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A Transient *GCN4* mRNA Destabilization Follows *GCN4* Translational Derepression*

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Studies based on experimental strategies that utilized either inhibitors or structural alterations point to the existence of an inverse relationship between translation and stability of a given mRNA. In this study we have investigated the potential link between translation and stability of the yeast *GCN4* mRNA whose translational rates change with respect to amino acid availability. We observed that under conditions favoring its translation, the steady state levels of the *GCN4* mRNA were decreased, but this was not due to a measurable alternation in its decay rate. We have demonstrated that an extensive destabilization of this message is intimately coupled with its increased access to heavy polysomes, which occurs transiently in the process of translational derepression. This transient change in the stability is what readjusts the steady state levels of the *GCN4* mRNA. This study demonstrates *in vivo* the existence of a mechanism of mRNA degradation that is coupled with the process of translation.

The process of genetic information flow involves, among other things, the turnover of mRNA, the template for translation. In the last few years, several studies have revealed an underlying relationship between the decay rates of a large variety of different mRNAs and their respective translational efficiencies (for reviews, see Refs. 1–3).

Using one experimental approach, it was shown that inhibition of protein synthesis by antibiotics results in stabilization of many mRNAs as is the case of histone (4), tubulin (5), transferrin receptor (6), c-*myc* (7), c-*fos* (8), and granulocyte macrophage colony-stimulating factor (9) mRNAs. In yeast, it has been shown that almost all mRNAs are stabilized in the presence of the translation inhibitor, cycloheximide (10, 11). The above studies point to the existence of a mechanism that serves to couple the intrinsic rate of mRNA decay with its rate of translation. Two molecular mechanisms, not mutually exclusive, have been proposed to account for this coupling. First, a ribosome-associated nuclease or another degradation factor might be responsible, which becomes active when the ribosome interacts with destabilizing elements within the mRNA. Alternatively, the degradation mechanism may require labile cytoplasmic factor(s) that are rapidly lost upon protein synthesis inhibition.

A different experimental strategy employed alterations on the mRNA structure, that either introduce premature stop codons or block translation initiation. Premature translation termination has been shown to increase the rate of mRNA turnover in a number of cases, a phenomenon that has been described as nonsense-mediated mRNA decay (2, 12–14). Such studies have lead to the identification of the *UPF1* gene in yeast, which may be part of a cellular safeguarding system that prevents the accumulation of potentially deleterious fragmented polypeptides (12–14). Introduction of a strong secondary structure into the 5′-untranslated region (UTR)[1] immediately adjacent to the cap site of the mRNAs, blocks ribosome binding, and therefore inhibits translation in *cis*. Using this approach, it was shown that the selective degradation of the granulocyte macrophage colony-stimulating factor mRNA is coupled to ongoing translation of this mRNA (9).

All of the experimental strategies described so far have the common disadvantage of utilizing nonnatural conditions, either drugs or mutated mRNAs. Therefore, it is not surprising to encounter cases of apparently contradictory results as in the case of c-*fos* mRNA (15, 8). In order to overcome this major disadvantage, we decided to investigate the decay rates of an mRNA that is translationally regulated *in vivo*. The yeast *GCN4* mRNA is under translational regulation imposed by its primary structure. This message is poorly translated when cells are grown in media containing all amino acids (rich media) due to the negative effects on translation of the four small open reading frames (ORFs) located in its 5′-UTR (16–18). However, when the cells are grown under amino acid limitation conditions, *GCN4* mRNA is translationally derepressed. Therefore, this mRNA offered us the opportunity to investigate *in vivo* the relationship of mRNA stability with translational rates. We have demonstrated that the *GCN4* mRNA is transiently destabilized when its translation rate is increased.

EXPERIMENTAL PROCEDURES

Strains and Media—All yeast strains used in this study were derivatives of *S288C* cured for the *GAL2* deficiency. In detail, these were as follows: *MATa ura3–52 leu2–2*, *MATa gcn2–11 ura3–52 leu2–2*, *MATa rpb1–1 ura3–52*. The *rpb1–1* strain was kindly provided by A. Jacobson. *GCN4* derepresing conditions were accomplished by growing leucine auxotroph cells in minimal medium for 6 h. Minimal medium contained yeast nitrogen base (Difco) and 2% glucose. All 20 amino acids were supplemented when necessary.

Plasmids—The construction of *GCN4-LacZ*, *ΔORFGCN4* and *ΔORFGCN4-LacZ* has been described elsewhere (16). The *DED1-LacZ* plasmid was constructed by inserting the *ScaI/SalI* DNA fragment from *ΔORFGCN4-LacZ*, which contains the *GCN4-LacZ* gene, from the initiation of transcription of *GCN4* to the end of *LacZ*, into the *Hin*dIII site of Ycp88, which is located just downstream of the promoter of *DED1* (19). The *GCREHIS3-LacZ* fusion contains the GCR element within unique *Bam*HI and *Eco*RI sites in the promoter of *HIS3-LacZ*, which was modified to include only the regulated TATA element 18 base pairs downstream. This reporter gene is on a centromeric yeast plasmid

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[1] The abbreviations used are: UTR, untranslated region; ORF, open reading frame.

carrying *URA3* for selection and was obtained from K. Struhl. Construction of Yep24-*GCN2* has been described previously (20).

RNA Procedures—Total yeast RNA was extracted from the indicated strains grown under the appropriate conditions as described previously (21). Northern blotting, hybridization, and signal quantitation have been described previously (21, 28). Total RNA loading and transfer were monitored by visualization of the ethidium bromide-stained RNA on the membrane after transfer. The DNA probes used where as follows: the 1.1-kilobase *Bam*HI-*Pvu*II DNA fragment of the *GCN4* gene and the 2.1-kilobase *Bam*HI-*Hin*dIII DNA fragment from pRIP1 (22) kindly provided by A. Jacobson, containing the *PGK1* gene. mRNA decay rates were measured in a strain harboring the *rpb1–1* allele as described previously (2, 10).

Other Methods—Transformations were carried out with the lithium acetate method (23). β-Galactosidase assays were performed as described previously (17).

RESULTS

GCN4 Translational Derepression Results in Decreased GCN4 Steady State mRNA Levels—To delineate the possible relationship between the *GCN4* mRNA translational status and the corresponding steady state mRNA levels, we took advantage of the available genetic means to affect *GCN4* mRNA translation. In total, three different approaches were undertaken.

First, we induced *GCN4* mRNA translation by overexpressing, in wild-type cells, the *GCN2* protein kinase that, by phosphorylating the α subunit of the eIF2, relieves the translational repression of the *GCN4* mRNA (20). This is shown in Fig. 1*C* (*wt*, *wt*/*HGCN2*, *+Leu*), where the translational status of the *GCN4* mRNA was monitored by assaying for β-galactosidase activity produced by a reporter gene driven by the *GCN4* promoter and bearing the 5′-UTR of *GCN4*. As shown in Fig. 1*A*, the *GCN4* mRNA levels were decreased approximately 3-fold (±0.2) in this genetic background as compared with a wild-type strain (*GCN4* mRNA, *lane 1 versus lane 2*).

Second, we investigated whether a blockage in the translation derepression of the *GCN4* mRNA had the opposite effect on its levels. The above described analysis was performed using a *gcn2* mutant strain, where the Gcn2 protein kinase has been inactivated. In such strains, translation of *GCN4* mRNA is low and cannot be derepressed (20, 24, Fig. 1*C*, *gcn2*, *+Leu*, *−Leu*). In this background, the steady state amount of *GCN4* mRNA was increased 2.5-fold (±0.3) above the levels of a wild-type strain (Fig. 1*B*, *lanes 1* and *3*).

Third, we introduced into a wild-type and a *gcn2* strain, a plasmid bearing a *GCN4* gene derivative deleted for the four upstream ORFs and thus relieved from translational repression (Δ*ORFGCN4*). As it can be seen in Fig. 2 (*GCN4-LacZ versus* Δ*ORFGCN4-LacZ*, *+Leu*), a *GCN4-LacZ* reporter gene devoid of its 5′-UTR produces under repressing conditions a 50-fold excess of β-galactosidase compared with the one containing the 5′-UTR. It was expected that the resulting increase in the amount of Gcn4 will activate transcription of the *GCN2* gene and thus will induce translational derepression of the *GCN4* mRNA (20). Indeed, in this Δ*ORFGCN4*-transformed wild-type strain, the endogenous *GCN4* mRNA was translationally derepressed (Fig. 1*C*, *wt*, *wt*/Δ*ORFGCN4*, *+Leu*). By contrast and as anticipated, the Δ*ORFGCN4*-induced translational derepression of the endogenous *GCN4* mRNA did not occur in a *gcn2* strain (Fig. 1*C*, *gcn2*, *gcn2*/Δ*ORFGCN4*, *+Leu*). We again observed that the endogenous *GCN4* mRNA was decreased approximately 2-fold (±0.1) in a Δ*ORFGCN4*-transformed wild-type, as compared with a *gcn2* strain (Fig. 1*B*, *lanes 2* and *4*). An interesting observation from this series of experiments was that the steady state levels of the highly translatable Δ*ORFGCN4* mRNA were 1.5-fold (±0.2) lower than those of the endogenous *GCN4* mRNA (Fig. 1*B*, *lanes 2* and *4*). The reduction in the *GCN4* mRNA levels could not be attributed to the Gcn2 protein kinase specifically, since the elevation of the Gcn2-independent *GCN4* mRNA translation that results from overexpression of a variant $tRNA^{Val}$ (25), also effected the same reduction in the *GCN4* mRNA levels (not shown).

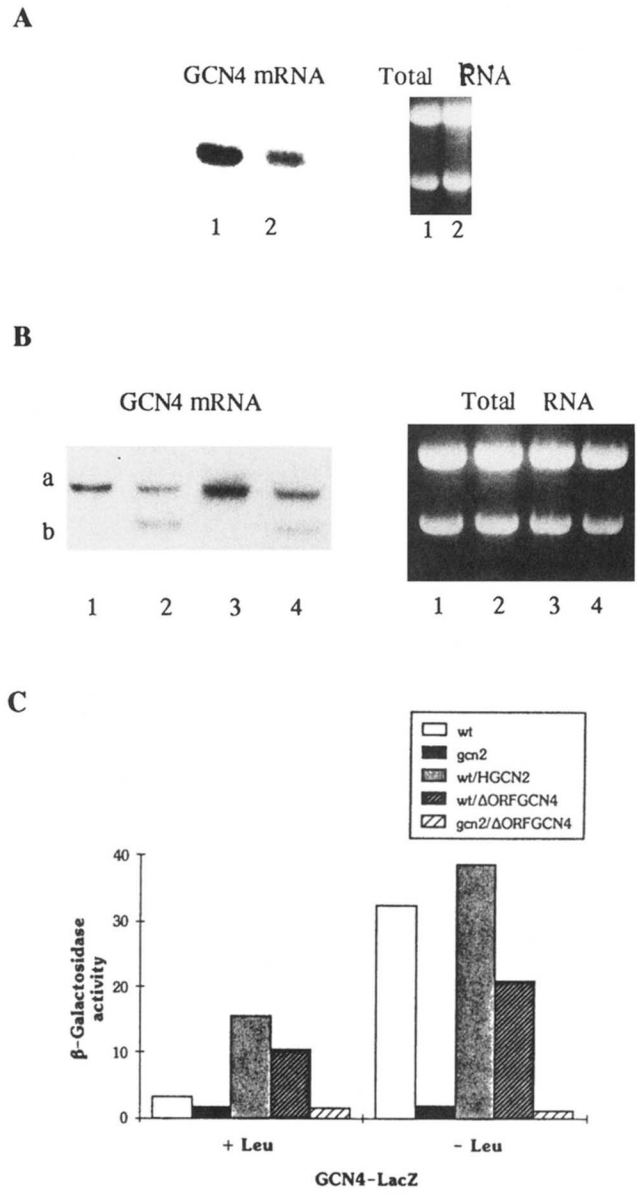

FIG. 1. **Steady state *GCN4* mRNA levels in relation to its translational status.** *A*, the relative amounts of the *GCN4* mRNA in wild-type cells either transformed with a plasmid overexpressing the *GCN2* gene (*HGCN2*, *lane 2*) or with the vector alone (*lane 1*) are shown. The amount of the total RNA blotted is also shown (*right panel*). *B*, wild-type (*lanes 1* and *2*) and *gcn2* (*lanes 3* and *4*) cells were either transformed with a plasmid bearing the Δ*ORFGCN4* gene derivative (*lanes 2* and *4*) or with the vector alone (*lanes 1* and *3*). The longer endogenous (*a*) *GCN4* mRNA migrates slower than the Δ*ORFGCN4* message (*b*). As in *A*, the total RNA amount is depicted in the *right panel*. *C*, graphic representation of the β-galactosidase activity from a *GCN4* based reporter fusion, in the genetic backgrounds depicted under rich (*+Leu*) or poor (*−Leu*), nutritional conditions. Typical experiments are presented. Values are Miller units and represent the average of three independent experiments.

The totality of the evidence presented above, revealed a relationship between the *GCN4* mRNA translational status and its steady state levels. Whenever *GCN4* mRNA was translationally derepressed, its mRNA levels were decreased.

The Transcription of the GCN4 Gene Is Not Affected by Induction of Its Translation—One possible explanation for the above observed variations in the *GCN4* mRNA levels is a de-

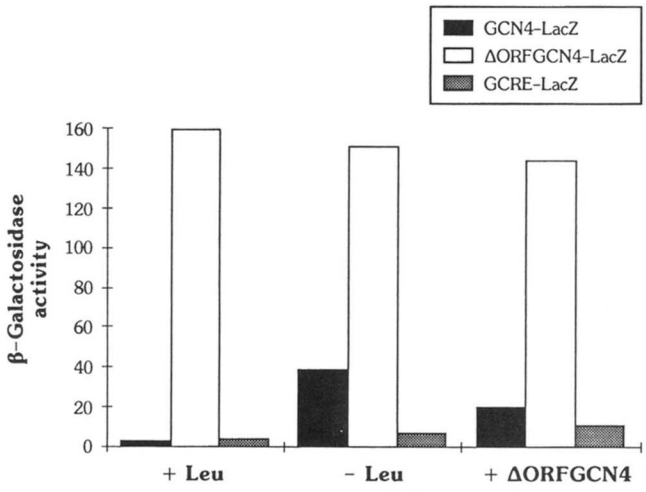

FIG. 2. **Transcription through the *GCN4* promoter in relation to the amount of Gcn4.** Graphic representation of the β-galactosidase activity from the depicted reporter gene fusions under either repressing (*+Leu*), or derepressing (*−Leu*), conditions. The levels of activity a strain transformed with the *ΔORFGCN4* derivative exhibits (*+ΔORFGCN4*), are also depicted. Values are Miller units and represent the average of three independent experiments.

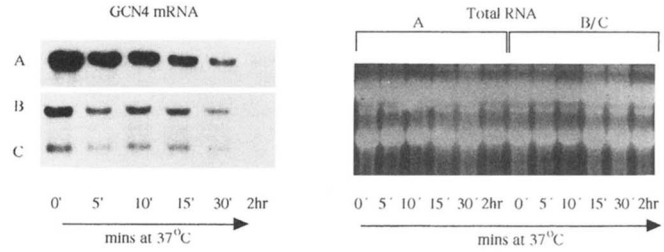

FIG. 3. **Decay rate of *GCN4* mRNA under low and moderate translation rates.** *Left panel*, relative levels of the *GCN4* mRNA were measured at different time points after a shift to 37 °C in a temperature-sensitive RNA polymerase II mutant (*rpb1–1*). *A*, the *GCN4* mRNA decay rate from wild-type cells; *B*, the endogenous *GCN4* mRNA decay rate from cells transformed with the *ΔORFGCN4* gene derivative; *C*, the exogenous *ΔORFGCN4* mRNA decay rate in the above transformed cells. *Right panel*; the amount of the total RNA loaded in each lane. A typical experiment is presented.

crease in the transcription of the *GCN4* gene effected by the known squelching properties of high levels of Gcn4 (26). In order to address this possibility, we examined the expression of a *LacZ* reporter gene driven by the *GCN4* promoter. Cells were grown under nonstarvation conditions for low, starvation conditions for moderate, and transformed with *ΔORFGCN4* for high Gcn4 levels. The intracellular levels of Gcn4 were indirectly monitored by assaying for β-galactosidase produced by a *LacZ* reporter driven by a promoter bearing the Gcn4 DNA binding site (*GCRE-LacZ*, Fig. 2). As shown in Fig. 2, the transcription driven by the *GCN4* promoter was only slightly affected by the intracellular levels of Gcn4. Therefore, the observed differences in the *GCN4* mRNA levels must, for the most part, be the consequence of alterations in the mRNA half-lives under the translation conditions imposed.

The Decay Rate of the GCN4 mRNA Is Not Affected by the GCN4 mRNA Translational Derepression—To determine the *GCN4* mRNA half-life when translated with low or moderate rates, we employed the *rpb1–1* mutant strain (kindly provided by Allan Jacobson). A time course analysis of the levels of a given mRNA in this strain, following a shift from 24 to 37 °C, provides an estimate for its decay rates (2). Two strains were used for such an analysis: the *rpb1–1* strain and the *rpb1–1* strain, transformed with the *ΔORFGCN4* gene derivative to induce the translation of the endogenous *GCN4* mRNA to moderate rates. As shown in Fig. 3, we did not observe any significant differences in the decay rates of the *GCN4* mRNA in these two strains. In both strains, the half-life was approximately 15 min (±1), as it has been reported previously (15). Interestingly enough, the *ΔORFGCN4* mRNA, although highly translated, had a decay rate similar to that of the wild-type message.

GCN4 mRNA Translational Derepression Results in Transient Destabilization of the GCN4 mRNA—Based on our previous results, reporting that during a nutritional downshift *GCN4* mRNA is transiently loaded with heavy polysomes (27), we envisioned a situation where a transient change in the decay rate of the *GCN4* mRNA would take place only immediately after the change in its translational status. When the translation rate would settle to a certain equilibrium, then the *GCN4* mRNA decay rate would be readjusted to its original value. This transient destabilization would then account for the decreased steady state levels.

We addressed the above hypothesis by monitoring the amount of the *GCN4* mRNA under conditions inducing rapid translational derepression. A shift from rich to minimal medium, which was previously shown to result in an acute increase in the translation of the *GCN4* mRNA (27), was imposed to wild-type cells. When the cells were transferred to minimal medium, the amount of *GCN4* mRNA detected within the first 3–5 min after the shift was decreased (Fig. 4*A*). In the reverse experiment, when the cells were returned to rich medium, the relative *GCN4* mRNA amount was increased within the first 5 min.

The short period of time (relative to the normal half-life) required to observe these changes implied an abrupt change in the decay rate of the message. In order to confirm this, we measured decay rates under the above conditions. *rpb1–1* mutant cells, growing at 24 °C in rich medium, were shifted to 37 °C and simultaneously to starvation medium. In a parallel control experiment, only the temperature shift was imposed to the cells. The amount of *GCN4* mRNA detected at the various time points after the double and the single shift is shown in Fig. 4*B*. When just the temperature shift was imposed, the typical decay rate (15 ± 1 min) was observed, as expected (Fig. 4*B*, *row a*). However, when the cells underwent an additional nutritional shift, the *GCN4* mRNA decay rate was increased 2.6-fold (±0.3) within the first 3–5 min (Fig. 4*B*, *row b*). This effect was not a general consequence of the nutritional shift since the decay rates of the *PGK1* mRNA were not similarly affected (Fig. 4*B*). We conclude that the *GCN4* mRNA is destabilized in parallel with a drastic increase in its translational rate.

DISCUSSION

We have revealed the existence of a link between the stability of the *GCN4* mRNA and a transient but drastic change in its translational status. This finding is in agreement with a plethora of published reports indicating that decreased rates of mRNA decay are coupled with a translational blockage imposed either through inhibitors or structural alterations. Our results present an example of a natural mRNA whose translational efficiency determines its decay rates and provide a new natural model through which the mechanism of translationally coupled mRNA decay rates can be studied.

A first indication for a link between translational and decay rates came from measuring the steady state levels of the *GCN4* mRNA. Moderate translational derepression was imposed by increasing *in vivo* the amount of the Gcn2 protein kinase. The alternative of imposing amino acid starvation conditions by utilizing inhibitors of amino acid biosynthesis such as 3-AT (17) was avoided since such treatment results in stabilization of mRNAs carrying nonsense codons (28). In all cases examined, an inverse influence of translation on the *GCN4* mRNA levels

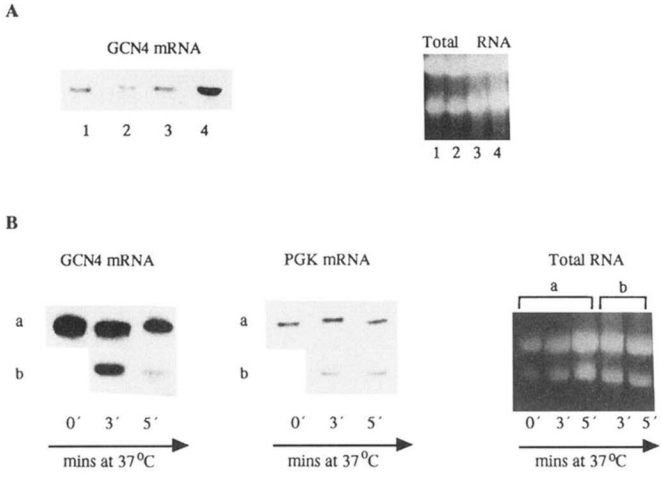

FIG. 4. **GCN4 mRNA levels and decay rates during a nutritional shift.** *A*, wild-type cells grown in rich medium (*lane 1*) were shifted to amino acid starvation medium, and RNA was isolated after 5 min (*lane 2*). In the reciprocal experiment, cells grown in starvation medium (*lane 3*) were shifted to rich medium, and RNA was isolated after 5 min (*lane 4*). The respective amount of the total RNA is depicted in the *right panel*. *B*, *rpb1–1* mutant cells grown in rich medium at 24 °C (*0′* common for *a* and *b* series), underwent either a single temperature shift to 37 °C (*3′* and *5′* for a series) or an additional nutritional shift to starvation medium (*3′* and *5′* for *b* series). *Left panel*, the *GCN4* mRNA levels after 0, 3, and 5 min at 37 °C are shown. *Central panel*, the *PGK1* mRNA levels were analyzed as in the *left panel*. In the *right panel*, the amount of the total RNA used for the *left* and *central panels*, is shown. Typical experiments are presented.

was observed, which was not the result of altered transcriptional rates. Such a drastic decrease in steady state levels was not easily justified as a consequence of a change in the translational status, since these conditions result in only a slight shift of the polysomal distribution of the *GCN4* mRNA toward heavier polysomes (26). Indeed, the measured decay rates of the *GCN4* mRNA were unaffected under conditions of low or moderate translation. The apparent paradox concerning the determinants of the *GCN4* mRNA steady state levels could be resolved only by considering a transient change in the decay rate that readjusts the final steady state levels.

In order to solve this problem, we followed the *GCN4* mRNA decay rates in a temporal manner. It was known that a transient and dramatic increase in the rate of the *GCN4* mRNA translation is effected rapidly after the removal of amino acids from the growth medium (27). Indeed, by using such a temporal analysis during a nutritional shift, we revealed that the decay rate of the *GCN4* mRNA dramatically increases within a very short period of time following the initiation of translational derepression. Therefore, based on the sensitivity of the method employed, we may conclude that a transient destabilization of the *GCN4* mRNA is tightly coupled with its shift to heavy polysomes. The possibility that the observed transient phenomenon could be due to unrelated physiological changes is unlikely, since the decay rates of the reference mRNA (*PGK1*) remained constant during the experiment.

Two major conclusions can be drawn from the combined interpretation of the above experimental results. One is that the apparent increased decay rate of *GCN4* mRNA is coupled with its increased access to heavy polysomes. The second is that the apparent decrease in the steady state levels of this message under conditions of moderate translation should be the consequence of a transient destabilization inherited in the physiology of translational derepression of the *GCN4* mRNA. This interpretation emphasizes the fact that the understanding of steady state phenomena should consider the dynamic process of their establishment.

It is worthwhile to discuss our observations concerning the decay rates of the Δ*ORFGCN4 mRNA*. This message is degraded with the same rate as the endogenous *GCN4* mRNA, even though it is always associated with a larger number of ribosomes (27). It should be noted however, that this is a different mRNA, lacking most of the 5′-UTR containing the upstream ORFs. One could consider that this region contains a destabilizing element that adds to the observed decay rate of the *GCN4* mRNA, whereas the Δ*ORFGCN4* mRNA is subjected mainly to the ribosome associated degradation machinery. It is tempting to speculate that this instability is mediated by the proposed nonsense mRNA decay mechanism (29) due to the presence of the four small ORFs. We suggest that the net effect of these two opposing mechanisms contributes to the overall decay rate of the *GCN4* mRNA.

The coupling of translational derepression with changes in mRNA decay rates poses several questions regarding the nature of this phenomenon. Is it just the consequence of a physical association between the *GCN4* mRNA and the ribosomes on which the decay machinery might reside, or is *GCN4* mRNA degradation an additional step in *GCN4* regulation? Given the body of information that has been accumulated, we believe that the former is true. The apparent contradiction in the biology of the response between the translational derepression and the increased decay rates for the overall increase in the amount of Gcn4, could be resolved by considering the recently demonstrated increased stability of this protein under amino acid starvation conditions (30).

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