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Translational Activation of GCN4 mRNA in a Cell-Free System Is Triggered by Uncharged tRNAs

GEORG KRUPITZA AND GEORGE THIREOS*

Foundation for Research and Technology, Institute of Molecular Biology and Biotechnology, P.O. Box 1527, Heraklion 711 10, Crete, Greece

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Translation of GCN4 mRNA is activated when yeast cells are grown under conditions of amino acid limitation. In this study, we established the conditions through which translation of the GCN4 mRNA could be activated in a homologous in vitro system. This activation paralleled the in vivo situation: it required the small open reading frames located in the 5′ untranslated region of the GCN4 mRNA, and it was coupled with reduced rates of 43S preinitiation complex formation. Translational derepression in vitro was triggered by uncharged tRNA molecules, demonstrating that deacylated tRNAs are more proximal signals for translational activation of the GCN4 mRNA.

The GCN4 protein activates the transcription of amino acid biosynthetic enzymes in yeast cells (4). In cells grown under amino acid limitation conditions, translation of the GCN4 mRNA is increased severalfold through a mechanism that neutralizes the negative effects exerted by the four small open reading frames (ORFs) located in its 5′ untranslated region (3, 12). Translational activation of the GCN4 mRNA is maintained by the action of the GCN2 protein kinase (11), but it can also be elicited transiently, immediately after amino acids are removed from the growth medium in a GCN2-independent manner (15). Under both conditions, the rate of general polypeptide chain initiation is reduced as a result of decreased rates of formation of 43S preinitiation complexes (15). This coupling suggests that the mechanism that activates translation of the GCN4 mRNA is dependent on the partial inactivation of the process in protein synthesis involved in the charging of 40S ribosomal subunits with Met-tRNA.

Essential for the operation of this regulatory mechanism is the translation of the 5′ most proximal ORF (ORF1), which exerts only a mild negative effect on translation downstream (10, 14). By contrast, translation of ORF4 has a strong negative effect and suppresses translation of the GCN4-coding frame under any growth condition unless it is preceded by ORF1 (17). The sum of the evidence suggests that translational activation of GCN4 mRNA is based on the increased ability of the ribosomal subunits that have been used for the translation of ORF1 to resume scanning and reinitiate translation at downstream AUGs (9, 16).

As a step toward the direct biochemical confirmation of this proposed mechanism, we have developed the appropriate conditions by which a homologous in vitro translation system faithfully reproduces the in vivo translational activation of the GCN4 mRNA. The acquisition of such a system enabled us to demonstrate directly that uncharged tRNA molecules are more proximal signals for this activation.

In vitro translation assays. Yeast cells were grown in YPD medium, and cell extracts from wild-type or *gcn2* strains depleted of small molecules (amino acids, salts, etc.) were prepared as described by Hussain and Leibowitz (5), with the inclusion of the 100S centrifugation step and the micrococcal nuclease treatment described by Tuite and Plesset (13). The translation reaction was assembled as described previously (13) and was supplemented either with 1 μg of poly(A)-containing yeast mRNA or with 200 ng of in vitro-synthesized capped mRNAs produced by the SP6 promoter system (7). After the addition of [^{35}S]methionine, the reaction mixture was incubated at 20°C for 2 h. The translation products were analyzed by sodium dodecyl sulfate-gel electrophoresis and fluorography. The amount of GCN4 protein synthesized was used as a measure of the translational efficiency of GCN4 mRNA and its derivatives. The plasmid constructions used in this study are shown in Fig. 1.

Effects of the upstream ORFs on basal-level translation of the GCN4 mRNA in vitro. In vitro translation of the wild-type (WT) GCN4 mRNA was very poor compared with that of its derivative lacking all upstream ORFs (Fig. 2, lanes WT and ΔORF). In addition, the negative effect on translation of ORF1 when it solely preceded the GCN4-coding ORF was less than that exerted by ORF4 placed in the same configuration (Fig. 2, lanes 1+5 versus 4+5). These in vitro translation results paralleled the results obtained from the in vivo experiments, which demonstrated that the cell-free system has the same *cis* requirements for basal-level translation of the GCN4-coding ORF.

Translational activation of GCN4 mRNA in vitro. Translation reactions were supplemented with decreasing amounts of amino acids (only [^{35}S]methionine was kept constant), and the extent of translation of various mRNA molecules was monitored. Decreasing amounts of amino acids resulted in a marked increase in the translation of WT mRNA, whereas translation of the ΔORF mRNA was slightly reduced and that of total yeast mRNA was severely reduced (Fig. 3). Low concentrations of amino acids in the translation reaction reduced the translation of the 4+5 GCN4 mRNA derivative, whereas translation of the 1+5 mRNA was less affected (Fig. 4A).

To monitor the in vitro requirement of the GCN2 protein for translational activation, the translation reaction was supplemented with cell extracts derived from a *gcn2* mutant strain. With use of these extracts, the basal-level translation of the GCN4 mRNA derivatives was the same as for extracts from the WT strain (data not shown), and translational activation of the GCN4 mRNA occurred in vitro upon amino acid limitation (Fig. 4B). Finally, the extent of formation of 43S preinitiation complexes was measured after fractiona-

* Corresponding author.

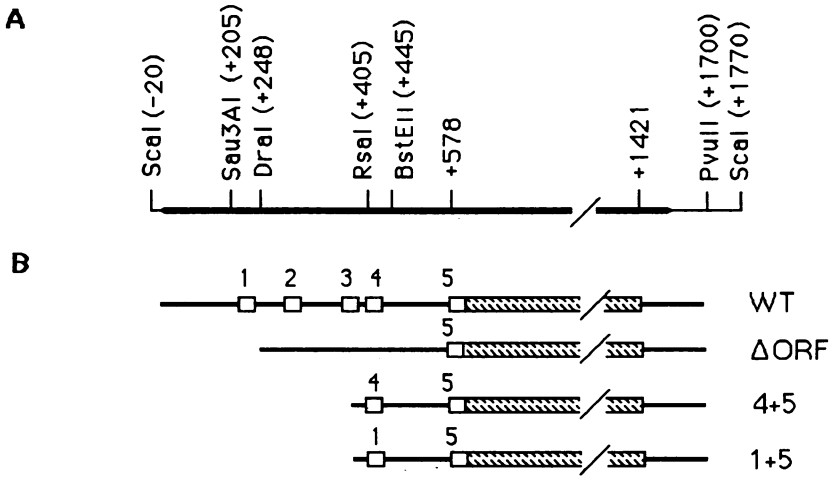

FIG. 1. (A) Partial restriction endonuclease map of the DNA region containing the *GCN4* gene (▬) showing the positions of cleavage sites used in this study. The numbered positions are relative to the transcription initiation site (12). As indicated, the GCN4-coding frame lies between positions +578 and +1421. (B) Schematic representation of the synthetic GCN4 mRNA derivatives used in this study. The 1,790-base-pair *Sca*I DNA fragment containing the GCN4 region was cloned into the *Sma*I site of plasmid SP65. The WT synthetic mRNA was obtained through in vitro transcription after linearization of the plasmid at the *Pvu*II site 3′ to the *GCN4* gene. The ΔORF transcript was obtained from a derivative of this construct that was deleted for the *Sau*3AI-*Bst*EII region. The 4+5 transcript was obtained from another derivative that was deleted for the *Sca*I-*Rsa*I region. Finally, the 1+5 transcript was obtained from a derivative in which the *Sca*I-*Bst*EII fragment was substituted with the *Sau*3AI-*Dra*I fragment. Symbols: □, upstream ORFs; ▨, GCN4-coding ORF.

tion of the translation extracts on sucrose gradients (6). This process was severely affected when translations were performed under amino acid limitation conditions. At amino acid concentrations of 200 and 5 μM, 43S formation (expressed as the total amount of [^{35}S]Met-tRNA associated with the 40S peak of the gradient, measured according to Legon et al. [6], normalized for the integrated absorbance of this peak at 260 nm) totaled 240 and 20 cpm per unit of optical density at 260 nm, respectively.

These results demonstrated that as in the in vivo situation, in vitro translational activation of the GCN4 mRNA (i) is triggered by amino acid limitation and (ii) is coupled with decreased rates of polypeptide chain initiation, a consequence of the reduction of 43S preinitiation complex formation, and (iii) translational repression by ORF4 is overcome by the presence in *cis* of the other ORFs. The independence from the GCN2 protein kinase parallels the same independence of the GCN4 mRNA translational activation observed in vivo when yeast cells are shifted from rich medium to medium lacking all amino acids (15).

Uncharged tRNAs activate translation of GCN4 mRNA in vitro. Translational assays were performed without any added amino acids (but with [^{35}S]methionine) or by adding charged tRNAs. Translation of the WT GCN4 mRNA was higher in the absence of any added charged yeast tRNAs (using those already existing in the cell extract), whereas

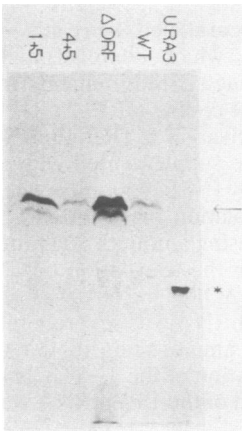

FIG. 2. Dependence of in vitro basal-level synthesis of the GCN4 protein on the upstream ORF configuration of the GCN4 mRNA. The fluorogram of the translation products directed by equal amounts (as determined by Northern [RNA] blot analysis) of the indicated synthetic mRNAs is shown. The arrow indicates the position of the GCN4 protein, which migrates with an apparent size of 44 kilodaltons, as has been observed previously (4); faster-migrating products were due to premature translation termination. The asterisk indicates the translation product of a *URA3* SP65-derived mRNA, used as a control.

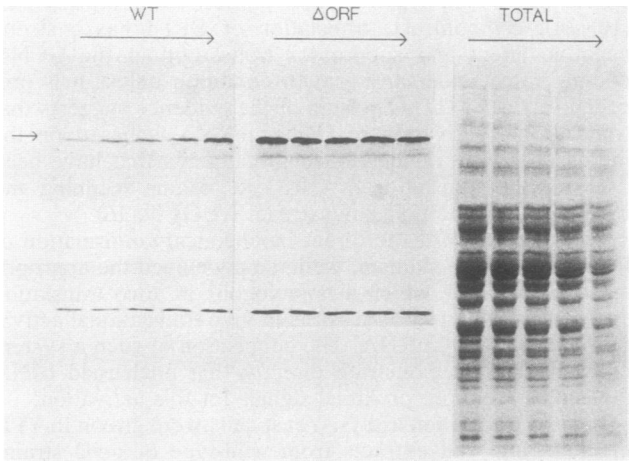

FIG. 3. In vitro activation of GCN4 mRNA translation. In vitro translations using WT, ΔORF, or total yeast mRNA were performed by providing the system with decreasing amounts of amino acids (indicated by the direction of the long arrows). In this experiment, the system was supplemented with 200, 100, 50, 10, and 5 μM amino acids. The arrow on the left indicates the position of the GCN4 protein.

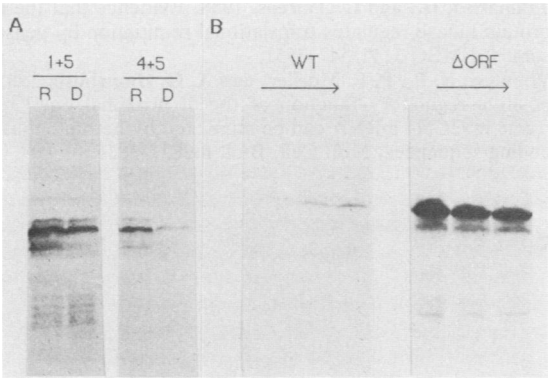

FIG. 4. Effects of *cis*- and *trans*-acting mutants on the in vitro translational activation of the GCN4 mRNA. (A) In vitro synthesis of the GCN4 protein as directed by the indicated synthetic mRNAs in the presence of 100 μM (R) or 5 μM (D) amino acids. (B) In vitro translation of WT or ΔORF mRNA in a cell-free system prepared from a *gcn2* strain and supplemented with decreasing amounts of amino acids (100, 10, and 5 μM).

translation of ΔORF mRNA was low (Fig. 5A). These results suggested that when the ratio of uncharged to charged tRNAs was high, translation of the GCN4 mRNA was activated in vitro. A more direct demonstration of the role of uncharged tRNAs in signaling translational activation of the GCN4 mRNA was achieved by introducing into the cell-free system tRNA molecules that were deacylated in vitro as described by Hill and Struhl (2). Translational assays were performed without any added amino acids to ensure no recharging of added deacylated tRNAs. Addition of discharged tRNAs activated translation of the WT mRNA, whereas translation of the ΔORF mRNA was reduced (Fig. 5B).

The involvement of uncharged tRNAs as more proximal signals for the derepression of the transcription of amino acid biosynthetic genes has been previously inferred from in vivo studies using strains carrying temperature-sensitive mutations for genes encoding tRNA synthetases (8). Using

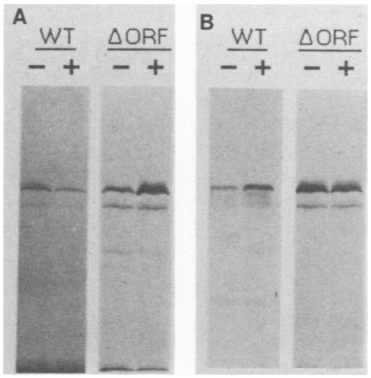

FIG. 5. Demonstration that the presence of deacylated tRNAs activate translation of GCN4 mRNA in vitro. (A) In vitro translations were performed without added amino acids in the absence (−) or presence (+) of 8 μg of charged yeast tRNAs. The translation products directed by the WT and ΔORF synthetic mRNAs are shown. (B) In vitro translations were performed without added amino acids in the absence (−) or presence (+) of 4 μg of uncharged yeast tRNAs, and the translation products of the WT and ΔORF mRNAs were monitored.

the developed in vitro translation system, we have shown directly that deacylated tRNAs signal the activation of translation of the GCN4 mRNA and a concomitant reduction of overall protein synthesis. It is noteworthy that transduction of this signal does not require the GCN2 protein kinase. It is possible that the presence of large amounts of deacylated tRNAs affect directly the same process in protein synthesis initiation that is affected by the GCN2 protein kinase in more mild (and more naturally occurring) amino acid limitation conditions.

We have described the conditions through which a homologous cell-free translation system reproduces faithfully the in vivo requirements for translational activation of the GCN4 mRNA. The combined use of genetic (in vivo) and biochemical (in vitro) approaches is now possible and will facilitate the elucidation of the mechanisms involved.

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