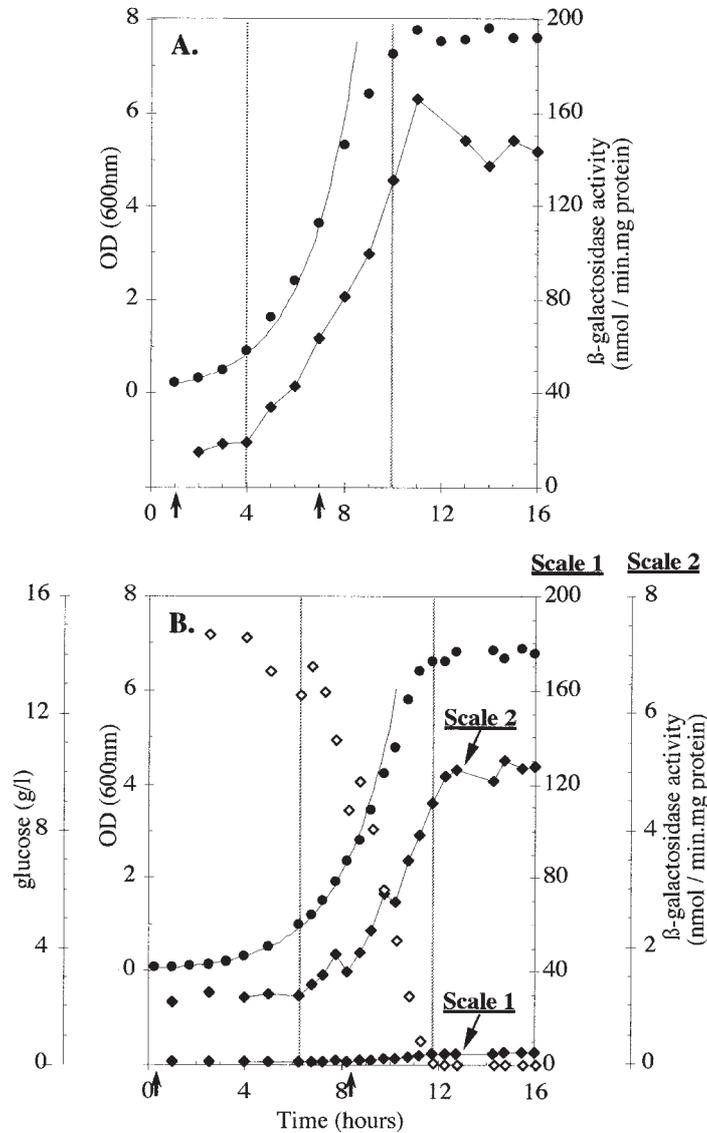


Figure 3. Expression patterns of *GSY2-lacZ* and  $\Delta$ -*STRE-gsy2-lacZ* gene fusion during diauxic growth on glucose. Strains JLP5-1A (wild-type construct, panel A) and JLP94-4B (mutated construct, panel B) were grown on YPD with 10 g/l glucose. In (B), both Scale 1 (0–200 units, the same as in panel A) and Scale 2 (0–8 units) were relative to  $\beta$ -galactosidase activity. Parameters from the equation of the fitting curve (continuous line) calculated from points between the arrows:  $\mu=0.48$ ,  $r^2=0.9951$  (A) and  $\mu=0.47$ ,  $r^2=0.9984$  (B). ●, Cell growth; ◇, residual glucose; and ◆,  $\beta$ -galactosidase activity.

*lacZ* construct integrated as a single copy at the *ura3* locus of a wild-type strain was monitored during growth in glucose-limited medium. As described previously (Parrou *et al.*, 1999), the

wild-type *GSY2-lacZ* construct was induced well before the diauxic shift with an induction ratio of about six-fold between exponential and diauxic phases (Figure 3A, scale 1), and the same

induction ratio was observed with the mutated promoter (Figure 3B, scale 2) despite a 20-fold drop in steady-state promoter activity (cf. scale 1 and scale 2 in Figure 3B). The STRE-independent induction of *GSY2* during the diauxic growth was further illustrated by Northern analysis of *lacZ* transcripts from *GSY2-lacZ* and  $\Delta$ -*STRE-gsy2-lacZ* integrated constructs (Figure 4). In these experiments, the levels of *lacZ* transcripts and of the endogenous *GSY2* mRNA in strains bearing the different constructs were determined along the growth and were reported to the levels of *ACT1* messenger, which was taken as the internal control. These ratios obtained at each time point were then divided by those of *lacZ-ACT1* and *GSY2-ACT1* determined at the first time point, which were normalized to 1. This calculation method could easily estimate the induction factor for *lacZ* and *GSY2* mRNAs and could be compared with the increase of  $\beta$ -galactosidase activity from *lacZ* constructs. According to this method, it was interesting to note that the  $\beta$ -galactosidase activity from *ACT1-lacZ* increased by about 1.5 fold (from 160 to 250 nmol/min/mg protein) as cells entered the diauxic shift. Therefore, this factor of 1.5 can be taken as the threshold value from which gene induction becomes significant, owing to the fact that *ACT1* is a constitutively expressed gene. Taking this into account, it can be seen in Figure 4A, B that the endogenous *GSY2* transcript was increased by about three- to five-fold at diauxic shift and this increase closely matched that of  $\beta$ -galactosidase activity from the *GSY2-lacZ* fusion gene. By contrast, the induction factor of *lacZ* transcripts driven by *GSY2* promoter was always lower (cf. open and hatched bars in Figure 4B). In agreement with results from Figure 3, the removal of the two STREs from *GSY2* promoter did not abolish the increase of  $\beta$ -galactosidase and of the *lacZ* mRNA from a  $\Delta$ -*STRE-gsy2-lacZ* construct. Note that, as expected, the absolute levels of *lacZ* messenger had been reduced by ca. 10- to 20-fold upon removal of the STREs (not shown), almost proportionally to the reduction of  $\beta$ -galactosidase activity. However, while the pattern of  $\beta$ -galactosidase activity from this  $\Delta$ -*STRE-gsy2-lacZ* fusion gene closely matched that of the endogenous *GSY2* mRNA during the growth, the rise of *lacZ* mRNA was slightly delayed, suggesting that the removal of STREs may somehow affect the timing of induction. Finally, to remove any doubt about the fact that the sudden rise in *GSY2* mRNA could result from a change in mess-

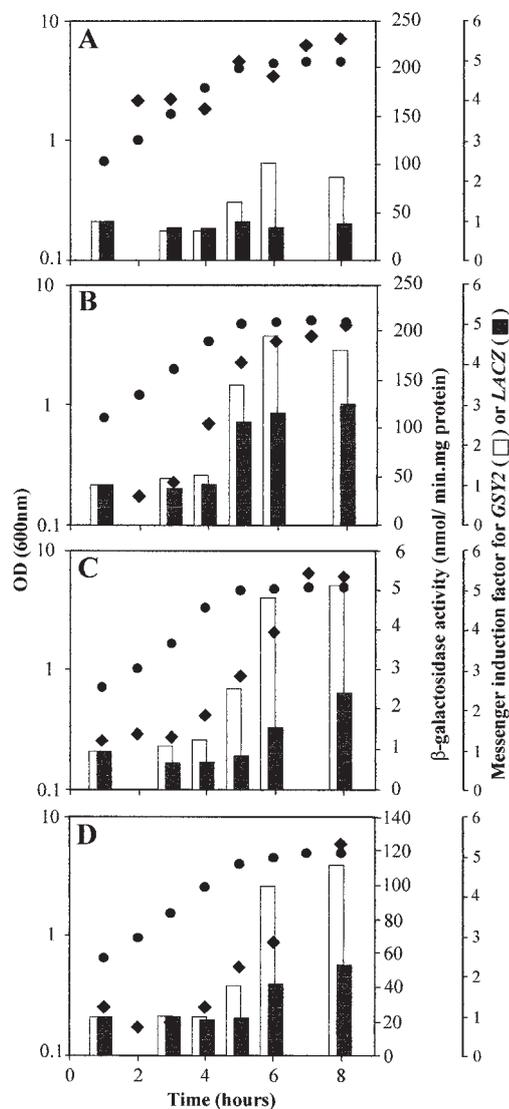


Figure 4. Northern analysis and  $\beta$ -galactosidase activity from *ACT1-lacZ* (A), *GSY2-lacZ*(B),  $\Delta$ -*STRE-gsy2-lacZ* (C) and  $\Delta$ -*STRE-gsy2p[ACT1<sup>5'UTR</sup>]-lacZ* (D) constructs during the diauxic growth on glucose. Strains JF1087 (A), JLP5-1A (B), JF1121 (C) and JF1120 (D) were cultivated on YEP with 10 g/l glucose. ●, cell growth; ◆,  $\beta$ -galactosidase. Autoradiographic films from Northern analysis were quantified as described in Material and Methods. At each time point, the values of *lacZ* and *GSY2* mRNAs were reported to that of *ACT1* taken as the internal control, and these ratios were again divided by the ratio of *lacZ:ACT1* and *GSY2:ACT1* from the first time point, which was normalized to 1. This calculation method allowed expression of the changes of *GSY2* (open bars) and *lacZ* (hatched bars) mRNAs in term of induction factor.

enger stability rather than from a transcriptional activation, the 5' UTR sequence of *GSY2* was

replaced by that of the *ACT1* gene (Munholland *et al.*, 1990). As shown in Figure 4D, this substitution did not modify the expression pattern of the new construct but it caused in a 10-fold rise in the steady-state levels of  $\beta$ -galactosidase. This strong increase might be due to a better stability of *lacZ* mRNAs, an unexpected enhancement which further supports the fact that the induction is possible in the absence of STREs.

Taken together, these results led to the suggestion that the *GSY2* promoter may contain a *cis*-element specific for this induction pattern. To verify this assertion, we have inserted a DNA fragment taken from the  $\Delta$ -*STRE-gsy2* promoter (–390 to –170 relative to the ATG) upstream to the TATA box of a *CYCI-lacZ* lacking its UAS elements (plasmid pLG669-ZX; Vincent and Gancedo, 1995). The activity of  $\beta$ -galactosidase from this hybrid *lacZ* construct exhibited an induction pattern comparable to that obtained with a wild-type *GSY2-lacZ*, while the activity of the reporter plasmid pLG669-ZX only increased by 1.5-fold as cells entered the diauxic shift (Figure 5). It is interesting that the effect of introducing a 223 bp fragment of the  $\Delta$ -*STRE-gsy2* promoter into the *CYCI-lacZ* has reduced by five-fold the activity of this hybrid promoter during the exponential growth without affecting its maximal activity at the end of growth. This result tends to reveal that the putative *cis*-element required for gene induction is actually a repressing sequence.

#### *STREs are absolutely required when cells are exposed to certain typical stress conditions*

While STREs do not seem to be involved in the growth-related transcriptional induction, we show in Figure 6 that the induction of *GSY2-lacZ* fusion gene in response to a temperature shift from 27°C to 37°C, to nitrogen and to carbon source starvations was completely abolished after mutation of both STRE/A and STRE/B. However, ethanol and pH shocks activated neither *GSY2* (not illustrated) nor *TPS1* (Alexandre *et al.*, 1998; our unpublished results), while these two stress conditions have been shown to activate the expression of other stress-responsive genes, such as *CTT1*, *HSP12* and *DDR2* (Preakelt and Meacock, 1990; Schüller *et al.*, 1994; Martinez-Pastor *et al.*, 1996; Treger *et al.*, 1998). Using an artificial *STRE-lacZ* fusion gene, Treger *et al.* (1998) demonstrated that STRE was by itself sufficient to confer a multistress response. However, at least in the

case of *GSY2*, we showed that STREs were actually the primary determinants for gene activation under a subset of stress conditions, but they do not confer a complete multistress response in their native promoter environment, probably because of antagonistic actions on STRE-mediated activation.

#### *The Ras-cAMP signalling pathway does not trigger the growth-related induction of GSY2, and does not exert its negative effect solely through STREs*

Compelling data suggest that the reduction of the cAMP-signalling pathway activity could serve as a cellular switch mechanism for the general stress response, including adaptation of yeast cells to the 'stationary phase' (Boy-Marcotte *et al.*, 1996, 1998; Werner-Washburne *et al.*, 1996). Since this adaptation was accompanied by the activation of stress-responsive genes, we wondered whether a *bcy1* mutant, which results in a hyperactive and almost constitutive kinase A (Toda *et al.*, 1987; Cannon *et al.*, 1987), might prevent the transcriptional induction of *GSY2*. For this purpose, we used a strain bearing a deletion of 42 bp in the *BCY1* gene (Cannon *et al.*, 1990) instead of a null mutation, since in this latter, many spontaneous revertants arose on galactose or acetate plates, which would prevent any reliable conclusion. As shown in Figure 7, the growth-related transcriptional induction of *GSY2-lacZ* was not abolished in this *bcy1* mutant, although this strain exhibited all the other traits associated with an hyperactive PKA, including the absence of glycogen accumulation, a high sensitivity to heat shock, and an inability to stop budding upon nutrient starvation. However, this experiment showed that the induction ratio of *GSY2* between the exponential phase and the exhaustion of glucose was only five-fold in the *bcy1* mutant, instead of 15-fold as in the wild-type strain (Figure 7). This result supports the notion that a lowering of the protein kinase A activity is required for full derepression of genes at the diauxic shift (Boy-Marcotte *et al.*, 1996). To further illustrate this negative control of gene expression by the PKA, Figure 8 shows that all the genes involved in the synthesis (*GSY2*, *GAC1*, *TPS1*, *TPS2*) and in the degradation (*GPH1*, *NTH1*) of glycogen and trehalose were similarly affected by mutations that activate (*bcy1*) or reduce (*ras2*) the RAS-cAMP pathway. Interestingly, when the  $\Delta$ -*STRE-gsy2-lacZ* construct was introduced into *ras2* and *bcy1* mutant strains, these

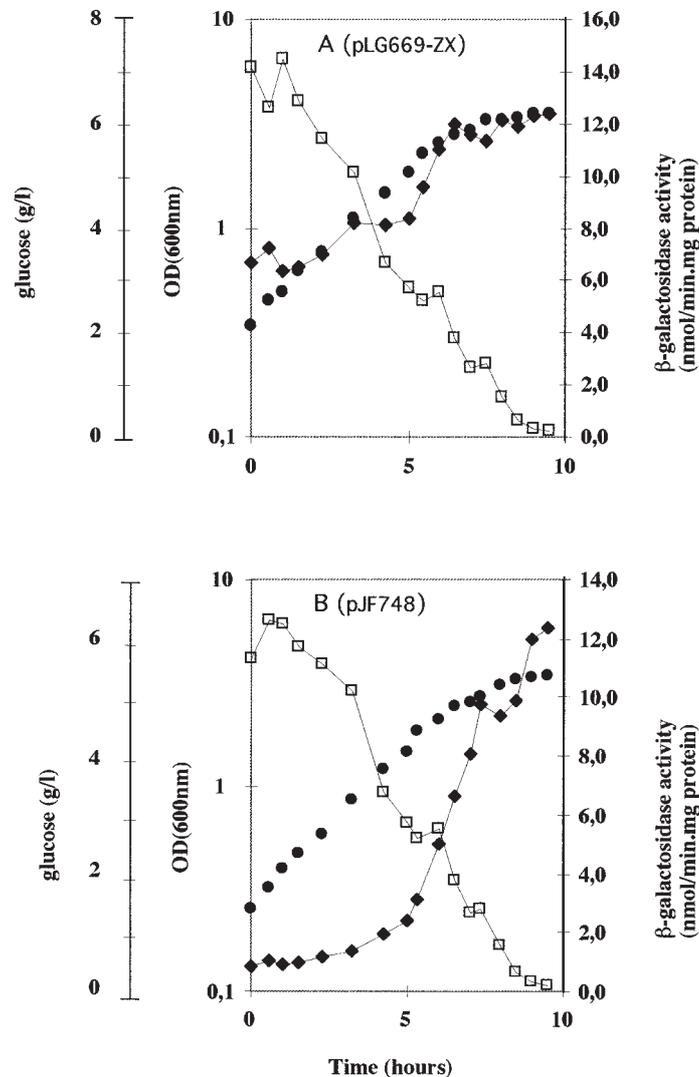


Figure 5. Expression patterns of *CYC1-lacZ* bearing DNA fragment from  $\Delta$ STRE-*gsy2* promoter upstream to *CYC1* TATA box. Control plasmids (pLG669-ZX; (A) and pJF748 (B) were transformed in strain KT1112.  $\beta$ -galactosidase was determined during the growth on YNB medium with 10 g/l glucose and supplemented with 100 mg/l of histidine and leucine. ●, Cell growth; ◆,  $\beta$ -galactosidase; □, residual glucose.

mutations identically affected the expression of both wild-type and mutated promoters, indicating that the repressive effect of the Ras-cAMP pathway on the *GSY2* promoter may not be mediated entirely through STREs.

## DISCUSSION

In this paper we provide new insights into the molecular mechanisms implicated in the

adaptation of the yeast *S. cerevisiae* to the diauxic transition of growth on glucose. We initially demonstrated that *GSY2*, one of the genes activated during this adaptation, was induced early before the diauxic shift, while glucose and other nutrients were still plentiful in the medium (Parrou *et al.*, 1999). By substituting the 5' UTR of *GSY2* by that of *ACT1*, we excluded that the sharp increase in *GSY2* transcripts resulted from a sudden change in the stability of these messengers. A site-directed

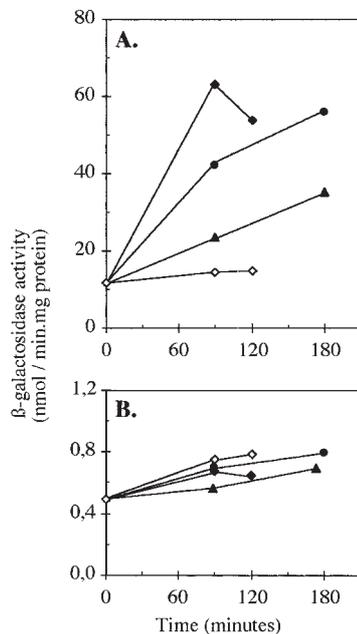


Figure 6. Response of *GSY2-lacZ* and *STRE-gsy2-lacZ* constructs to various stress. Strains JLP5-1A (wild-type construct, panel A) and JLP94-4B (mutated construct, panel B). Exponential growing cells in YNB ( $OD_{600}$  0.5) were subjected at time zero to a heat shock from 25°C to 37°C (◆), or were transferred to a carbon (●)- or a nitrogen (▲)-starved medium.  $\beta$ -galactosidase activity from the fusion genes was determined during 3 h after exposition to these stresses.

mutagenesis of the different consensus elements present in *GSY2* promoter, including both STREs, showed that none of these consensuses mediate the growth-related transcriptional induction. Northern analysis confirmed our  $\beta$ -galactosidase measurements, since the transcripts from a  $\Delta$ -*STRE-gsy2-lacZ* construct were still detectable and showed this typical induction. However, the absence of STREs led to a weaker promoter activity, and even to a slight delay in transcriptional induction, indicating that these *cis*-elements are powerful enhancers of expression that may work in synergy with other *cis*-elements. Taken together, these results indicate that, in the proper promoter context, other *cis*-acting elements mediate the specific growth-related—STRE independent—induction of *GSY2*. In favour of this hypothesis, we found that a 223 bp DNA sequence from  $\Delta$ -*STRE-gsy2* promoter was able to confer the growth-related induction of  $\beta$ -galactosidase when it was inserted upstream to the TATA box of a *CYC1-lacZ* fusion gene. This DNA fragment actually appears to contain a repressing sequence.

Work is currently under way to identify the consensus and to further characterize its role in the control of *GSY2* expression. Even if STREs are not necessary for diauxic growth-related induction, one or several STREs are absolutely required for the transcriptional activation of *GSY2* in response to heat shock and to starvation for carbon and nitrogen sources (Ni and Laporte, 1995; Timblin and Bergman, 1997; this study).

The second important message of this paper was to show that the growth-related induction of *GSY2* is not triggered by a reduction of the Ras-cAMP signalling pathway. Indeed, an hyperactive protein kinase A, which results from mutation in either *BCY1*, *IRA1* or *IRA2*, did not abolish this induction. However, we cannot rule out the possibility that the free catalytic subunits of PKA itself are directly implicated in this event, e.g. by being downregulated by some cellular factors during growth. *In vivo* determination of PKA activity in *bcy1* mutants during the diauxic growth should clarify this question. Even if the Ras-cAMP signalling pathway does not trigger this genetic event, it exerts a strong negative regulation, as indicated by the lower induction of *GSY2* in a *bcy1* mutant at the exhaustion of glucose. This observation supports the model that in a wild-type strain, reduction of cAMP levels (François *et al.*, 1987; Russel *et al.*, 1993)—and subsequent inactivation of protein kinase A—is necessary for the relief of cAMP-dependent repression and for the full stimulation of gene expression at the diauxic transition (Boy-Marcotte *et al.*, 1996, 1998).

The repressing effect of the cAMP-signalling pathway on genes involved in glycogen and trehalose metabolism (Toda *et al.*, 1987; François *et al.*, 1992; Hardy *et al.*, 1994; Gounalaki and Thireos, 1994; Winderickx *et al.*, 1996) is reminiscent of the one reported for *CTT1*, *HSP12* or *SOD2* (Praekelt and Meacock, 1990; Flattery-O'Brien *et al.*, 1997; Bissinger *et al.*, 1989; Belazzi *et al.*, 1991). Several sets of findings argued in favour of the model in which the negative regulation by cAMP is mediated by STREs (Marchler *et al.*, 1993; Ruis and Schüller, 1995; Varela *et al.*, 1995; Flattery-O'Brien *et al.*, 1997; Smith *et al.*, 1998) via the inhibition of Msn2p/Msn4p accessibility to these *cis*-elements (Görner *et al.*, 1998). Two sets of experimental findings complement this model. First, we found that  $\beta$ -galactosidase activity from *GSY2-lacZ* in a double *msn2-msn4* mutant was only three-fold reduced (unpublished data), whereas it was 10-fold lower in a *bcy1* mutant (see

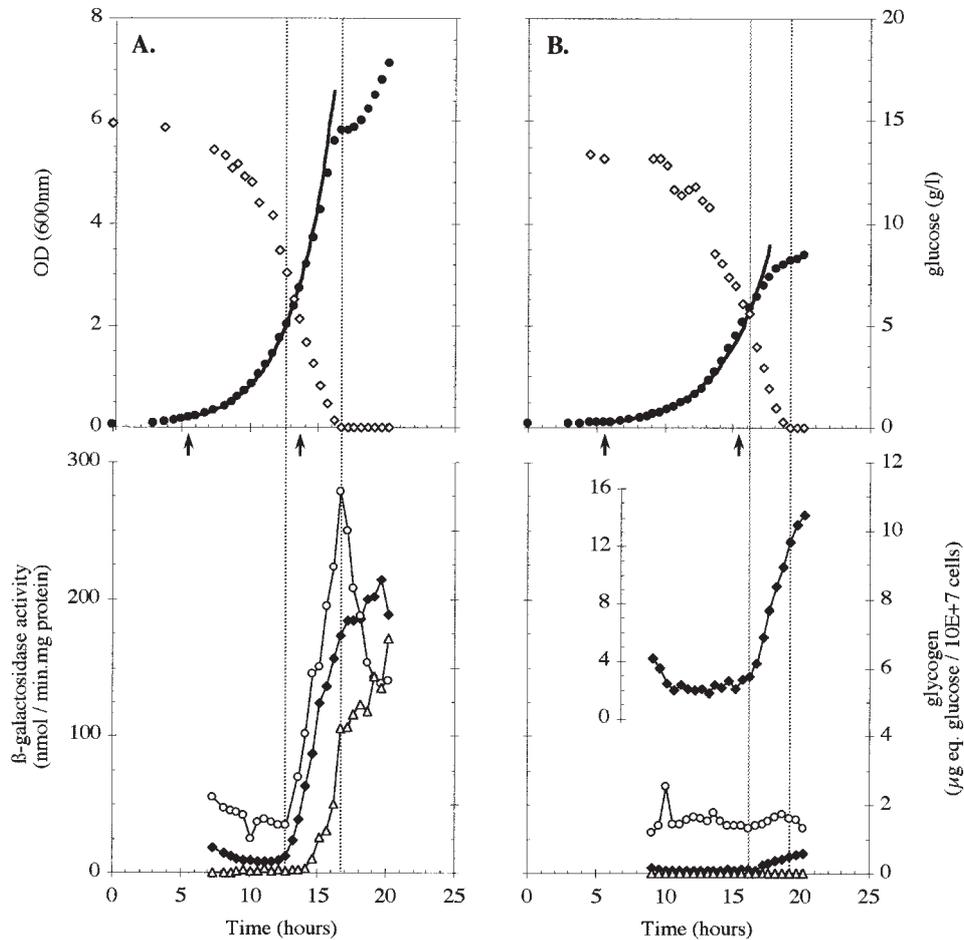


Figure 7. Expression pattern of *GSY2-lacZ* construct in wild-type and *bcy1* mutant strains during diauxic growth on glucose. Prototroph strains JLP48-3B (wild-type, panel A) and JLP86-3A (*bcy1* mutant, panel B) were grown on YNB with 10 g/l glucose. ●, Cell growth; ◇, residual glucose; ◆, β-galactosidase activity; ○, glycogen; △, and trehalose. Parameters from the equation of the fitting curve:  $\mu=0.34$ ,  $r^2=0.9992$  (A) and  $\mu=0.30$ ,  $r^2=0.9967$  (B). In (B), β-galactosidase activity was also plotted with an appropriate scale (0–16 units).

Figure 7), indicating that cAMP may act on additional targets other than Msn2p–Msn4p. The recent identification of *RIM15*, which encodes a protein kinase acting downstream of the PKA and which is needed for derepression of *SSA3*, *HSP12* and *HSP26* at diauxic growth on glucose (Reinders *et al.*, 1998), could be involved in this control. Recent data indicated that this kinase affects positively gene expression in a Msn2–Msn4p-independent way (De Virgilio, personal communication). Second, and more convincingly, both the strong reduction of *GSY2-lacZ* activity in a *bcy1* mutant and its increase in a *ras2* mutant,

were quantitatively conserved after mutation of both STREs, suggesting that other *cis*-acting element(s) confer the modulation of *GSY2* expression by the Ras–cAMP pathway. Nevertheless, our results do not refute the STRE-mediated activation of artificial promoters bearing one or several copies of oligonucleotides containing STREs (Marchler *et al.*, 1993; Martinez-Pastor *et al.*, 1996; Boy-Marcotte *et al.*, 1998; Treger *et al.*, 1998). To account for ‘derepression’ of these constructs at the end of growth, the reduction of Ras–cAMP pathway activity and the subsequent activation of Msn2p–Msn4p transactivators

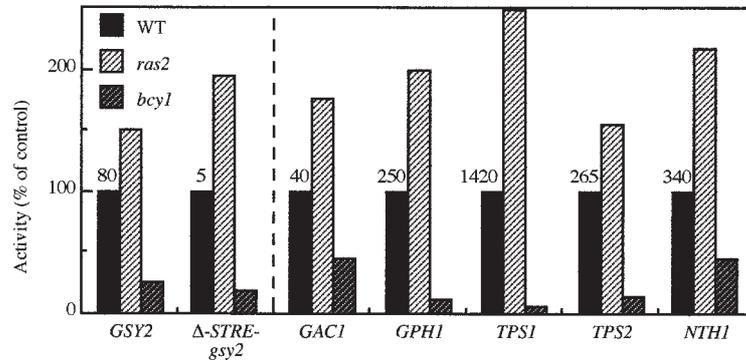


Figure 8. Effect of mutations in the Ras-cAMP signalling pathway on expression of genes involved in reserve carbohydrates metabolism. Wild-type and mutant strains carrying either *GSY2*, *GAC1*, *GPH1-lacZ* fusion genes or  $\Delta$ -*STRE-gsy2-lacZ* construct integrated as a single copy at the *ura3* locus were grown on YEP with 10 g/l glucose. *TPS1*, *TPS2* and *NTH1-lacZ* fusion genes on episomic vectors were transformed into wild-type and mutant strains which were grown on YNB with 10 g/l glucose and auxotrophic requirements. At the time of glucose exhaustion, five samples were taken for  $\beta$ -galactosidase assays. Absolute values (nmol/min.mg protein) are the averages of these five isolates from two independent experiments. Activities from wild-type strains are reported on the top of the histograms.

may activate transcription through the sole presence of STRE. Therefore, our results actually emphasize that reliable conclusions can be obtained solely with full-length gene promoter, being aware of misleading interpretation of complex gene regulation when working with artificial promoters.

In spite of a clear involvement of cAMP in transcriptional activation, there is evidence that another signalling pathway must exist to integrate variations in nutritional status and trigger this growth-related gene expression. Such a signalling pathway has been already inferred from the results of Cameron *et al.* (1988), who showed that cells without the regulatory subunit of PKA, and containing only attenuated PKA activity, exhibit apparently normal physiological processes during growth on glucose-limiting conditions, including glycogen induction. Furthermore, the existence of a system that couples growth control to nutrient levels was postulated by Russel *et al.* (1993), who reported that the expression of *CDC25* and *CDC35*, which belong to the Ras-cAMP system, strongly decreased as cells approached the diauxic shift of growth on glucose. More recently, Timblin and Bergman (1997) showed that the expression of *GSY2* is controlled in a STRE-independent fashion by the cyclin-dependent protein kinase Pho85. Whether Pho85 protein kinase belongs to

the second system or not remains to be investigated, but the close relationship between gene induction and growth control suggests that a pathway dependent upon cyclin activity may be a good candidate.

#### ACKNOWLEDGEMENTS

We thank P. Estruch (CSIC, Valencia, Spain) for providing strains and plasmids, and for fruitful discussion of this work. We are grateful to Dr Miguel A. Blazquez for his initial participation in this work through an 'Action Intégrée Picasso France-Espagne' and to Marie Ange Teste for her excellent technical assistance. This work was supported in part by the Commission of the European Union (Cell Factories program No. BIO4.CT95.132, to J.F.).

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