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Evidence that the *GCN2* protein kinase regulates reinitiation by yeast ribosomes

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The yeast gene *GCN4* produces an mRNA that has a long 5′ 'untranslated' region containing four small open reading frames (ORFs) preceding the protein coding frame. This configuration suppresses the rate by which *GCN4* protein is synthesized. However, translational derepression of the *GCN4* mRNA occurs when yeast cells are grown under conditions of amino acid limitation. Such translational derepression requires the *GCN2* protein kinase and the presence of the 5′ most proximal ORF. In this study we show that a functional coupling between the translation of the first ORF and the amount of the *GCN2* protein is responsible for the translational derepression of the *GCN4* mRNA. Our evidence suggests that this coupling involves an increase in the ability of 40S ribosomal subunits that have translated the first frame to resume scanning and reinitiate translation at a downstream AUG independently of the base sequence in the intervening region.

Key words: GCN4 mRNA/upstream ORFs/ribosome scanning

Introduction

The *GCN4* gene of the yeast *Saccharomyces cerevisiae* encodes a transcriptional activator of amino acid biosynthetic genes (Hope and Struhl, 1985). It produces an mRNA that belongs to a rare class of eukaryotic mRNAs: its long 5′ 'untranslated' region contains an array of four small open reading frames (ORFs) preceding the protein coding frame (Hinnebusch, 1984; Thireos *et al.*, 1984). Translation of these ORFs represses *GCN4* protein synthesis (Mueller and Hinnebusch, 1986), a fact consistent with the ribosome scanning mechanism for translation initiation in eukaryotes (Kozak, 1984). However, growth of yeast cells in media limited in amino acids results in a considerable translational derepression of the *GCN4* mRNA (Hinnebusch, 1984; Thireos *et al.*, 1984). Surprisingly, translation of ORF1 and to a lesser extent of ORF2 is required to suppress the negative effects of ORF3 and ORF4 on *GCN4* protein synthesis (Mueller and Hinnebusch, 1984; Tzamarias *et al.*, 1986).

The product of the *GCN2* gene is also essential for translational derepression of *GCN4* mRNA (Mueller and Hinnebusch, 1986; Tzamarias *et al.*, 1986; Hinnebusch, 1985). This gene encodes a protein kinase and its expression is transcriptionally regulated by the *GCN4* protein. This transcriptional–translational regulatory circuit is required to maintain translation of the *GCN4* mRNA derepressed, since at least one component of the *GCN2* protein function is quantitative: strains that lack the *GCN2* gene exhibit lower basal levels of *GCN4* mRNA translation, whereas strains that overexpress *GCN2* protein are derepressed even in rich media (Roussou *et al.*, 1988). Additional factors that are required for translational derepression of *GCN4* mRNA are the products of *GCN3* and *GCD1* genes (Hinnebusch, 1985) which have not yet been characterized.

All experimental evidence available so far favours a ribosome scanning mechanism for translational initiation in eukaryotes over the direct internal initiation that occurs in prokaryotes (Kozak, 1979, 1984; Hughes *et al.*, 1984; Liu *et al.*, 1984; Peabody *et al.*, 1986). According to this view, the 40S ribosomal subunit recognizes the 5′ end of a eukaryotic mRNA and translocates downstream until it encounters an AUG codon where it assembles with the 60S subunit to form an 80S ribosome. To account for messages with 5′ regions analogous to that of the *GCN4* mRNA, the ribosome scanning hypothesis has been modified by the addition of two concepts. One is that there exists an optimum sequence context around an AUG codon: ribosomes initiate with higher efficiencies when they encounter such an optimum context (Kozak, 1986). The second concept is reinitiation: 40S ribosomal subunits can resume scanning as soon as ribosomes terminate translation of an upstream ORF and can reinitiate at a downstream ORF. Although rigorous evidence for reinitiation is not available, studies on the *in vivo* translation of appropriately engineered mRNAs strongly suggest this concept. In bicistronic mRNAs, for example, translation of the 3′ ORF requires translation termination of the 5′ ORF (Peabody and Berg, 1986). A similar requirement is observed in mRNAs with upstream AUG codons: translation of the downstream ORF occurs only when an in-frame terminator follows the upstream AUG (Kozak, 1984). Finally there is an interesting inverse relationship between the translatability of a downstream ORF and the distance of an upstream small ORF (Kozak, 1987). This suggests that for ribosomes to become competent for reinitiation a minimum time translocating down the mRNA is required. Most arguments are against the only other alternative model, i.e. direct internal binding of 40S subunits, which is also contradicted by direct experimental evidence (reviewed in Kozak, 1984).

These considerations could suggest that the rate of translation of the *GCN4* mRNA is determined partly by the frequency by which ribosomes are used at the upstream AUGs, depending on the particular sequence context (leaky scanning), and partly by the efficiency of translation reinitiation (resumed scanning). Translational derepression could thus involve regulation of either one or both of these events. The observation that translation of ORF1 has a positive effect on the synthesis of the *GCN4* protein even in repressing conditions (Mueller and Hinnebusch, 1986; Tzamarias *et al.*, 1986) argues in favour of the idea that ribosomal subunits that translate ORF1 are able to reinitiate downstream. In this report we present further evidence

suggesting that modulation of reinitiation is at least one of the regulated steps in this translational control system: our results imply that 40S ribosomal subunits that have participated in the translation of ORF1 acquire an increased ability to reinitiate at a downstream AUG when the cellular amount of the *GCN2* protein is elevated.

Results

The levels of β-galactosidase activity produced by *GCN4−lacZ* fusion mRNAs, which contained either the wild-type untranslated region (Figure 1) or an ORF1 deleted version (with deletion endpoints from postitions 165−220), are presented in Figure 2 as a function of genetic background and growth conditions. Translational derepression of *GCN4−lacZ* mRNA was observed when cells were grown in amino-acid-limited conditions and this required both the function of the *GCN2* protein kinase and the presence of ORF1. Translational derepression also occurred in a strain transformed with a multiple-copy plasmid containing the *GCN2* gene ($GCN2^+$, Figure 2). High levels of *GCN2* protein expressed in this strain were detected immunologically (I.Roussou and G.Thireos, unpublished observations). The observed quantitative dependence of translation on *GCN2* was drastically reduced when ORF1 was deleted. The requirement for ORF1 translation was *cis*-dominant since translational derepression in the ORF1 deletion mutant was not restored by either the presence of the resident *GCN4* gene or a co-transformed, engineered gene that highly expressed an RNA containing only the upstream ORFs (data not shown).

In order to determine whether the ORF1-dependent translational derepression was coupled specifically to the *GCN4* initiating codon, we constructed a fusion gene in which the *lacZ* coding region was fused in frame to ORF3 (see Figure 1) and retained the remaining features of the 5′ region of *GCN4* mRNA. The negative effects of ORF1 and ORF2 on *ORF3−lacZ* mRNA translation were partially suppressed in amino-acid-limited growth conditions and in strains that expressed high levels of *GCN2* protein (Figure 3). Deletion of ORF1 rendered translation of *ORF3−lacZ* completely independent of the amount of *GNC2* protein present in the cell (Figure 3). The results of Figures 2 and 3 indicate that translational derepression requires a functional coupling between the short ORF1 and the presence of *GCN2* protein: both of these components are essential, and they can regulate translation of a downstream long ORF that differs from the *GCN4* coding ORF (cf. Mueller *et al.*, 1987).

The observed requirement of both ORF1 translation and the function of the *GCN2* protein kinase for translational derepression of a downstream ORF could suggest that translation of ORF1 is regulated by the amount of the *GCN2* protein. In order to monitor translation directed by ORF1 a fusion gene was constructed in which ORF1 was fused in frame with the *lacZ* gene (see Figure 1). This fusion gene produces an mRNA that contains the 5′ most proximal sequences of *GCN4* mRNA. Translation of the *ORF1−lacZ* mRNA was largely independent of growth conditions and of the amount of *GCN2* protein (Figure 3). Assuming that translation elongation and termination of ORF1 are not affected by such conditions (see Discussion), this result suggested that translation of ORF1 is not a regulated event.

If translation of ORF1 is not regulated, then the regulated step should occur following ORF1 translation. This could involve sequence-specific interactions along the region of the *GCN4* mRNA that is necessary and sufficient for translational regulation (positions 205−445, Thireos *et al.*, 1984) including the downstream small ORFs. When this region in its entirety was replaced with a heterologous, 114-base-long, sequence containing a small ORF at its 3′ end (ORF6, Figure 1B), translation of the *GCN4−lacZ* was severely suppressed, and *GCN2*-dependent regulation was not observed (Figure 4). However, when the same heterologous sequence replaced *GCN4* mRNA sequences from position 248 to position 445 (Figure 1), i.e. was preceded by an additional 43 bases containing ORF1, translational regulation was restored. As seen in Figure 4, relative to the basal repressed levels, translation of the *GCN4−lacZ* frame was increased 4-fold in amino-acid-limited growth conditions

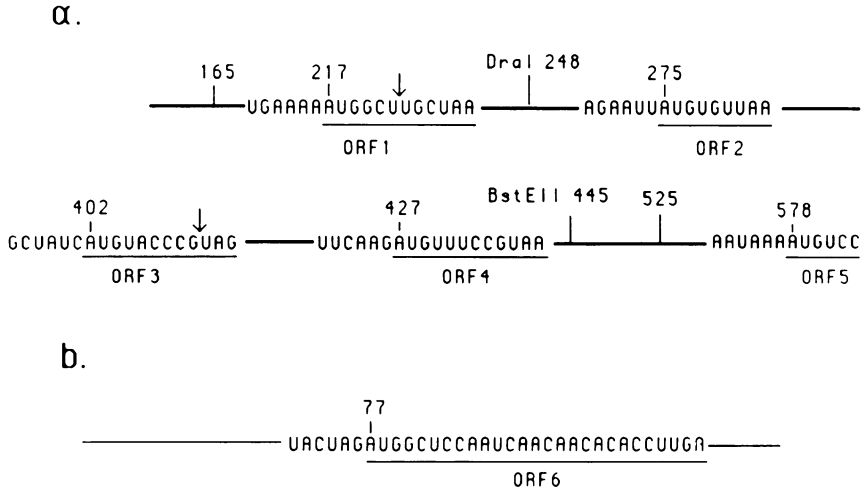

Fig. 1. (**A**) Schematic representation of the 5′ untranslated region of the *GCN4* mRNA including the base sequences of the four ORFs. The numbered position of each ORF is indicated relative to the 5′ end. Deletion endpoints as well as the sites of restriction enzyme cleavages that are used in this study are also indicated. Arrows indicate the exact sites of fusions of ORF1 and ORF3 to the coding region of the *E.coli lacZ* gene. (**B**) Schematic representation of a RNA produced by the heterologous DNA fragment used to replace sequences of the 5′ non-coding region of the *GCN4* gene. The relative position and the sequence of ORF6 present in this fragment is indicated.

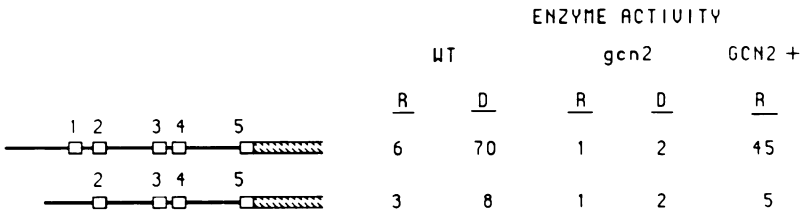

Fig. 2. Translational regulation of the *GCN4−lacZ* mRNA in the presence or absence of ORF1. The structures of the mRNAs that were monitored are shown schematically. Open boxes correspond to the upstream ORFs. Box 5 corresponds to the *GCN4−lacZ* coding frame. Part of the coding region of the *lacZ* gene is indicated by the shaded rectangle. Levels of β-galactosidase activity that were produced by each mRNA were measured in a wild-type strain (WT), in a *gcn2* strain and in a strain that overexpressed the *GCN2* gene (GCN2+), under rich (R) or under amino-acid-limited (D) growth conditions.

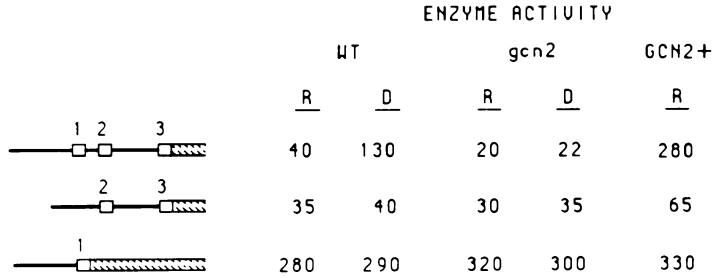

Fig. 3. Translational regulation of two mRNAs containing an *ORF3−lacZ* fusion and translational efficiency of the *ORF1−lacZ* mRNA. Diagrams of the constructs and β-galactosidase activities are presented as described in the legend to Figure 2.

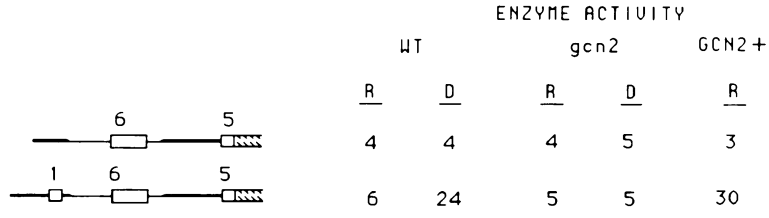

Fig. 4. The effects of a heterologous sequence containing ORF6 on the translation of *GCN4−lacZ* coding frame in the absence or presence of ORF1. Diagrams of the constructs and β-galactosidase activities are presented as described in the legend to Figure 2.

and 6-fold in the presence of increased amounts of *GCN2* protein. In the absence of *GCN2* gene function, the addition of ORF1 was not sufficient to overcome the negative effect of ORF6 on downstream translation. Thus, the presence of ORF1 and the function of the *GCN2* protein kinase can result in the partial suppression of the negative interference on translation of a heterologous ORF through largely heterologous sequences.

The fact that this translational regulation can be elicited through a heterologous ORF raised the possibility that, in order for the *GCN2* protein to regulate translation of *GCN4* mRNA, prior translation of ORF1 is sufficient even in the absence of any other small ORFs. Indeed, when ORF1 alone was placed 164 bases upstream of the *GCN4−lacZ* frame translational derepression still occurred in wild-type strains grown under derepressing conditions as well as in strains containing high amounts of GCN2 protein, but not in gcn2 strains (Figure 5). Two additional observations in this series of experiments deserve some attention: the fact that the translational efficiency of an ORF-less *GCN4−lacZ* mRNA was lowered in a strain that overexpresses GCN2; and the

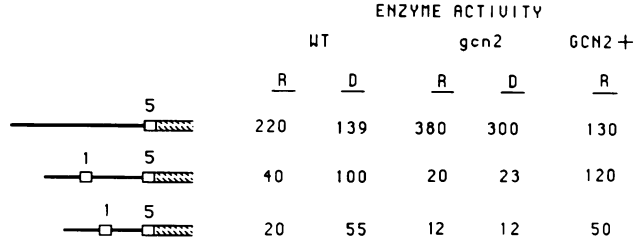

Fig. 5. The negative interference of ORF1 on the translation of the *GCN4−lacZ* coding frame as a function of distance and of the amount of the *GCN2* protein. Diagrams of the constructs and β-galactosidase activities are presented as described in the legend to Figure 2.

fact that the negative effect of ORF1 on downstream translation was less severe than that imposed by ORF6. Both these observations will be discussed below. When ORF1 was placed only 84 rather than 164 bases upstream of the *GCN4−lacZ* frame (see Figure 1), downstream translation was reduced to a larger extent but was still dependent on

the amount of *GCN2* protein. The fact that the negative effect of ORF1 on downstream translation was inversely related to the distance between the ORFs (even in the *gcn2* background) might be explained as due to the ability of the ribosomal subunits that had translated ORF1 to reinitiate downstream in a manner analogous to what has been observed in mammalian systems (see Discussion).

Translational derepression of the *GCN4* mRNA dependent on the presence of ORF1 was also observed in a *gcd1* strain (Hinnebusch, 1985; Tzamarias *et al.*, 1986). All the constructions used in this study when introduced in the *gcd1* strain showed translational efficiencies similar to strains overexpressing the *GCN2* gene and were dependent on the presence of ORF1. The positive effect of the absence of *GCD1* function on *GCN4* mRNA translation will be presented elsewhere (D.Tzamarias, I.Roussou and G.Thireos, in preparation).

Discussion

The data presented in this paper can be summarized as follows. When translation of ORF1 is allowed, the amount of *GCN2* protein can (i) modulate the rate of translation initiating at an AUG codon other than the *GCN4* start codon, (ii) suppress the negative effects on downstream translation imposed by the presence of a heterologous sequence containing a small ORF, (iii) increase the translation of the *GCN4* coding frame even in the absence of intervening ORFs, in a manner dependent on the distance between the two ORFs but (iv) has no effect on the rate of translation initiation at ORF1. These results reveal three major novel properties of this translational control system: translational regulation operates (i) if ORF2, ORF3 and ORF4 are all substituted with a heterologous ORF, (ii) if only ORF1 precedes the GCN4 coding ORF and finally (iii) independently of the rate of translation initiation at ORF1. These new results show that at least one of the regulated steps occurs following translation initiation at ORF1, is independent of downstream sequences and requires high levels of the *GCN2* protein kinase.

Following translation initiation at ORF1 there are three events that should take place: elongation, termination and release of the ORF1 tripeptide. Regulation by the *GCN2* protein could operate at any one of these steps. One model might suggest that the *GCN2* kinase releases an elongation or termination block on ORF1 translation and this in turn increases the number of 40S subunits that reach the ORF5 start codon (through leaky scanning). We consider this idea to be in conflict with the positive nature of ORF1: it slightly suppresses the negative effects of the downstream ORFs even on the basal level translation of the *GCN4* ORF in wild-type strains (Figures 2 and 4) and when it is the only upstream ORF, it has a less severe negative effect on the translation of ORF5, even in the *gcn2* strain (Figure 5), compared to ORF6 (Figure 4) and to ORF4 in the same position (shown in Mueller and Hinnebusch, 1986). In addition, it is difficult to explain how such a block on ORF1 translation could manifest the observed dependence on distance from ORF5 (Figure 5). Alternative models based on a function of the ORF1 tripeptide are very improbable, taking into account the *cis* dominance of the effects of ORF1 translation. The above considerations force us to consider the possibility of yet another event following ORF1 translation: ribosome reinitiation. The totality of the evidence leads us to favour a model in which translational regulation is accomplished by a *GCN2*-dependent increase in the ability of ribosomal subunits to reinitiate following translation of ORF1.

Final acceptance of this model will require demonstration of reinitiation following ORF1 translation. Rigorous proof requires the direct observation that the same ribosomal subunits that have translated ORF1 initiate translation at ORF5. Since we lack such direct proof we will consider the evidence that indirectly favours such a possibility. The positive nature of ORF1 translation, as summarized above, can be best explained if reinitiation operates. In contrast to the fate of ribosomes following translation of any other small ORF, ribosomes that translate ORF1 are apparently not totally discarded but can be reused for downstream initiation. This could explain the fact that translation of ORF1 can suppress the negative nature of the other ORFs and that it has a less severe negative effect on the translation of ORF5. The inverse relationship between the negative effects of ORF1 and its distance from ORF5 also argues in favour of ribosome reinitiation: it is analogous to a similar observation in mammalian systems (see Introduction) where this relationship has been extensively discussed as suggesting ribosome reinitiation over other alternatives (Kozak, 1987). In addition this inverse relationship suggests that yeast and mammals share similar time requirements to achieve reinitiation competence of 40S subunits.

If ribosome reinitiation following translation of ORF1 occurs, then the observation that translational derepression requires coupling of ORF1 translation with the amount of *GCN2* protein can be best explained if the *GCN2* kinase increases the rate of such reinitiation events. We should emphasize that this idea is tentative and is based solely on the unlikely nature of alternative explanations, as presented above. The activity of the *GCN2* protein kinase could increase the fraction of the 40S ribosomal subunits that remain bound on the message upon termination of ORF1 translation, or it could accelerate recharging of such subunits with factors essential for reinitiation. Alternatively, high amounts of *GCN2* protein might have more general effects on cellular protein synthesis which favour ribosome reinitiation. Suggestive of such a possibility is the fact that high amounts of *GCN2* protein have a negative effect on the translation of an ORF-less *GCN4–lacZ* mRNA (Figure 5) and on the translation of a *URA3–lacZ* mRNA (G.Thireos, unpublished observations). In addition, we have observed that strains which express high amounts of *GCN2* protein exhibit a phenotype of slow growth.

Whatever the function of the *GCN2* protein kinase, it can only be elicited in conjunction with the translation of ORF1. In a *GCN4* mRNA that contained any of the other ORFs or a heterologous ORF upstream of the protein coding ORF but lacked ORF1, no regulation of translation occurred and the negative ORF effects on downstream translation were severe. If our idea that ribosomes that have translated ORF1 reinitiate translation is correct, then there must be a mechanism by which these ribosomes are 'imprinted' for subsequent reinitiation. Such an imprinting might be determined by the sequence context of AUG1. Consistent with this is the fact that the sequence context of AUG1 has the closest match to the proposed optimum consensus for yeast mRNAs (Cigan and Donahue, 1987; Hamilton *et al.*, 1987). In addition, there is evidence that an extended sequence context might

be involved in the recognition of the first AUG. A deletion that removes an internal segment of the *GCN4* mRNA from positions 165 to position 206 (11 bases upstream of AUG1) severely impairs the ability of the *GCN4* mRNA to be derepressed translationally (Tzamarias *et al.*, 1986). It could be that an appropriate sequence context flanking an AUG determines a ribosomal subunit configuration that permits the 40S subunit to resume scanning after termination and/or to be recharged for reinitiation.

If regulation of *GCN4* protein synthesis requires translation of ORF1 and modulation of the ability of ribosomal subunits to reinitiate by the *GCN2* protein kinase, then what is the role of the other small ORFs in the *GCN4* mRNA? One major function should be to maintain low levels of *GCN4* protein when amino acids are available for growth, since strains that produce high levels of *GCN4* protein exhibit slow growth (G.Thireos, unpublished observations). In addition, they might contribute to the final extent of translational derepression. Ribosomal subunits that emerge following translation of ORF1 could be able to undergo multiple rounds of initiation–reinitiation at these ORFs which might result in an increased stability of the scanning 40S subunits for downstream initiation. Evaluation of these possibilities awaits the direct demonstration and measurements of reinitiation rates.

Materials and methods

Strains and media

The yeast strains used were derivatives of the wild-type strain S288C. These were the *ura3-52*, the *gcn2-15, ura3-52* and the *leu2-2, ura3-52* strains. Rich media were minimum-dextrose media supplemented with all 20 amino acids; amino-acid-limited media were minimal-dextrose supplemented with 10 mM 3-amino-1,2,4-triazole, which elicited histidine starvation.

Construction of fusion genes

All gene constructions described below contain 650 bp of the 5′ non-transcribed *GCN4* sequences necessary for proper transcriptional initiation and at least the first 165 bp of the 5′ untranslated sequences. The in-frame fusion of the *GCN4* coding region to the *Escherichia coli lacZ* gene has been described elsewhere (Thireos *et al.*, 1984). In-frame fusions of upstream ORF1 and ORF3 to the coding region of the *lacZ* gene were obtained by joining 5′ deleted DNA fragments [randomly deleted from the *Bst*EII site (Figure 1)] to the eighth codon of the *lacZ* gene carried on a YCp50 centromeric yeast–*E.coli* shuttle vector. Such fusion constructs were used to transform *E.coli* and transformants expressing β-galactosidase were selected. The fusion point of the selected plasmid DNA was determined by the chain termination sequencing method using a DNA primer hybridizing to the 5′ end of the *lacZ* gene sequence. To obtain a gene fusion to ORF3 that lacked ORF1, random deletions were generated starting from the *GCN4* derivative deleted for a small region that includes ORF1 (positions 165–220) described in Tzamarias *et al.* (1986).

The heterologous DNA fragment was the 114-bp-long sequence which contains a small ORF in its 3′ end (S1 substitution in Tzamarias *et al.*, 1986). This fragment was used to substitute for all upstream ORFs (positions 174–445) or a region containing the three 3′ proximal ORFs (positions 248–445).

A *GCN4–lacZ* fusion gene that contained only ORF1 in its 5′ region was constructed by deleting the DNA fragment from position +248 to position +445. This construct placed ORF1 164 bases upstream from the start of the *GCN4* coding frame. Similarly a deletion from position +248 to position +525 placed ORF1 84 bases upstream from the start of the *GCN4* coding frame.

Translation assays

All the gene fusions described above were inserted in the yeast centromeric plasmid YCp50. Such recombinant plasmids were used to transform the designated yeast strains. Translational efficiencies of the resulting mRNAs were monitored by measuring β-galactosidase activities that were produced when these strains were grown in rich or amino-acid-starved media (Thireos *et al.*, 1984). Yeast cells overproducing *GCN2* protein were *leu2-2,ura3-52* strains transformed with the YEp13 multiple-copy yeast plasmid carrying the *GCN2* gene (Roussou *et al.*, 1988). Such strains were co-transformed separately with each one of the *lacZ* fusion genes. To account for co-transformation effects the levels of β-galactosidase activity were corrected for the levels obtained from strains co-transformed with the YEp13 vector plasmid and each one of the fusion constructs.

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References

Cigan,M.A. and Donahue,T.F. (1987) *Gene*, **59**, 1–18.
Hamilton,R., Watanabe,C.K. and deBoer,H.A. (1987) *Nucleic Acids Res.*, **15**, 3581–3593.
Hinnebusch,A.G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6442–6446.
Hinnebusch,A.G. (1985) *Mol. Cell Biol.*, **5**, 2349–2360.
Hope,I.A. and Struhl,K. (1985) *Cell*, **43**, 177–188.
Hughes,S., Mellstrom,K., Kosik,E., Tamanoi,F. and Brugge,J. (1984) *Mol. Cell Biol.*, **4**, 1738–1746.
Kozak,M. (1979) *Nature*, **280**, 82–85.
Kozak,M. (1984) *Nucleic Acids Res.*, **12**, 3873–3893.
Kozak,M. (1986) *Cell*, **44**, 283–292.
Kozak,M. (1987) *Mol. Cell Biol.*, **7**, 3438–3445.
Liu,C., Simonsen,C.C. and Levinson,A.D. (1984) *Nature*, **309**, 82–85.
Mueller,P.P. and Hinnebusch,A.G. (1986) *Cell*, **45**, 201–207.
Mueller,P.P., Harashima,S. and Hinnebusch,A.G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2863–2867.
Peabody,D.S. and Berg,P. (1986) *Mol. Cell Biol.*, **6**, 2695–2703.
Peabody,D.S., Subramani,S. and Berg,P. (1986) *Mol. Cell Biol.*, **6**, 2704–2711.
Roussou,I., Thireos,G. and Hauge,B.M. (1988) *Mol. Cell Biol.*, **8**, 2132–2139.
Thireos,G., Penn,M.D. and Greer,H. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5096–5100.
Tzamarias,D., Alexandraki,D. and Thireos,G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4849–4853.

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