

## Gene overexpression reveals alternative mechanisms that induce *GCN4* mRNA translation

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### Abstract

The *Saccharomyces cerevisiae GCN4* gene which encodes the transcription activator Gcn4, is under translational regulation. Derepression of *GCN4* mRNA translation is mediated by the Gcn2 protein kinase which phosphorylates the  $\alpha$  subunit of eIF-2, upon amino-acid starvation. Here, we report that overexpression of certain *Saccharomyces cerevisiae* genes generates intracellular conditions that alleviate the requirement for a functional Gcn2 kinase to induce *GCN4* mRNA translation. Our findings, combined with the fact that Gcn2 kinase is dispensable during the initiation phase of the cellular response to amino-acid limitation, provide the grounds to further elucidate the mechanisms underlying the physiology of this homeostatic response.

**Keywords:** Amino-acid starvation; eIF-2; *GCN2*; *NME1*; Phosphorylation; *TPK2*; tRNA

### 1. Introduction

*Saccharomyces cerevisiae* copes with extracellular amino-acid limitation by inducing the transcription of genes coding for amino-acid biosynthetic enzymes through the action of the dedicated, Gcn4 transcription activator (reviewed by Jones and Fink, 1985). Amino-acid starvation signals converge to the modulation of the synthesis of Gcn4, that occurs at the level of translation of *GCN4* mRNA (reviewed by Hinnebusch, 1990).

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Abbreviations: arg, arginine; 3-AT, 3-amino 1,2,4-triazole; cAMP, cyclic adenosine 3',5'-monophosphate;  $\Delta$ , deletion; eIF-2, eukaryotic initiation factor-2; gly, glycine; *GCN2*, gene encoding Gcn2 protein kinase; *GCN4*, gene encoding Gcn4 transcription activator; *HIS3*, gene encoding imidazole-glycerol-phosphate dehydratase; Min, minimal growth medium; MRP, mitochondrial RNA processing ribonuclease; *NME1*, gene encoding the RNA component of MRP ribonuclease; PKA, protein kinase A; ser, serine; *SUI2*, gene encoding eIF-2 $\alpha$ ; *SUI3*, gene encoding eIF-2 $\beta$ ; *TPK2*, gene encoding the Tpk2 protein kinase A; val, valine; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside.

An additional consequence of this response, is a decline in the general protein synthesis during the initial phase of the response and a concomitant reduction in the consumption of amino acids (Tzamarias et al., 1989; Williams et al., 1989).

Under steady conditions, the key molecule that mediates this dual adjustment, is the Gcn2 protein kinase that upon depletion of amino acids, phosphorylates the  $\alpha$  subunit of eIF-2 at ser-51 (Dever et al., 1992). This modification inhibits initiation of polypeptide chain synthesis, but favours productive translation of the *GCN4* message which is translationally inert under normal conditions (Mueller and Hinnebusch, 1986; Tzamarias et al., 1986). Apart from this mechanism yeast cells, immediately after withdrawal of a single amino acid from the culture medium, display an acute and transient increase in *GCN4* mRNA translation, coupled with an extensive protein synthesis shut down. However, both phenomena are independent of Gcn2 function (Tzamarias et al., 1989). In addition, the assimilation of *GCN4* mRNA translational derepression in vitro, does not require the Gcn2 kinase (Krupitza and Thireos, 1990).

Since the coordinated, acute response involves activation of operator(s) different from Gcn2, we searched for

such molecules by isolating genes capable of suppressing *gcn2* null mutations, when overexpressed. The rationale supporting this approach, was that it should be possible to mimic activation of a molecule by overexpressing the corresponding gene (Kanazawa et al., 1988; Roussou et al., 1988; Wek et al., 1992). Here we demonstrate that overexpression of certain *Saccharomyces cerevisiae* genes, overcomes the requirement for a functional Gcn2 kinase to induce *GCN4* mRNA translation and that phosphorylation of eIF-2 $\alpha$  at ser-51 is not the sole regulatory event underlying amino-acid homeostasis. Rather, alternative mechanisms are employed. We suggest that these might account for the Gcn2-independent situation that operates during the initial phase of the physiological rehabilitation to amino-acid limitation conditions.

## 2. Experimental and discussion

### 2.1. Isolation of high-copy suppressor genes of a *gcn2* null mutation

To identify genes involved in the stimulation of *GCN4* mRNA translation in the absence of the Gcn2 kinase, we conducted a genetic screen aiming to suppress the effects of a *GCN2* deletion by overexpression of yeast genes. A genomic library derived from a *gcn2* deleted strain (*gcn2 $\Delta$* ), was constructed in the high-copy episomal vector YEp352 and introduced into a *gcn2 $\Delta$*  stain, carrying a *GCN4-lacZ* reporter gene. The resulting transformants (approximately 20,000) were screened for resistance to 10 mM 3-AT which is a competitive inhibitor of Imidazole-Glycerol-Phosphate Dehydratase, the product of the *HIS3* gene (Klopotoski and Wiater, 1965). *gcn* strains fail to survive histidine starvation imposed by this selection, due to their inability to induce translation of *GCN4* mRNA and consequently transcription of the *HIS3* gene (Driscoll Penn et al., 1983). Out of the 20,000 initial transformants, 37 were resistant to 3-AT and in 24 of those, this resistance was accompanied by induction of *GCN4* expression, as evidenced by the blue colony colour on XGal plates. Application of this double selection strategy enabled to discard genes irrelevant to *GCN4* mRNA translational derepression such as those involved in the detoxification of the cell from 3-AT (Kanazawa et al., 1988).

Following rescue and retransformation, in 19 instances both phenotypes (resistance and colour) co-segregated with the library plasmid. Using restriction analysis, the plasmids were grouped into six categories. Partial sequencing of the relevant DNA fragments of representative plasmids from each category, revealed the isolation of six previously cloned genes. These were: (1) the *SUP61* gene encoding a tRNA<sup>ser</sup> (UCA) (Baker et al., 1982), (2) a tRNA<sup>val\*</sup> (AAC) pseudo-gene harbouring an A-to-G transition at the extreme 3' nucleotide, previously shown

to interfere with the *GCN4* mRNA translation when overexpressed (Vazquez de Aldana et al., 1994), (3) the *NME1* gene coding for the RNA component of the MRP ribonuclease (Schmitt and Clayton, 1992), (4) the *SUI3* gene for the  $\beta$  subunit of eIF-2 (Donahue et al., 1988), (5) a truncated derivative (*sui2- $\Delta$ C*) of the *SUI2* gene encoding the  $\alpha$  subunit of eIF-2 (Cigan et al., 1989), which was generated during the library construction procedure, and finally, (6) the *TPK2* gene, one of the three yeast genes for the cAMP regulated, catalytic subunit of PKA (Toda et al., 1987).

The growth properties of yeast strains harbouring these plasmids are summarised in Table 1A. None of the above genes affected transcription of the *GCN4* gene, as assessed by employing a reporter gene (*DORFGCN4-lacZ*; Tzamarias et al., 1986) driven by the *GCN4* promoter (Table 2). Furthermore, the steady-state *GCN4* mRNA levels were not altered under the same conditions indicating that changes in the stability of the *GCN4* message are not involved in establishing the observed effects on *GCN4* expression (Fig. 1).

### 2.2. Excess amounts of certain tRNAs and the *NME1* small RNA, interfere with *GCN4* expression

Overproduction of a tRNA<sup>ser</sup> (UCA), a pseudo-tRNA<sup>val\*</sup> (AAC), and the small RNA component of ribonuclease MRP, stimulated *GCN4* mRNA translation 3, 4 and 5-fold, respectively, in strains deleted for the *GCN2* gene, irrespective of nutritional conditions (Fig. 2A and B and Fig. 3). The physiological substrate for the Gcn2 enzyme is the  $\alpha$  subunit of eIF-2. In the absence of this enzyme, a yet unidentified additional eIF-2 kinase, might be triggered, that would account for the observed effects. To exploit this possibility, we introduced the plasmids in strains carrying a mutation in the *SUI2* gene, that replaces ser-51 with alanine (*sui2-S51A*). This substitution has been shown to prevent regulatory phosphorylation of the  $\alpha$  subunit (Dever et al., 1992). As shown in Fig. 2A and B and Fig. 3, the effects of overexpression persisted in these strains, a fact demonstrating that they are independent of eIF-2 $\alpha$  phosphorylation at ser-51. We next sought to determine whether overexpression of other tRNA genes interferes with *GCN4* mRNA translation. Upon similar examination of the wt genes for the tRNA<sup>val</sup> (AAC), tRNA<sup>arg</sup> (ACG) and tRNA<sup>gly</sup> (UCC) (Baker et al., 1982), no effect was observed under a comparable extent of overexpression (Fig. 2A and B). In all instances the growth properties of the transformed strains paralleled the levels of *GCN4* expression (Table 1A and B). The levels of expression for these three tRNAs were comparable to those of the suppressor tRNA<sup>val\*</sup> and tRNA<sup>ser</sup>, as shown in Fig. 1B. Therefore, suppressor activity is not a general property of all tRNAs but rather a characteristic of a defined tRNA set. In accordance, a wt tRNA<sup>his</sup>, reported to

Table 1  
Plate growth assays of wt, *gcn2Δ* and *sui2-S51A* yeast strains transformed with the indicated overexpressing constructs

	Nutritional condition <sup>b</sup> :	Growth <sup>a</sup>					
		Min			3-AT		
		wt	<i>gcn2Δ</i>	<i>sui2-S51A</i>	wt	<i>gcn2Δ</i>	<i>sui2-S51A</i>
A	Strain <sup>c</sup> : Overexpressed gene <sup>d</sup>						
	tRNA <sup>ser</sup>	+++	+++	+++	+++	+	+
	tRNA <sup>val*</sup>	+++	+++	+++	+++	++	++
	<i>NME1</i>	++	++	++	++	++	++
	<i>SUI3</i>	+++	+++	+++	+++	++	++
	<i>sui2-ΔC</i>	+++	+++	+++	+++	++	++
	<i>TPK2</i>	+	+	+++	+	+	–
	vector	+++	+++	+++	+++	–	–
B	tRNA <sup>val</sup>	+++	+++	+++	+++	–	–
	<i>SUI2</i>	+++	+++	+++	++	–	++

<sup>a</sup> +++ indicates normal growth, – indicates no growth, + or ++ indicate different strengths of leaky growth. Plates were scored after incubation for 3 days at 30°C.

<sup>b</sup> Min is *S. cerevisiae* minimal medium that does not induce a response of the general control of amino-acid biosynthesis. 3-AT is Min supplemented with 10 mM 3-amino-1,2,4-triazole, which simulates amino-acid limitation.

<sup>c</sup> wt is *leu2-112, ura3-52 S288C* derivative complemented with plasmid pRS315[*GCN4-lacZ*] and the indicated overexpressing constructs. *gcn2Δ* and *sui2-S51A* strains carry a deletion of the *GCN2* gene (Roussou et al., 1988) and a mutation in the *SUI2* gene replacing serine-51 of eIF-2 with alanine (Dever et al., 1992), respectively. These strains are otherwise isogenic to the wt strain.

<sup>d</sup> (A) Overexpression of the six isolated genes. Growth of strains transformed with only the high-copy (vector) plasmid, are also depicted, for control. (B) Effects of the wild type tRNA<sup>val</sup> and *SUI2* genes. Standard protocols were used for the manipulation of DNA molecules and yeast strains (Ausubel et al., 1987).

Table 2  
The transcription of *GCN4* is not affected by overexpression of genes that suppress of *gcn2* deletion

	Nutritional condition <sup>b</sup> :	β-Galactosidase units <sup>a</sup>			
		Min		3-AT	
		wt	<i>gcn2Δ</i>	wt	<i>gcn2Δ</i>
A	Strain <sup>c</sup> : Overexpressed gene <sup>d</sup>				
	tRNA <sup>ser</sup>	159.6	164.1	143.2	157.6
	tRNA <sup>val*</sup>	155.3	158.6	148.8	149.1
	<i>NME1</i>	151.8	153.2	151.7	153.0
	<i>SUI3</i>	162.3	167.8	156.9	158.2
	<i>sui2-ΔC</i>	150.9	155.4	149.0	154.3
	<i>TPK2</i>	143.8	148.2	138.2	136.2
	vector	157.7	160.8	148.5	157.4
B	tRNA <sup>val</sup>	148.3	161.7	139.6	149.6
	<i>SUI2</i>	151.7	164.6	147.8	158.4

<sup>a</sup> β-Galactosidase assays were done as described (Tzamarias et al., 1986). Values are Miller units and represent the average of three independent experiments with less than 10% deviation.

<sup>b</sup> Culture conditions were as described in Table 1.

<sup>c</sup> wt is a *leu2-112, ura3-52 S288C* derivative complemented with plasmid pRS315[*ΔORFGCN4-lacZ*] (Tzamarias et al., 1986) and the indicated overexpressing constructs. *gcn2Δ* carries a deletion of the *GCN2* gene (Roussou et al., 1988) and is otherwise isogenic to the wt strain.

<sup>d</sup> (A) Effect of overexpression of the six isolated genes on the transcription of *GCN4*. (B) Examination of the same effects for the wt tRNA<sup>val</sup> and *SUI2* genes.

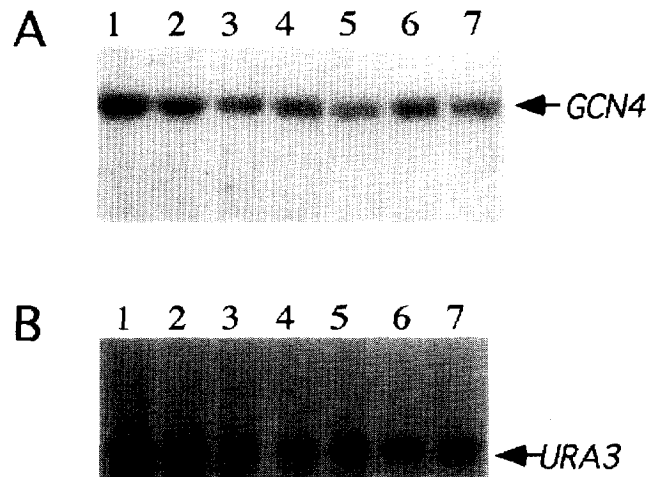


Fig. 1. Accumulation of *GCN4* message is not affected by overexpression of genes isolated as high-copy suppressors of *gcn2*. (A) *GCN4* mRNA levels of *Dgcn2* strains transformed with overexpressing constructs bearing genes tRNA<sup>ser</sup>, tRNA<sup>val\*</sup>, *NME1*, *SUI3*, *sui2-ΔC*, *TPK2* and library vector serving as control (lanes 1–7, respectively). Similar analysis of a wt strain harbouring the above plasmids did not reveal any difference in *GCN4* mRNA levels (not shown). (B) *URA3* was utilised as control for the amount of RNA subjected to northern analysis (lanes 1–7 as in A). Northern analysis was performed as described by Ausubel et al. (1987).

induce *GCN4* translation in a manner mostly *Gcn2*-dependent (Vazquez de Aldana et al., 1994), was not isolated in our screenings.

The *gcn2Δ* suppressing function of excessive amounts

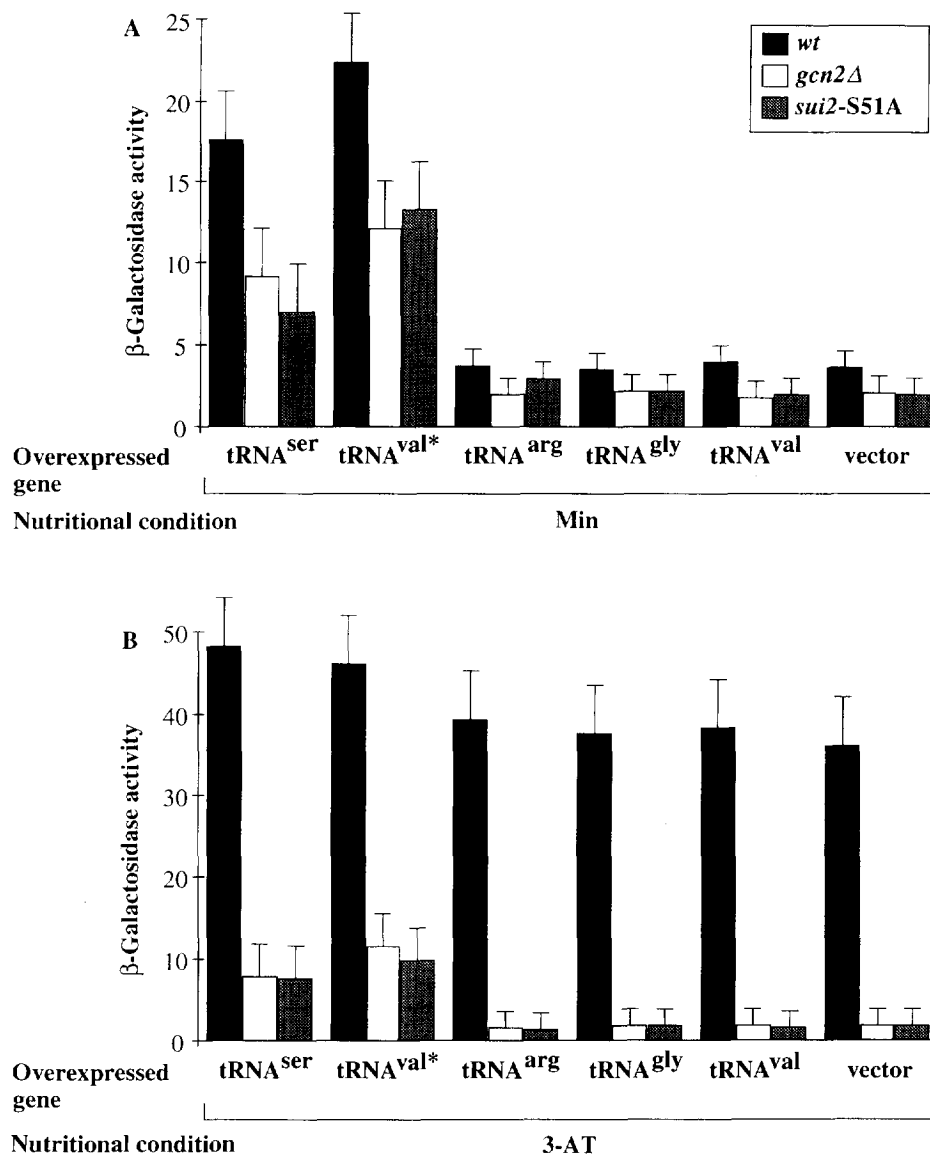


Fig. 2. Graphic representation of the effects of overproduction of the indicated tRNAs, on the translation of *GCN4* mRNA, compared to the vector alone (vector). Genetic backgrounds have been described in the footnote to Table 1. Cells were co-transformed with a centromeric plasmid (pRS315) carrying the *GCN4-lacZ* reporter gene (Tzamarias et al., 1986) and a high-copy plasmid (YEp352) harbouring each tRNA gene. 10 mM 3-AT were used to impose histidine starvation (shown in **B**), while Min maintained repressing *GCN4* mRNA translation, nutritional conditions (shown in **A**).  $\beta$ -Galactosidase assays were done as described (Tzamarias et al., 1986). Values are Miller units and represent the average of three independent experiments with less than 10% deviation.

of specific tRNAs could be attributed to limited aminoacylation by their cognate aminoacyl-tRNA synthetases (Normanly and Abelson, 1989). In agreement with this, the pseudo-tRNA<sup>val\*</sup> could not be aminoacylated in vivo (D.A., unpublished observations). Uncharged tRNAs might then act as a secondary, Gcn2 bypassing starvation signal that directly modifies the protein synthesis apparatus in a still unknown manner. A consequence of the affected expression of the *NME1* gene, is the improper maturation of the 5.8S ribosomal RNA (Chu et al., 1994). Therefore, overexpression of this gene

could impair aspects of ribosomal biogenesis, a process known to affect translational initiation (Rotenberg et al., 1988), thus favouring *GCN4* mRNA translation.

### 2.3. Overexpression of wt and mutant subunits of eIF-2 stimulates Gcn4 synthesis

Elevated expression of the *SUI3* gene encoding the  $\beta$  subunit of eIF-2, permitted growth of yeast cells under histidine starvation conditions, in the absence of Gcn2 (Table 1A). This effect was due to the derepression of

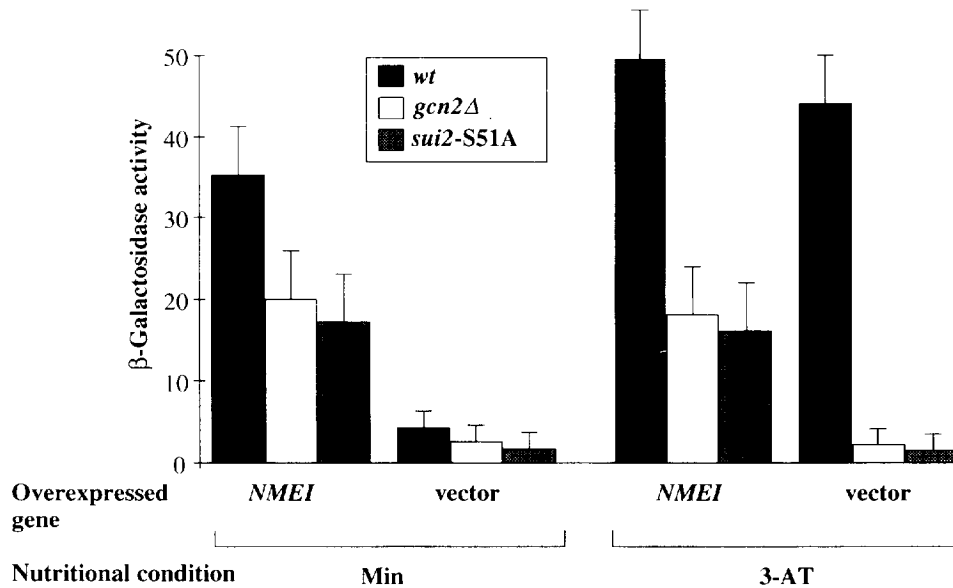


Fig. 3. Graphic representation of the effects of overexpression of the *NME1* gene on *GCN4* mRNA translation, in the three genetic backgrounds indicated, compared to the vector alone (vector). β-Galactosidase assays were carried out as described in the legend to Fig. 2 and under the same nutritional conditions. Values are Miller units and represent the average of three independent experiments with less than 10% deviation.

*GCN4* mRNA translation which occurred independently of phosphorylation of eIF-2α at ser-51 (Fig. 4). In addition, overexpression of a truncated derivative of the *sui2* gene that codes for eIF-2α (*sui2-ΔC*), imposed a similar effect on *GCN4* mRNA translation (Table 1A and Fig. 4). This clone lacks the carboxy-terminal amino acids 293 to 305, due to ligation of the *Bgl*II site within the *SUI2* gene (Cigan et al., 1989) directly into the *Bam*HI site of the library vector. Strains in which the wt *SUI2* gene has been replaced by *sui2-ΔC* were viable

and exhibited the same phenotypes as the corresponding, *sui2-ΔC* overexpressing ones, indicating that eIF-2α molecules carrying such a deletion are not totally incapacitated (not shown).

By contrast, oversynthesis of the wt eIF-2α subunit, did not reproduce the effects of the truncated derivative but it prevented *GCN4* mRNA translational derepression to full extent, in wt yeast cells (Fig. 4). As a result, the growth of these cells, under starvation conditions, was partially impaired (shown in Table 1B).

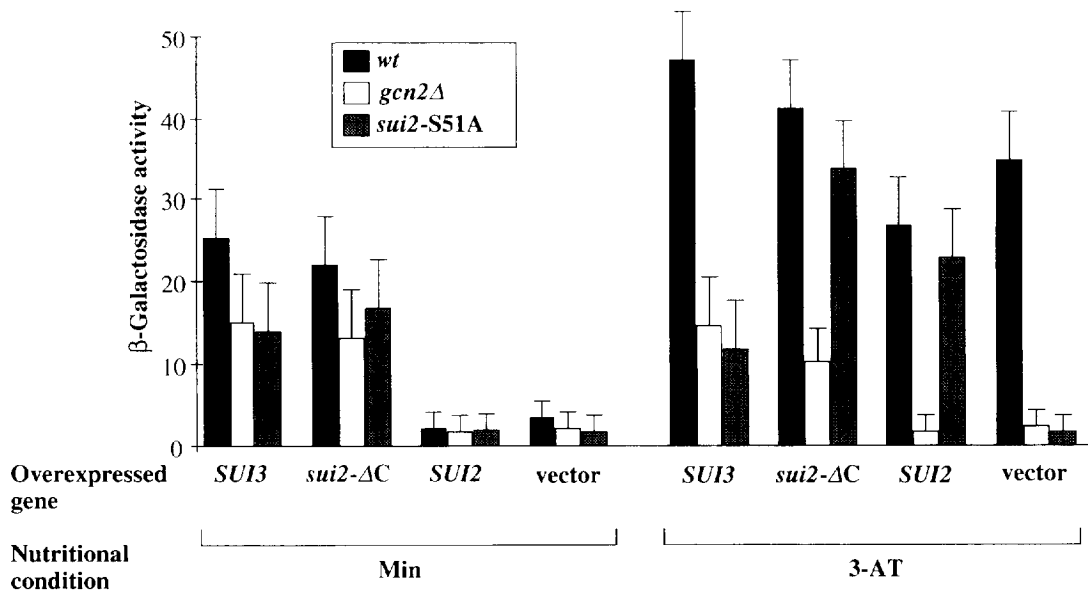


Fig. 4. Graphic representation of the effects of overexpression of the three indicated genes, on the translation of *GCN4* mRNA. Genetic backgrounds and growth conditions were as in Fig. 2. β-Galactosidase assays were carried out as described in the legend to Fig. 2. Values are Miller units and represent the average of three independent experiments with less than 10% deviation.

Mutations in the *SUI3* and *SUI2* genes have been shown to affect translation initiation (Donahue et al., 1988; Cigan et al., 1989). We propose that an excess of  $\beta$  subunits alters ribosomal scanning of the *GCN4* message to favour Gcn4 production, while deletion of the eIF-2 $\alpha$  carboxy-terminus partially impairs the function of the  $\alpha$  subunit, resulting in a similar net effect. However, overexpression of the wt *SUI2* gene dilutes the consequences of phosphorylation of eIF-2 $\alpha$  and reduces the magnitude of *GCN4* mRNA translational derepression.

#### 2.4. *Tpk2* overproduction requires phosphorylation of eIF-2 $\beta$ at ser-51 to induce Gcn2-independent translation of *GCN4* mRNA

A *gcn2 $\Delta$*  strain transformed with a high-copy plasmid carrying the *TPK2* gene grew poorly on minimal plates and was partially resistant to 10 mM 3-AT (Table 1A). As shown in Fig. 5, *GCN4* mRNA translation increased 2-fold in this strain. None of these effects was observed in a *sui2-S51A* strain transformed with the same plasmid (Fig. 5). Therefore, the effects of *TPK2* overexpression are mediated by phosphorylation of eIF-2 $\alpha$  at ser-51.

We hypothesise that Tpk2 might phosphorylate the  $\alpha$  subunit either directly or via an intermediate kinase. This suggestion is in agreement with previous studies indicating activation of PKA by nitrogen depletion which can be a consequence of amino-acid starvation, since amino acids can also be utilized as nitrogen sources (reviewed by Thevelein, 1994). In addition, PKA has been implicated in the acute initial response of the cell

to amino-acid starvation (Engelberg et al., 1994). Given the Gcn2-independent mechanism of the initial response (Tzamarias et al., 1989) and the *TPK2* overexpression effects, it is possible that such overexpression is mimicking the conditions present in the early stages of amino-acid deprivation rather than resembling steady limitation which requires Gcn2 for the manifestation of *GCN4* induction.

### 3. Conclusions

- (1) It has been previously shown that the function of Gcn2 is not a prerequisite for the initiation of *GCN4* mRNA translational derepression, in vivo or its simulation in vitro (Tzamarias et al., 1989; Krupitza and Thireos, 1990). In accordance to this, we report that overexpression of a set of yeast genes stimulates translation of *GCN4* mRNA in the absence of the Gcn2 protein kinase. Therefore, alternative mechanisms of adaptation to limited amino-acid provision, that do not involve this kinase, are also at operation.
- (2) Elevated expression of the *SUP61* gene, a pseudogene for the tRNA<sup>val\*</sup> (AAC) and the *NME1* gene, induced translational derepression of *GCN4* mRNA in strains deleted for *GCN2*. These effects on translation did not require phosphorylation of eIF-2 $\alpha$  at ser-51 and are not a general property of overexpression of tRNA genes. Gcn4 production was similarly affected when the *SUI3* gene, as well as the truncated *sui2- $\Delta$ C* derivative, were overexpressed.
- (3) Multiple copies of the PKA gene *TPK2*, partially

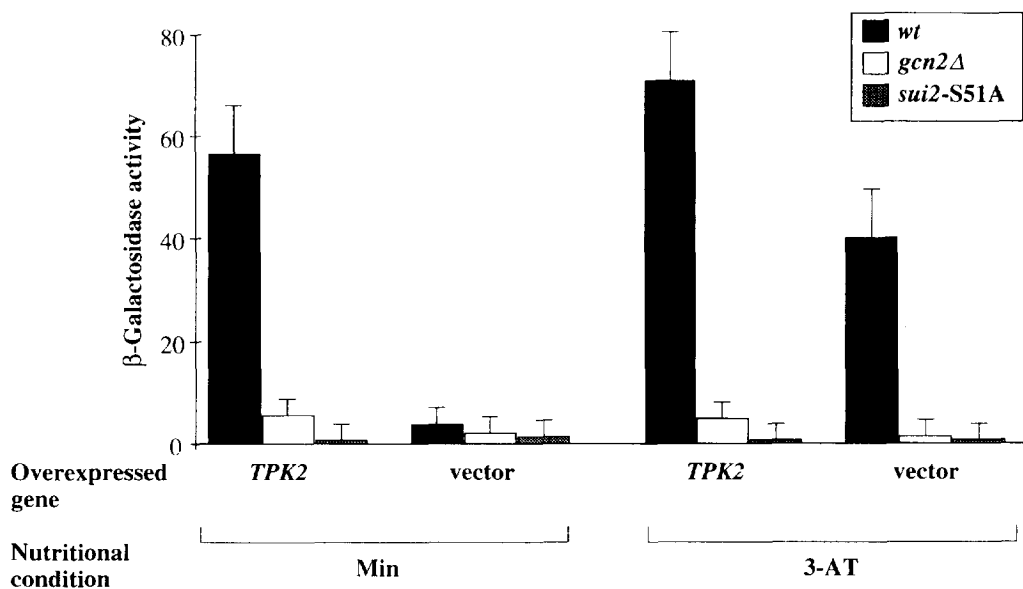


Fig. 5. Graphic representation of the translation of *GCN4* mRNA in the indicated yeast strains transformed either with a multi-copy plasmid harbouring the *TPK2* gene, or with the vector alone (vector). Cells were grown under nutritional conditions identical to those of Fig. 2.  $\beta$ -Galactosidase assays were carried out as described in the legend to Fig. 2. Values are Miller units and represent the average of three independent experiments with less than 10% deviation.

suppressed the amino-acid starvation sensitive phenotype of a *gcn2Δ* strain, by inducing *GCN4* mRNA translation. Additionally, a severe growth inhibition was imposed to the cells, suggesting interference of the high levels of Tpk2 with general protein synthesis. However, in contrast with the above described cases, these effects were reversed by a conservative substitution of ser-51 with alanine, in the  $\alpha$  subunit of eIF-2.

- (4) The presence of the Gcn2 kinase in all cases exacerbated the effects of gene overexpression, i.e. the magnitude of *GCN4* expression related to the overexpression of the above genes, was to an extent amplified by a functional Gcn2 molecule (Figs. 2–5). Given the positive feedback regulation circuit that exists between *GCN4* and *GCN2* genes (Roussou et al., 1988), we propose that the Gcn2 kinase participates in the establishment and the maintenance of the response to amino-acid limitation, rather than in its initiation. Further studies will reveal whether there exists a connection between the mechanisms involved in the initiation of the response and the effects induced by gene overexpression.

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