

***Saccharomyces cerevisiae* Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1**

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The *Saccharomyces cerevisiae* protein kinase Rim15 was identified previously as a component of the Ras/cAMP pathway acting immediately downstream of cAMP-dependent protein kinase (cAPK) to control a broad range of adaptations in response to nutrient limitation. Here, we show that the zinc finger protein Gis1 acts as a dosage-dependent suppressor of the *rim15Δ* defect in nutrient limitation-induced transcriptional derepression of *SSA3*. Loss of Gis1 results in a defect in transcriptional derepression upon nutrient limitation of various genes that are negatively regulated by the Ras/cAMP pathway (e.g. *SSA3*, *HSP12* and *HSP26*). Tests of epistasis as well as transcriptional analyses of Gis1-dependent expression indicate that Gis1 acts in this pathway downstream of Rim15 to mediate transcription from the previously identified post-diauxic shift (PDS) element. Accordingly, deletion of *GIS1* partially suppresses, and overexpression of *GIS1* exacerbates the growth defect of mutant cells that are compromised for cAPK activity. Moreover, PDS element-driven expression, which is negatively regulated by the Ras/cAMP pathway and which is induced upon nutrient limitation, is almost entirely dependent on the presence of Gis1.

Keywords: cAPK/Gis1/nutrient signaling/PDS element/yeast

Introduction

The yeast *Saccharomyces cerevisiae* copes with diverse environmental conditions by coordinated regulation of growth, cell cycle progression and metabolic activities. The Ras/cAMP pathway is essential for the control and integration of these processes, in particular with respect to the nutritional status. The dramatic reprogramming of the metabolism at the diauxic transition, when glucose becomes limiting, as well as some of the subsequent adaptations during both the post-diauxic phase, when cells grow respiratively on ethanol, and during entry into stationary phase, are negatively controlled by the Ras/cAMP pathway. Thus, cells with elevated cAMP-dependent protein kinase (cAPK) activity fail to accumulate carbohydrate reserves, to undergo a proper diauxic transition and to arrest in G₁ upon nutrient limitation. As a result, such cells remain highly sensitive to heat stress and rapidly lose viability in stationary phase. In contrast,

cAPK-deficient cells exhibit physiological changes normally associated with nutrient limitation, such as G₁ cell cycle arrest, accumulation of storage carbohydrates and increased resistance towards heat and oxidative stress (for reviews and further details on the Ras/cAMP pathway, see Tatchell, 1986; Broach and Deschenes, 1990; Thevelein and de Winde, 1999; for a review on stationary phase, see Werner-Washburne *et al.*, 1993).

The mechanism by which cAPK controls growth is still an issue of conjecture. One mechanism by which cells may control proliferation has recently been suggested to be based on cAPK-mediated regulation of G₁ cyclin translation (Hall *et al.*, 1998). In addition, it was found that modulation of Rap1 transcriptional activity by cAPK mediates growth-regulated expression of ribosomal protein genes (Klein and Struhl, 1994). However, positive cAPK-mediated regulation of ribosomal protein synthesis may not be the only reason for the dependence of growth on cAPK (Klein and Struhl, 1994; Neumann-Silberberg *et al.*, 1995) and some of the growth-related effects of cAPK may be due to changes in additional transcriptional control mechanisms. For instance, transcriptional derepression/activation of a large number of yeast genes (e.g. *CTT1*, *DDR1*, *HSP12* and *TPS2*) is negatively regulated by cAPK through one or more AG₄ stress-responsive elements (STREs), which confer transcriptional activation in response to a wide range of stresses, including heat, oxidative and osmotic shocks, and nutrient limitation (for reviews, see Mager and De Kruijff, 1995; Ruis and Schüller, 1995). Control of STRE-driven gene expression by cAPK is mediated via Msn2 and Msn4, a pair of partially redundant zinc finger transcription factors that recognize and bind to STRE (Estruch and Carlson, 1993; Martínez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996; Görner *et al.*, 1998). Surprisingly, loss of Msn2 and Msn4 renders cells largely independent of cAPK activity, suggesting that an essential function of cAPK is to inhibit expression of growth-inhibitory genes that are under the control of Msn2 and Msn4 (Smith *et al.*, 1998). In agreement with this suggestion, Msn2/Msn4 function is also required for expression of *YAK1*, a gene whose product was shown previously to antagonize cAPK-dependent growth (Garrett and Broach, 1989; Garrett *et al.*, 1991; Smith *et al.*, 1998). cAPK-mediated control of gene expression is also exerted via additional transcriptional regulators. Accordingly, expression of the glucose-repressible *ADH2* gene has been found to be regulated mainly by cAPK-dependent inactivation of the transcriptional activator Adr1 (Cherry *et al.*, 1989), while expression of *GAC1* and *SSA3* was reported possibly to be regulated by cAPK-dependent activation of the transcriptional repressor Sok2 (Ward *et al.*, 1995). Since overexpression of *SOK2* can partially relieve dependence on cAPK function, Sok2 acts as a cAPK-dependent repressor

of one or more genes whose products inhibit cell proliferation.

Several observations suggest the existence of additional cAPK-dependent transcriptional control mechanisms. For instance, expression of *GSY2* was recently found to be partially repressed by the cAPK pathway through an unknown STRE-independent mechanism (Parrou *et al.*, 1999). Similarly, a large-scale analysis of gene expression in a *msn2 msn4* double mutant (Boy-Marcotte *et al.*, 1998), as well as a screen for cAMP-repressible genes (Tadi *et al.*, 1999), revealed a subset of Msn2/Msn4-independent genes whose expression is negatively controlled by cAMP. One cAPK-regulated element that may confer Msn2/Msn4-independent transcriptional control is the T(T/A)AG₃AT post-diauxic shift (PDS) element, which mediates transcriptional activation in response to nutritional limitation (Boorstein and Craig, 1990). Unlike the very similar STRE, it does not mediate transcriptional activation in response to other stresses such as heat shock (Boorstein and Craig, 1990), indicating that STRE and PDS elements may be controlled by different cAPK-dependent mechanisms. In agreement with such a model, Msn2 and Msn4 were found to be dispensable for the nutrient limitation-induced transcriptional activation of *SSA3*, a gene with several PDS elements, but no conserved STRE in its promoter region (Martínez-Pastor *et al.*, 1996). In this context, it is interesting to note that transcriptional derepression/activation of *SSA3* at the diauxic transition is almost entirely dependent on the presence of the protein kinase Rim15, which has been identified as a component of the Ras/cAMP pathway acting immediately downstream and under negative control of cAPK to control a broad range of adaptations in response to nutrient limitation (Reinders *et al.*, 1998).

Here we describe the identification of *GIS1* as a dosage-dependent suppressor of the *rim15Δ* defect to derepress/activate *SSA3* upon nutrient limitation. *GIS1* encodes a zinc finger transcription factor, which has recently been isolated as a damage-responsive repressor of the photolyase-encoding *PHR1* gene (Jang *et al.*, 1999), as well as a high-copy suppressor of the Gal⁻ phenotype of a *snf1 mig1 srb8* mutant (Balciunas and Ronne, 1999). Loss of *Gis1* results in a defective response of mutant cells to nutrient limitation, including a defect in transcriptional derepression/activation of *SSA3*. Tests of epistasis as well as transcriptional analyses of *Gis1*-dependent expression suggest that *Gis1* acts in the Ras/cAMP pathway downstream of Rim15 specifically to control transcriptional activation through the PDS element. Interestingly, overexpression of *GIS1* prevents growth, and loss of *Gis1* partially relieves the dependence on cAPK function, indicating that *Gis1* activates one or more genes whose products inhibit cell proliferation.

Results

Identification of *GIS1* as dosage suppressor of *rim15Δ*

We have previously reported that loss of Rim15 results in a defective response to nutrient limitation: mutant cells lacking Rim15 fail to accumulate trehalose, to derepress *HSP12*, *HSP26* and *SSA3*, to induce thermotolerance and to arrest properly in G₁ (Reinders *et al.*, 1998). To identify

potential downstream effectors of Rim15, we performed a screen for dosage-dependent suppressors of the *rim15Δ* defect in nutrient limitation-induced transcriptional derepression of *SSA3*. Accordingly, strain AR2 (*rim15Δ/rim15Δ*) containing the reporter plasmid pWB204Δ-236 (*SSA3-lacZ*; Boorstein and Craig, 1990) was transformed with a genomic DNA library under the control of the strong, inducible *GAL1* promoter (Ramer *et al.*, 1992). We rescued library plasmids from cells in which the reporter gene showed galactose-dependent transcription after replica-plating and incubation for 2 days on selective X-gal plates (containing 2% galactose and 1% raffinose), and assigned the plasmids to 48 different classes by restriction analysis. To isolate specifically downstream effectors of the Ras/cAMP/Rim15 pathway, we further tested the positive plasmids for their ability to exacerbate the growth defect of a strain with attenuated cAPK activity. To this end, one representative plasmid of each class was assayed for galactose-dependent inhibition of growth at 31.5°C of a *tpk2^{ts}* strain (SGY446). Partial sequencing of a plasmid from one of the six remaining positive classes showed it to contain the entire *GIS1* open reading frame, including 470 bp of the 5'-untranslated region, fused to the *GAL1* promoter of the library plasmid. *GIS1* encodes a 90 kDa protein, which contains one classical C₂H₂ zinc finger followed by an alternative C₂HC zinc finger at the C-terminal end (Böhm *et al.*, 1997). Comparison of the predicted *Gis1* sequence with all other deduced amino acid sequences in yeast revealed a second gene product, Rph1, with particularly high homology to the zinc finger-containing C-terminal region of *Gis1* (Balciunas and Ronne, 1999; Jang *et al.*, 1999). Interestingly, while genetic studies suggested different roles for *Gis1* and Rph1 (Balciunas and Ronne, 1999), *in vivo* footprinting, binding competition and transcriptional studies indicated that *Gis1* and Rph1 may act as functionally redundant transcriptional repressors of an STRE-like upstream repressing sequence (URS_{PHR1}; Jang *et al.*, 1999).

***Gis1* regulates nutrient limitation-induced transcription of several cAPK/Rim15-dependent genes**

To determine the consequences of the loss of *Gis1*, we replaced the complete *GIS1* coding region by a PCR-based gene deletion method (Baudin *et al.*, 1993; Wach *et al.*, 1994; see Materials and methods). In agreement with previous studies (Balciunas and Ronne, 1999; Jang *et al.*, 1999), deletion of *GIS1* caused no obvious defect in germination of spores, or in exponential growth on YPD at 30°C. Thus, *GIS1* is not essential for growth or germination. The effect of *gis1Δ* on *SSA3* transcription was assessed by using the *SSA3-lacZ* reporter plasmid pWB204Δ-236. Comparison with wild-type cells revealed that *gis1Δ* cells, similarly to *rim15Δ* cells, were strongly defective for nutrient limitation-induced derepression of *SSA3-lacZ* (Table I). Overexpression of *GIS1*, in contrast, was able not only to suppress fully the *SSA3-lacZ* derepression defect of *rim15Δ* cells, but also to increase *SSA3-lacZ* induction significantly in wild-type, *rim15Δ* and *gis1Δ* mutant cells when compared with the control plasmid-containing wild-type strain. Finally, the increase in *SSA3-lacZ* induction mediated by *RIM15* overexpression was found to be almost entirely dependent

Table I. Epistatic relationship between *RIM15* and *GIS1*

Plasmid	Strain		
	Wild type	<i>rim15Δ</i>	<i>gis1Δ</i>
YCpIF2	153.0 ± 23.1	33.3 ± 9.0	22.1 ± 4.0
YCpIF2- <i>RIM15</i>	280.9 ± 54.6	249.6 ± 58.5	34.1 ± 2.9
YCpIF2- <i>GIS1</i>	264.5 ± 45.8	281.5 ± 54.4	258.7 ± 36.3

Transformants of wild-type (AR1-1A), *rim15Δ* (AR1-1C) and *gis1Δ* (CDV100-10D) cells were grown to early stationary phase (2 days) on SD medium containing 2% galactose and 1% raffinose to induce *GAL1*-driven transcription of *RIM15* and *GIS1* in the corresponding YCpIF2-derived plasmids. β -galactosidase activities were measured (as described in Miller, 1972) to monitor the induction of an *SSA3-lacZ* fusion gene (from plasmid pWB204Δ-236). Notably, all YCpIF2-*GIS1* transformants reached much lower cell densities (<30%) than corresponding YCpIF2 and YCpIF2-*RIM15* transformants. Values represent means \pm SDs of β -galactosidase activities (in Miller units) of three independent experiments.

on the presence of Gis1 (Table I). The simplest interpretation of these results is that Gis1 may act downstream of Rim15 to mediate one or more Rim15-controlled, nutrient limitation-induced responses. Therefore, we also analyzed whether deletion of *GIS1* causes additional defects in response to nutrient limitation. As is seen in Figure 1, *SSA3*, *HSP12* and *HSP26* were repressed in exponentially growing wild-type cells and induced upon glucose starvation. Under the same conditions, *gis1Δ* mutant cells, similarly to *rim15Δ* and *gis1Δ rim15Δ* double mutant cells, were almost entirely defective for derepression of *SSA3* and partially defective for the maintenance of derepression of *HSP12* (Figure 1, lane 4). Derepression of *HSP26*, in contrast, was found to be only slightly defective in *gis1Δ* mutant cells, while being strongly defective in *rim15Δ* and *rim15Δ gis1Δ* double mutant cells (Figure 1, lanes 3 and 4). Notably, glucose limitation-induced *TPS2* transcription remained largely unaffected by *gis1Δ* and/or *rim15Δ*. As a control for the physiological status of the cells, we also examined the expression pattern of a gene, *SSB1*, the transcription of which is repressed upon nutrient limitation (Werner-Washburne *et al.*, 1989). Transcript levels of *SSB1* were high in exponentially growing cells and very low in glucose-starved cells of all four strains (Figure 1, lanes 1–4). Together, these results show that Gis1 not only is involved in nutrient limitation-induced derepression of *SSA3*, but also partially regulates expression of additional cAPK-repressible genes such as *HSP12* and *HSP26* (Engelberg *et al.*, 1994; Varela *et al.*, 1995).

The strong defect in *HSP26* derepression observed in *rim15Δ* cells is only partially mimicked by loss of Gis1, indicating that Gis1 mediates only a subset of Rim15-controlled responses. Therefore, diploid *gis1Δ gis1Δ* cells were also analyzed for a variety of other phenotypic traits that are characteristic of *rim15Δ* cells (e.g. the defects in trehalose accumulation, in proper G₁ arrest, in long-term stationary phase survival and in sporulation; Vidan and Mitchell, 1997; Reinders *et al.*, 1998). When compared with isogenic wild-type cells (YEF473), 4-day-old stationary phase *gis1Δ gis1Δ* cells (CDV101) exhibited normal trehalose levels (0.207 \pm 0.029 g/g protein versus 0.176 \pm 0.008 g/g protein for *GIS1/GIS1* cells) and were only slightly defective for proper G₁ arrest (3.5 \pm 0.4%

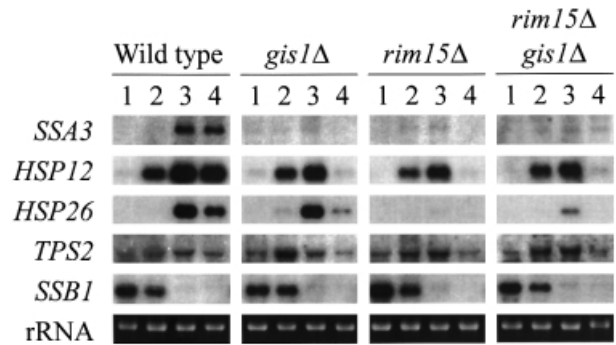


Fig. 1. Effects of *gis1Δ*, *rim15Δ* and *gis1Δ rim15Δ* on the expression of various genes following glucose starvation. Homozygous wild-type (YEF473), and *gis1Δ gis1Δ* (CDV101), *rim15Δ rim15Δ* (AR2) and *gis1Δ gis1Δ rim15Δ rim15Δ* (CDV104) mutant cells were harvested by centrifugation and resuspended in S medium containing 0.1% glucose. Total RNAs were extracted immediately after resuspension (1) and following further incubation for 90 (2), 180 (3) and 270 min (4). Equal amounts of RNAs (10 μ g) were probed with *SSA3*, *HSP12*, *HSP26*, *TPS2* and *SSB1* fragments after electrophoresis and blotting. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

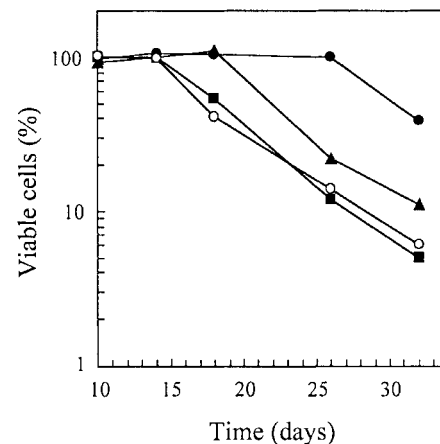


Fig. 2. Effects of *gis1Δ*, *rim15Δ* and *gis1Δ rim15Δ* on stationary phase survival. Homozygous wild-type (YEF473; filled circle) and *gis1Δ gis1Δ* (CDV101; filled triangle), *rim15Δ rim15Δ* (AR2; open circle) and *gis1Δ gis1Δ rim15Δ rim15Δ* (CDV104; filled square) mutant cells were grown to stationary phase on YPD medium. The percentage of viable cells was determined by the colony-forming efficiency on YPD agar at the times indicated.

budded cells versus 0.8 \pm 0.1% budded cells for *GIS1/GIS1* cells). In addition, *gis1Δ gis1Δ* cells exhibited no obvious sporulation defect (data not shown). However, *gis1Δ gis1Δ* cells were found to be significantly more sensitive than wild-type cells, and only slightly less sensitive than *rim15Δ* and *rim15Δ gis1Δ* cells, to prolonged nutrient starvation (Figure 2). These results suggest that Gis1 controls transcriptional activation of a subset of Rim15-dependent genes, the expression of which is important for long-term survival under conditions of nutrient starvation. In agreement with such a role for Gis1, we found Gis1 protein levels to be very low in exponentially growing cells, as estimated by immune blots of myc13-tagged Gis1 (Figure 3, lane 1), and to increase following glucose exhaustion at the diauxic transition

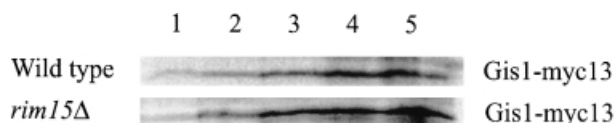


Fig. 3. Immunoblot analysis of Gis1-myc13 in wild-type (NB14) and *rim15Δ* mutant (NB15) cells entering the diauxic transition phase. Samples were taken 2 h before glucose exhaustion (1), at the time of glucose exhaustion (2), and 2 (3), 4 (4) and 6 h (5) following glucose exhaustion. Twenty micrograms of extract protein were loaded in each lane.

(Figure 3, lanes 2–5). Interestingly, Gis1 protein levels were induced similarly in *rim15Δ* cells entering the diauxic transition (Figure 3), indicating that potential Rim15-dependent regulation of Gis1 activity is exerted at a post-transcriptional level.

Loss of *Gis1* partially suppresses *cdc25^{ts}*, *cdc35^{ts}* and *tpk^{ts}*

The data presented above suggest that Gis1 may act in the Ras/cAMP pathway downstream of Rim15 to control transcriptional activation upon nutrient limitation. To determine the potential contribution of Gis1 to cAPK-dependent growth, we tested whether loss of Gis1 could suppress growth defects that are associated with attenuated cAPK activity. To this end, temperature-sensitive Ras GTP-exchange factor and adenylate cyclase mutant strains, harboring corresponding *cdc25^{ts}* and *cdc35^{ts}* mutations, respectively, as well as a temperature-sensitive cAPK mutant strain with only one functional *tpk2^{ts}* gene, were transformed with the *gis1Δ::kanMX2* cassette. In all three mutant strains, deletion of *GIS1* did not restore growth at the non-permissive temperature, indicating that *gis1Δ* is not able to suppress the total loss of cAPK activity (data not shown). However, when tested at a semi-permissive temperature (i.e. at 34°C), the relatively low growth rates of each of the three temperature-sensitive mutant strains were significantly increased by loss of Gis1. Accordingly, deletion of *GIS1* caused growth rates of *cdc25^{ts}*, *cdc35^{ts}* and *tpk2^{ts}* cells to increase from 0.158 ± 0.008 (OL86) to 0.205 ± 0.006 h⁻¹ (NB19), from 0.147 ± 0.007 (PD6517) to 0.181 ± 0.008 h⁻¹ (NB21) and from 0.151 ± 0.005 (SGY446) to 0.191 ± 0.002 h⁻¹ (NB23), respectively. Together, these results show that loss of Gis1 partially relieves dependence on cAPK function.

GIS1 overproduction exacerbates the growth defect of *cdc25^{ts}*, *cdc35^{ts}* and *tpk^{ts}* strains

In order to determine whether Gis1, as suggested by the results presented above, may antagonize cAPK-dependent growth, we tested whether *GIS1* overexpression could exacerbate the growth defect associated with attenuated cAPK activity. Overexpression of *GIS1* from the galactose-inducible *GAL1* promoter inhibited growth even in wild-type cells (Figure 4A; see also Table I). In contrast, wild-type cells overexpressing *GIS1* from the constitutive *ADHI* promoter had only a slight growth defect, which was apparent after growth for 1 day on plates (data not shown), but not when plates were incubated for >2 days (Figure 4A). In accordance with this result, assessment of growth in liquid cultures revealed that the growth rate of

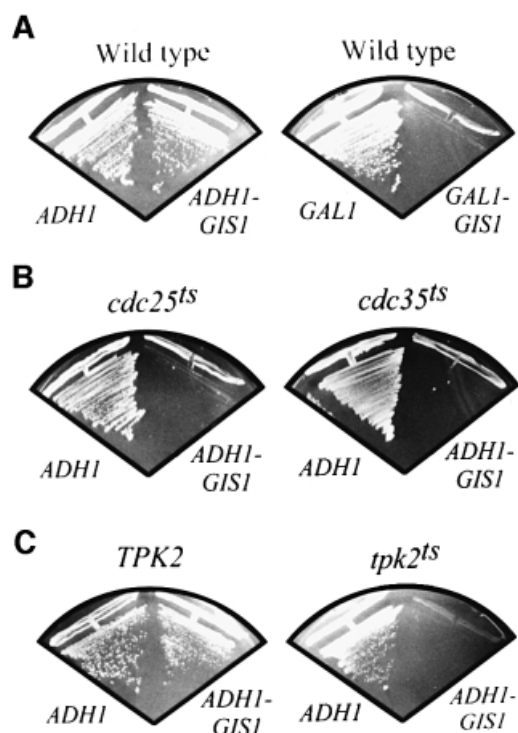


Fig. 4. *GIS1* overproduction inhibits growth of wild-type cells and exacerbates the growth defect of *cdc25^{ts}*, *cdc35^{ts}* and *tpk2^{ts}* strains. (A) Wild-type strain YEF473 was transformed with plasmids YCp*ADHI* (*ADHI*), YCp*ADHI-GIS1* (*ADHI-GIS1*), YCpIF2 (*GAL1*) and YCpIF2-*GIS1* (*GAL1-GIS1*), streaked on glucose- (*ADHI* plasmids) or galactose-containing (*GAL1* plasmids) selective plates and incubated for 3 days at 30°C. (B) Strains OL86 (*cdc25^{ts}*) and PD6517 (*cdc35^{ts}*) were transformed with plasmids YCp*ADHI* and YCp*ADHI-GIS1*, streaked on glucose-containing SD plates and incubated for 3 days at 27°C. (C) Strain SGY446 (*tpk2^{ts}*) and its isogenic wild-type strain ASY18 (*TPK2*) were transformed with plasmids YCp*ADHI* and YCp*ADHI-GIS1*, streaked on glucose-containing SD plates and incubated for 3 days at 27°C.

ADHI-GIS1-overexpressing wild-type cells was reduced by ~50% in comparison with control cells (data not shown). When transformed into *cdc25^{ts}* and *cdc35^{ts}* mutants, the *GIS1* overexpression plasmid YCp*ADHI-GIS1*, but not the corresponding control plasmid YCp*ADHI*, seriously compromised both mutants for growth even at the permissive temperature of 27°C (Figure 4B). Similarly, overexpression of *GIS1* from the *ADHI* promoter inhibited growth of *tpk2^{ts}* cells at the permissive temperature of 27°C, while it had little effect on the growth of isogenic *TPK2* wild-type cells. Thus, Gis1 overproduction partially inhibits growth in wild-type cells and exacerbates the growth defects of cAPK-compromised mutants.

To study further the effects of *GIS1* overexpression in wild-type cells, and to compare these effects with overexpression of *RIM15* and the *GIS1* homolog *RPH1*, strain YEF473 was transformed with YCp*ADHI*, YCp*ADHI-RIM15*, YCp*ADHI-GIS1* and YCp*ADHI-RPH1*, and analyzed for transcription of *SSA3*, *HSP12*, *HSP26*, *TPS2* and *SSB1* in exponentially growing cells. Transcript levels of *SSA3*, *HSP12* and *HSP26* were barely detectable in wild-type cells growing exponentially on glucose-containing medium, but were strongly induced following

overexpression of *GIS1* under the same conditions (Figure 5, lanes 1 and 3). Similarly, albeit to a significantly lower extent, overexpression of *RIM15* was found to cause transcriptional induction of *HSP12* and *HSP26*, while transcription of the same genes was not induced by overexpression of *RPH1* (Figure 5, lanes 1, 2 and 4). In contrast, the level of *TPS2* expression detected in exponentially growing cells remained largely unaffected by *GIS1* and *RIM15* overexpression, but was significantly reduced by overexpression of *RPH1*, suggesting that Rph1 may function as a transcriptional repressor of *TPS2* under these conditions (Figure 5, lane 4). Together, these results show that overexpression of *GIS1* allows derepression/activation of cAPK-repressible genes even under repressing conditions.

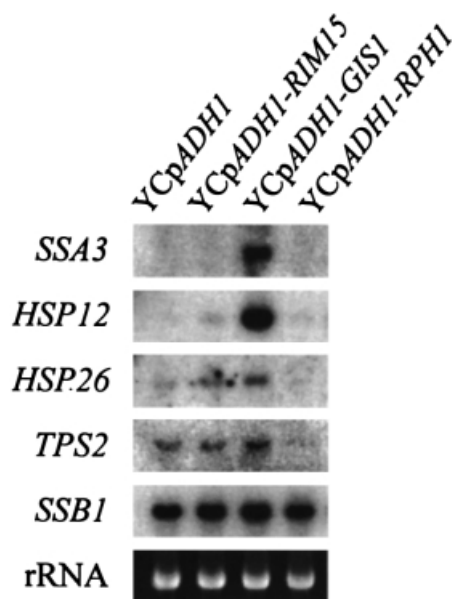


Fig. 5. Overexpression of *GIS1*, but not of *RPH1*, induces transcriptional activation of *SSA3*, *HSP12* and *HSP26*. Wild-type (YEF473) cells transformed with either YCpADH1, YCpADH1-*RIM15*, YCpADH1-*GIS1* or YCpADH1-*RPH1* were grown to exponential phase on SD media containing 2% glucose. Total RNAs from exponentially growing transformants were extracted and equal amounts of RNAs (10 μ g) were probed with *SSA3*, *HSP12*, *HSP26*, *TPS2* and *SSB1* fragments after electrophoresis and blotting. The application and transfer of equal amounts of RNA were verified by ethidium bromide staining.

Transcriptional activation of the PDS element, but not of STRE, is dependent on Gis1

The data presented above suggest that Gis1 functions as a transcriptional activator of PDS element- and/or STRE-controlled genes. Therefore, we next determined whether Gis1 acts as an activator of transcription using the one-hybrid technique. To this end, we fused the entire Gis1 to the LexA DNA-binding domain (DBD) in plasmid pEG202 (see Materials and methods). This fusion protein was able to activate transcription strongly from a promoter containing eight *lexA*-binding sites (Table II). This is in agreement with a previously reported observation that a partial Gis1 protein, lacking the C-terminal zinc finger domain, can activate transcription in a one-hybrid assay (Balciunas and Ronne, 1999). Interestingly, we were not able to detect transcriptional activity of a corresponding LexA-Rph1 fusion protein when assayed under the same conditions. Moreover, transcriptional activity of Gis1, measured by the one-hybrid assay, was equally strong in exponentially growing as in stationary phase wild-type cells, but slightly reduced in corresponding *rim15* Δ cells (Table II). These results show that Gis1 can act as a transcriptional activator that, under the conditions of the one-hybrid assay, is unresponsive to the nutritional status and only partially dependent on the presence of Rim15. These results may be explained if overexpression of *GIS1* from the strong *ADH1* promoter in pEG202 results in protein levels that exceed a certain threshold beyond which Gis1 becomes largely independent of activation by upstream regulatory proteins (see Discussion).

Gis1-dependent transcriptional activation may be mediated via STREs and/or PDS elements. To assess quantitatively the role of Gis1 for STRE- and PDS element-driven expression, nutrient limitation-induced expression of heterologous *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ* genes was tested in different strain backgrounds. As expected, nutrient limitation significantly induced both STRE- and PDS element-driven expression in wild-type cells (Figure 6A and B). The induction ratio (defined as the ratio of nutrient limitation-induced expression to basal level expression) of the *STRE-LEU2-lacZ* reporter was reduced slightly in *rim15* Δ cells (26.8% decrease), remained unchanged in *gis1* Δ and *rph1* Δ cells, and was strongly reduced in both *msn2 msn4* (89.8% decrease) and *msn2 msn4 gis1* Δ (94.1% decrease) cells when compared with the induction ratio in wild-type cells (Figure 6A). These results show that STRE-driven expression, while being strongly dependent on the presence of Msn2 and Msn4, is completely independent of

Table II. Transcriptional activation by LexA-Gis1

Expressed proteins	Wild type		<i>rim15</i> Δ	
	LOG	STAT	LOG	STAT
LexA	1.1 \pm 0.1	7.1 \pm 2.8	5.1 \pm 0.1	1.6 \pm 0.3
LexA-Gis1	1268.4 \pm 65.6	1157.0 \pm 167.1	981.9 \pm 256.7	922.3 \pm 25.0
LexA-Rph1	9.5 \pm 2.5	11.5 \pm 5.0	25.2 \pm 2.9	25.0 \pm 5.1

Proteins were expressed from plasmids pEG202-*GIS1* and pEG202-*RPH1* and from vector pEG202 (Zervos *et al.*, 1993). Target plasmid was pSH18-34 containing eight *LexA* operators (Gyuris *et al.*, 1993). Transformants of wild-type (AR1-1A) and *rim15* Δ (AR1-1C) cells were grown with selection for both plasmids. β -galactosidase activities were measured (as described in Miller, 1972) to monitor the induction of the *LexAops-lacZ* fusion gene (from plasmid pSH18-34). Values represent means \pm SDs of β -galactosidase activities (in Miller units) of three independent transformants.

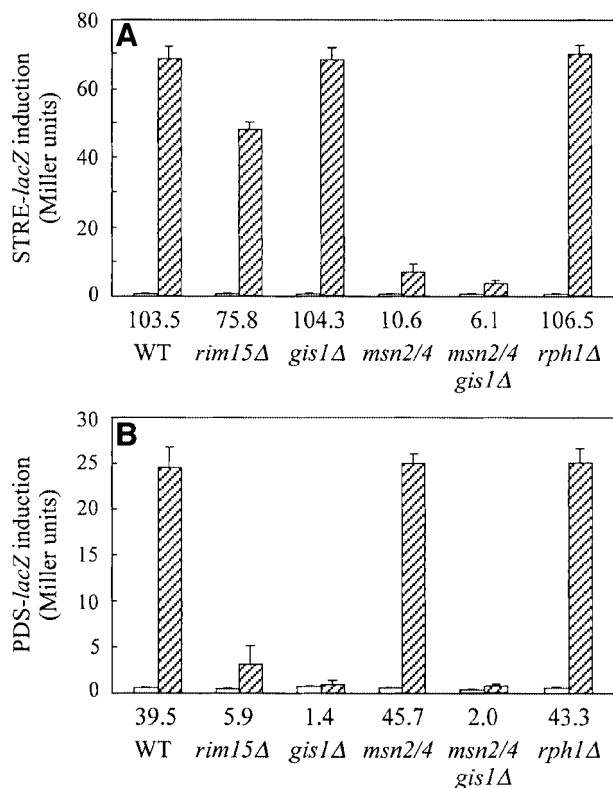


Fig. 6. Effects of *rim15Δ*, *gis1Δ*, *msn2 msn4*, *msn2 msn4 gis1Δ* and *rph1Δ* mutations on the basal level expression and nutrient limitation-induced expression of STRE- and PDS element-driven *LEU2-lacZ* reporter genes. Wild-type, *rim15Δ*, *gis1Δ*, *msn2 msn4*, *msn2 msn4 gis1Δ* and *rph1Δ* cells carrying a single chromosomally integrated STRE-*LEU2-lacZ* reporter gene (strains CDV120, CDV121, CDV122, CDV123, CDV124 and IP17, respectively) (A) or a corresponding PDS-*LEU2-lacZ* reporter gene (strains CDV125, CDV126, CDV127, CDV128, CDV129 and IP13, respectively) (B) were grown to exponential (open bars) and to early stationary phase (2 days; cross-hatched bars) on YPD medium. The chromosomal genotypes are indicated below the ordinate; the induction ratios following nutrient limitation are indicated above the chromosomal genotypes. β -galactosidase activities were measured (as described in Miller, 1972) to monitor the induction of the STRE-*LEU2-lacZ* and PDS-*LEU2-lacZ* fusion genes. Values represent means \pm SDs of β -galactosidase activities (in Miller units) of three independent experiments.

Gis1 and Rph1, and only partially dependent on Rim15. The induction ratio of the *PDS-LEU2-lacZ* reporter, in contrast, was strongly reduced in *rim15Δ* (85.1% decrease), *gis1Δ* (96.5% decrease) and *msn2 msn4 gis1Δ* (95.0% decrease) cells, while it remained virtually unchanged in *msn2 msn4* and *rph1Δ* cells when compared with the induction ratio in wild-type cells (Figure 6B). Thus, PDS element-driven expression is completely independent of Msn2, Msn4 and Rph1, strongly dependent on Rim15, and almost entirely dependent on the transcriptional activator Gis1.

Finally, STRE-driven basal level expression has previously been reported to be enhanced when cells were growing on rich medium containing ethanol instead of glucose as carbon source (Martínez-Pastor *et al.*, 1996). In this context, we found that expression from both the heterologous *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ* genes, while being very low in wild-type cells growing exponentially on glucose (0.5 ± 0.1 and 0.6 ± 0.1 Miller

units for *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ*, respectively), was significantly enhanced in cells growing exponentially on ethanol (30.0 ± 5.1 and 15.3 ± 3.8 Miller units for *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ*, respectively) or glycerol (46.8 ± 15.3 and 25.0 ± 7.8 Miller units for *STRE-LEU2-lacZ* and *PDS-LEU2-lacZ*, respectively). Thus, both STRE- and PDS element-driven expression are repressed by the presence of glucose.

Discussion

We have identified the zinc finger protein Gis1 in a screen for dosage-dependent suppressors of the *rim15Δ* defect in nutrient limitation-induced transcriptional derepression of *SSA3*. Our results are interpreted most simply in a model in which Gis1 functions in the Ras/cAMP pathway downstream of Rim15 to control transcription of a set of genes whose products are essential for long-term survival following nutrient limitation. Several observations support such a model. First, loss of Gis1 causes a defect in transcriptional derepression following nutrient limitation of various genes, including *SSA3*, whose expression is known to be negatively regulated by the Ras/cAMP pathway through Rim15. As a result, *gis1Δ* cells are seriously defective in induction of starvation resistance upon entry into stationary phase. Secondly, deletion of *GIS1* partially suppresses the growth defect at elevated temperatures of mutant cells that are compromised for cAPK activity (e.g. *cdc25^{ts}*, *cdc35^{ts}* and *tpk2^{ts}*). Thirdly, overexpression of *GIS1* suppresses the defect in transcriptional derepression of *SSA3* in nutrient-limited *rim15Δ* cells, induces transcription of cAPK-repressible genes (e.g. *SSA3*, *HSP12* and *HSP26*) in exponentially growing wild-type cells and exacerbates the growth defect of strains compromised for cAPK activity. Fourthly, expression driven by the PDS element, which has previously been shown to be negatively regulated by the Ras/cAMP pathway and which confers transcriptional activation following nutrient limitation at the diauxic transition and in stationary phase, is almost entirely dependent on the presence of Gis1.

It was reported recently that most of the growth-related effects of cAPK might be accounted for by Msn2/Msn4-controlled transcription (Smith *et al.*, 1998). In this context, our result showing that loss of Gis1 only partially suppresses the growth defect of cAPK-compromised mutant cells suggests that Gis1 may antagonize cAPK-dependent growth rather weakly. Nevertheless, overexpression of *GIS1*, in particular under conditions of presumably reduced activity of the Ras/cAMP pathway (e.g. on galactose-containing medium), was found to inhibit growth strongly. This indicates that one role for Gis1 is indeed to stimulate the expression of growth-inhibitory genes and that cAPK-dependent control of growth is the result of a coordinated regulation of various, different transcription factors. Accordingly, cAPK might regulate growth through its direct and/or indirect modulation of transcriptional activators including Msn2, Msn4 and Gis1, as well as by its action on transcriptional repressors such as, for instance, Sok2 (Ward *et al.*, 1995; Smith *et al.*, 1998). Interestingly, although there is as yet no evidence that cAPK phosphorylates Msn2 directly, recent results suggest that cAPK exerts its effect on Msn2-

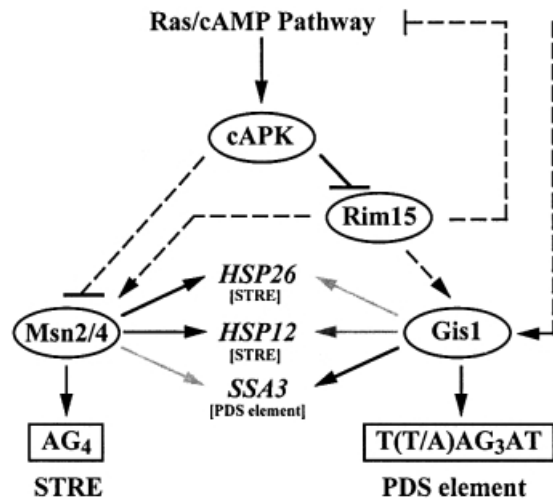


Fig. 7. Model for cAPK regulation of nutrient limitation-induced gene expression. The relative importance of Gis1 and Msn2/Msn4 for transcriptional activation of *HSP12*, *HSP26* and *SSA3* upon nutrient limitation is illustrated by the different shading (i.e. darker shading representing stronger dependence on the corresponding zinc finger protein; for studies of Msn2/Msn4-dependent transcription, see Martínez-Pastor *et al.*, 1996). Notably, *HSP12* and *HSP26* have several STREs in their promoter regions, while the *SSA3* promoter contains no conserved STRE, but several PDS elements (indicated in brackets). Arrows and bars denote positive and negative interactions, respectively. Dashed arrows and bars refer to potential interactions. For further details, see the text.

dependent gene expression by directly inhibiting Msn2 function (Görner *et al.*, 1998). In this context, our model posits that Gis1 acts downstream of a protein kinase, Rim15, which is negatively controlled by cAPK-dependent phosphorylation (Reinders *et al.*, 1998). One might predict, therefore, that Gis1 activity is modulated directly by Rim15-dependent phosphorylation (Figure 7). In accordance with such a model, we found that transcriptional activity of a LexA-Gis1 hybrid protein is partially dependent on the presence of Rim15. Nevertheless, both the one-hybrid assay and the *rim15Δ* suppression experiments indicate that Gis1 may become largely independent of Rim15 when overproduced from the strong constitutive *ADHI* or inducible *GALI* promoters. This effect may be due to the fact that *GIS1* overexpression yields protein levels that exceed a certain threshold value beyond which Gis1 becomes independent of upstream regulatory proteins. Alternatively, Gis1 may be controlled via additional Ras/cAMP/Rim15-independent mechanisms (Figure 7). Further studies with particular focus on the biochemical characterization of Gis1, which thus far has been hampered by excessive proteolysis of Gis1 (data not shown; see also Jang *et al.*, 1999), should allow a more detailed assessment of this issue.

Loss of Gis1 causes a defect in nutrient limitation-induced derepression/activation of *SSA3*, *HSP12* and *HSP26*. Thus, while Gis1 has recently been found to function as a damage-responsive repressor of *PHR1* (Jang *et al.*, 1999), we show here that it formally functions as an activator of *SSA3*, *HSP12* and *HSP26*. Such a dual role in transcriptional activation/repression is not uncommon amongst transcriptional regulators and may be based on specific protein-protein interactions, promoter sequence

contexts or physiological conditions (e.g. the repressor-activator Ume6; Krishnamurthy *et al.*, 1997; Sweet *et al.*, 1997). Notably, in this context, both our one-hybrid experiments and our overexpression studies suggest that Rph1, unlike its homolog Gis1, may function as a repressor of STRE- and PDS element-controlled genes. Together, these findings support the previous notion that Rph1 and Gis1, in addition to their common role in regulation of *PHR1* expression, may also exert distinct roles in transcriptional control (Balciunas and Ronne, 1999).

Interestingly, *in vitro* footprinting and binding competition studies indicated that Rph1 and Gis1 bind, like Msn2 and Msn4, to STRE-like sequences, suggesting that Gis1 and Msn2/Msn4 may overlap functionally *in vivo* (Treger *et al.*, 1998; Jang *et al.*, 1999). Surprisingly, however, we found that both *msn2 msn4* and *gis1Δ* mutants were defective specifically for STRE-driven and PDS element-driven expression, respectively. Thus, Msn2/Msn4 and Gis1 are not functionally equivalent *in vivo* and are likely to discriminate between the two very similar STRE and PDS promoter elements. Such a conclusion is supported further by our findings that *gis1Δ* mutants are seriously defective for nutrient limitation-induced transcriptional activation of the PDS element-controlled *SSA3* gene, while exhibiting only a minor defect in transcriptional activation of the STRE-controlled *HSP12* and *HSP26* genes under the same conditions. Nevertheless, the observed minor effect of loss of Gis1 on *HSP12* and *HSP26* expression suggests that Gis1 can, possibly in a proper promoter context, also mediate partial STRE-dependent activation. Such context specificity may be masked in heterologous promoter studies because of the absence of potential ancillary sequences that modulate STRE-driven transcription. Therefore, detailed reinvestigation of the *HSP12* and *HSP26* promoters in the context of the present results should allow us to understand the relationship between STREs and their dependence upon the Msn2, Msn4 and Gis1 transcriptional regulators following nutrient limitation.

A further interesting point with respect to the heterologous promoter studies is the finding that *rim15Δ* mutants were partially reduced for nutrient limitation-induced activation of STRE-*lacZ*. One explanation for this result may be that Rim15 is involved in Gis1-independent, possibly Msn2/Msn4-dependent, control of STRE. Alternatively, Rim15 could, as suggested previously (Reinders *et al.*, 1998), be involved in mechanisms of feedback inhibition of the Ras/cAMP pathway. In this scenario, loss of Rim15 could cause overactivation of the Ras/cAMP pathway and, consequently, inhibition of Msn2/4 function. Notably, both explanations are consistent with our model that Rim15 acts upstream of Gis1 and may overall, therefore, cause a more serious physiological defect than loss of Gis1 (Figure 7).

A final issue that remains to be addressed is that Gis1 has previously been implicated in a mechanism of positive regulation of the Ras/cAMP pathway (Balciunas and Ronne, 1999). Accordingly, and at variance with the results presented here, the authors found that overexpression of *GIS1* suppressed a *cdc25-5^{ts}* mutation. It must be noted, however, that the experimental set-up in that report differed in at least two important aspects from our studies.

Table III. List of yeast strains

Strain	Genotype	Source	
YEF473	<i>MATa/α</i>	<i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3</i>	Bi and Pringle (1996)
AR1	<i>MATa/α</i>	<i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/RIM15</i>	Reinders <i>et al.</i> (1998)
AR1-1A	<i>MATa</i>	<i>his3 leu2 lys2 trp1 ura3</i>	segregant from AR1
AR1-1C	<i>MATa</i>	<i>his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2</i>	segregant from AR1
AR2	<i>MATa/α</i>	<i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rim15Δ::kanMX2/rim15Δ::kanMX2</i>	Reinders <i>et al.</i> (1998)
OL86	<i>MATα</i>	<i>leu2 trp1 ade2 cdc25-5</i>	Camonis <i>et al.</i> (1986)
PD6517	<i>MATα</i>	<i>ade8 leu2 trp1 cdc35-10</i>	Becher dos Passos <i>et al.</i> (1992)
SGY446	<i>MATα</i>	<i>tpk1Δ::ADE8 tpk2-63(Ts) tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8</i>	Smith <i>et al.</i> (1998)
ASY18	<i>MATα</i>	<i>tpk1Δ::ADE8 TPK2 tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8</i>	Smith <i>et al.</i> (1998)
NB19	<i>MATα</i>	<i>leu2 trp1 ade2 cdc25-5 gis1Δ::kanMX2</i>	this study
NB21	<i>MATα</i>	<i>ade8 leu2 trp1 cdc35-10 gis1Δ::kanMX2</i>	this study
NB23	<i>MATα</i>	<i>tpk1Δ::ADE8 tpk2-63(Ts) tpk3::TRP1 ura3-52 his3 leu2-3,112 trp1 ade8 gis1Δ::kanMX2</i>	this study
IP6	<i>MATa/α</i>	<i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/GIS1 rim15Δ::kanMX2/RIM15</i>	this study
CDV100	<i>MATa/α</i>	<i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/GIS1 rim15Δ::kanMX2/RIM15 rph1Δ::TRP1/RPH1</i>	this study
CDV100-10D	<i>MATa</i>	<i>his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3</i>	segregant from CDV100
CDV100-4D	<i>MATα</i>	<i>his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3</i>	segregant from CDV100
CDV100-6C	<i>MATa</i>	<i>his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3 rim15Δ::kanMX2</i>	segregant from CDV100
CDV100-10A	<i>MATα</i>	<i>his3 leu2 lys2 trp1 ura3 gis1Δ::HIS3 rim15Δ::kanMX2</i>	segregant from CDV100
CDV101	<i>MATa/α</i>	<i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/gis1Δ::HIS3</i>	CDV100-10D × CDV100-4D
CDV104	<i>MATa/α</i>	<i>his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 gis1Δ::HIS3/gis1Δ::HIS3 rim15Δ::kanMX2/rim15Δ::kanMX2</i>	CDV100-6C × CDV100-10A
NB14	<i>MATa</i>	<i>his3 leu2 lys2 trp1 ura3 GIS1-Myc13:TRP1</i>	this study
NB15	<i>MATa</i>	<i>his3 leu2 lys2 trp1 ura3 rim15Δ::kanMX2 GIS1-Myc13:TRP1</i>	this study
W303-1A	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Thomas and Rothstein (1989)
Wmsn2msn4	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 msn2-Δ3::HIS3 msn4-1::TRP1</i>	Martínez-Pastor <i>et al.</i> (1996)
CDV120	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ</i>	this study
CDV121	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ rim15Δ::kanMX2</i>	this study
CDV122	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ gis1Δ::kanMX2</i>	this study
CDV123	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ msn2-Δ3::HIS3 msn4-1::TRP1</i>	this study
CDV124	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ msn2-Δ3::HIS3 msn4-1::TRP1 gis1Δ::kanMX2</i>	this study
IP17	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::STRE-lacZ rph1Δ::TRP1</i>	this study
CDV125	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ</i>	this study
CDV126	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ rim15Δ::kanMX2</i>	this study
CDV127	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ gis1Δ::kanMX2</i>	this study
CDV128	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ msn2-Δ3::HIS3 msn4-1::TRP1</i>	this study
CDV129	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ msn2-Δ3::HIS3 msn4-1::TRP1 gis1Δ::kanMX2</i>	this study
IP13	<i>MATa</i>	<i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 URA3::PDS-lacZ rph1Δ::TRP1</i>	this study

First, the *cdc25-5^{ts}* mutant under study, in contrast to the one used here, harbored an additional mutation in the *PDE2* gene whose product, phosphodiesterase, negatively regulates Ras/cAMP signaling (Sass *et al.*, 1986). Secondly, overexpression of *GIS1* was achieved by expression of *GIS1* under its own promoter from a 2 μ plasmid. Presumably, this may yield much lower Gis1 protein levels than expression of *GIS1* from the strong *ADHI* and *GALI* promoters, as done in the present study. One might speculate, therefore, that moderate overproduction of Gis1 in a *cdc25-5^{ts} pde2* mutant, while not reaching the threshold level for growth inhibition, causes transcriptional activation of one or more genes that act as specific suppressors of the *cdc25-5^{ts}* mutation. One such candidate gene encodes Tfs1, which, when overproduced, shows allele-specific suppression of *cdc25-1^{ts}* and *cdc25-5^{ts}* (Robinson and Tatchell, 1991). Intriguingly, we found the *TFS1* gene to contain a PDS element consensus site at position -136 in its promoter region, which may confer the reported induction upon nutrient limitation via the transcriptional activator Gis1 (DeRisi *et al.*, 1997). Despite the admittedly speculative nature of this model, it may reconcile the obviously contradictory observations illustrated above, and leads to several predictions that can be tested in future studies.

Materials and methods

Strains, media, and microbiological and recombinant DNA methods

The *S. cerevisiae* strains used in this study are listed in Table III. To construct *gis1 Δ* , *rph1 Δ* and *rim15 Δ* mutants, the complete *GIS1*, *RPH1* and *RIM15* coding regions were deleted by the PCR method (Baudin *et al.*, 1993) using the Expand™ High Fidelity PCR System (Roche Diagnostics) and either plasmid pRS303 (*HIS3*; Sikorski and Hieter, 1989), pRS304 (*TRP1*; Sikorski and Hieter, 1989) or pFA6a (*kanMX2*; Wach *et al.*, 1994) as template. The PCR products that contained flanking sequences of *GIS1*, *RPH1* and *RIM15* separated by the *HIS3*, *TRP1* or *kanMX2* modules were extracted with phenol/chloroform, precipitated and used for transformations. The *gis1 Δ ::HIS3* deletion cassette was transformed into strain AR2 to construct IP6; the *gis1 Δ ::kanMX2* deletion cassette was transformed into strains OL86, PD6517 and SGY446 to construct NB19, NB21 and NB23, respectively; and the *rph1 Δ ::TRP1* deletion cassette was transformed into strain IP6 to construct CDV100. Strains CDV101 and CDV104 were constructed by crossing haploid segregants from CDV100 as follows: CDV100-10D \times CDV100-4D (CDV101) and CDV100-6C \times CDV100-10A (CDV104).

Linearized, *NcoI*-cut integrative vectors pCTT1-18/7x and pLS9-PDS were transformed into strain W303-1A to construct CDV120 and CDV125, respectively, co-transformed with the *rim15 Δ ::kanMX2* deletion cassette into strain W303-1A to construct CDV121 and CDV126, respectively, co-transformed with the *gis1 Δ ::kanMX2* deletion cassette into strain W303-1A to construct CDV122 and CDV127, respectively, transformed into strain Wmsn2msn4 to construct CDV123 and CDV128, respectively, co-transformed with the *gis1 Δ ::kanMX2* deletion cassette into strain Wmsn2msn4 to construct CDV124 and CDV129, and co-transformed with the *rph1 Δ ::TRP1* deletion cassette into strain W303-1A to construct IP17 and IP13, respectively.

Transformants that had *GIS1* replaced by *gis1 Δ ::HIS3* or *gis1 Δ ::kanMX2*, *RPH1* replaced by *rph1 Δ ::TRP1*, or *RIM15* replaced by *rim15 Δ ::kanMX2* were confirmed by PCR and/or Southern blot analysis (data not shown). NB14 and NB15 were generated by PCR-based, chromosomal *myc13* tagging of *GIS1* in AR1-1A and AR1-1C, respectively, using pFA6a-13Myc-TRP1 as template (Longtine *et al.*, 1998).

Plasmid manipulations were performed in *Escherichia coli* strain DH5 α (Gibco-BRL) using standard procedures (Sambrook *et al.*, 1989). Yeast and *E. coli* media, including the rich, glucose-containing medium (YPD), the defined media (SD with appropriate supplements) and sporulation medium, were prepared by standard recipes (Sambrook *et al.*,

1989; Rose *et al.*, 1990). Standard procedures of yeast genetics and molecular biology were used (Sambrook *et al.*, 1989; Guthrie and Fink, 1991). Yeast transformations were performed using a modification of the Li⁺ ion method (Gietz *et al.*, 1992). Sporulation experiments were performed essentially as described (Reinders *et al.*, 1998).

Plasmid constructions

For construction of the galactose-inducible *GALI-GIS1* and *GALI-RPH1* alleles, the full-length *GIS1* and *RPH1* coding sequences were amplified using the Expand Long Template PCR System (Boehringer Mannheim) and genomic DNA of strain AR2 (Table III) as template. *SalI* restriction sites were introduced immediately upstream of both ATG start codons, and *NotI* restriction sites were introduced 227 and 220 bp downstream of the *GIS1* and *RPH1* stop codons, respectively. The PCR products were cloned at the *SalI-NotI* sites of YCpIF2 (Foreman and Davis, 1994) to yield YCpIF2-*GIS1* and YCpIF2-*RPH1*. Plasmids YCpADHI-*GIS1* and YCpADHI-*RPH1* were constructed by replacing the *GALI* promoter-containing *ApaI-SalI* fragments of YCpIF2-*GIS1* and YCpIF2-*RPH1*, respectively, with a PCR-generated *ApaI-SalI* fragment containing the 854 nucleotides upstream of and including the *ADHI* start codon. Plasmids YCpIF2-*RIM15*, YCpADHI and YCpADHI-*RIM15* were described earlier (Reinders *et al.*, 1998). To fuse Gis1 and Rph1 to the LexA DBD coding sequence in plasmid pEG202 (Zervos *et al.*, 1993), *GIS1* and *RPH1* full-length coding sequences were amplified as described above and cloned at the *SalI-NotI* sites of a modified version of plasmid pEG202 that contains a unique *SalI* site in its polylinker. The constructs contain four (EFVD) additional amino acids between the LexA DBD and the first amino acid (M) of the fused protein. Plasmid pLS9-PDS was constructed by cloning a synthetic double-stranded oligonucleotide consisting of 37 bp identical to the SSA3-PDS region (-206 to -170), plus AATT overhangs and an additional *HindIII* restriction site upstream of the SSA3-PDS region, into the *EcoRI* site of pLS9 (Sarokin and Carlson, 1986). Plasmid pCTT1-18/7x has been described in Marchler *et al.* (1993).

Preparation and northern analysis of RNA

Extraction of total cellular RNA was performed as described previously (Piper, 1994). For northern analysis, 10 μ g of total RNA were separated on 1.1% agarose gels containing 0.65 M formaldehyde, transferred to nitrocellulose membranes (Protran BA 83, 0.2 μ m; Schleicher and Schuell, Germany) in 20 \times SSC and hybridized with [³²P]dATP-labeled DNA fragments that were amplified by PCR from genomic DNA (of strain AR2; Table III) and labeled by using the Prime-It Random Primer Labeling Kit (Stratagene). The primers used for PCR to generate SSA3, *HSP12*, *HSP12* and *SSB1* DNA fragments have been described in Reinders *et al.* (1998); those used to generate the 2.78 kb TSP2 DNA fragment were as follows: 5'-ACCACTGCCCCGGGCAATTCTCCA-AAGAAGAGACAG-3' (forward) and 5'-ACTATTATATATATGCCCCGGGTCGCAACACCACATA-3' (reverse).

Protein analyses

Protein concentrations were measured either by the Bio-Rad protein assay according to the manufacturer's instructions or by a modified Lowry assay (Peterson, 1977) using bovine serum albumin (BSA) as standard. For immunodetection of Gis1-myc13, total cellular proteins were extracted by vortexing cells in lysis buffer [0.2 M Tricine (Na⁺) pH 7.0, and one tablet of complete protease inhibitor cocktail (Boehringer Mannheim) per 50 ml] in the presence of acid-washed glass beads (0.4 mm diameter; Merck), run on SDS-polyacrylamide gels (10% w/v acrylamide) and transferred electrophoretically to nitrocellulose membranes as described previously (Reinders *et al.*, 1999). Blots were stained with Ponceau S to ensure equal protein loading. The membranes were incubated with 1:5000 diluted monoclonal mouse anti-myc (MIgG₁) antibodies (Invitrogen), followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG secondary antibodies (Biosource, USA). Reactive bands were visualized by an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Fluka, Switzerland).

Acknowledgements

We thank P.Estruch, H.Ronne and S.Garrett for strains, and E.A.Craig, M.Carlsen and H.Ruis for plasmids. We are grateful to D.Balciunas, T.Boller and A.Wiemken for critically reading the manuscript. This work was supported by the Swiss National Science Foundation, grant 3100-052245.97/1 to C.D.V.

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Received February 22, 2000; revised and accepted April 11, 2000