An upstream ORF with non-AUG start codon is translated in vivo but dispensable for translational control of GCN4 mRNA

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ABSTRACT

Genome-wide analysis of ribosome locations in mRNAs of Saccharomyces cerevisiae has revealed the translation of upstream open reading frames that initiate with near-cognate start codons in many transcripts. Two such non-translation initiation codon (AUG)-initiated upstream open reading frames (uORFs) (nAuORFs 1 and 2) occur in GCN4 mRNA upstream of the four AUG-initiated uORFs (uORFs 1–4) that regulate GCN4 translation. We verified that nAuORF2 is translated in vivo by demonstrating β-galactosidase production from lacZ coding sequences fused to nAuORF2, in a manner abolished by replacing its non-AUG initiation codon (AUA) start codon with the non-cognate triplet AAA, whereas translation of nAuORF1 was not detected. Importantly, replacing the near-cognate start codons of both nAuORFs with non-cognate triplets had little or no effect on the repression of GCN4 translation in non-starved cells, nor on its derepression in response to histidine limitation, nutritional shift-down or treatment with rapamycin, hydrogen peroxide or methyl methanesulfonate. Additionally, we found no evidence that initiation from the AUA codon of nAuORF2 is substantially elevated, or dependent on Gcn2, the sole eIF2α kinase of yeast, in histidine-deprived cells. Thus, although nAuORF2 is translated in vivo, it appears that this event is not stimulated by eIF2α phosphorylation nor significantly influences GCN4 translational induction under various starvation or stress conditions.

INTRODUCTION

Regulated expression of the GCN4 gene in Saccharomyces cerevisiae provides an evolutionarily conserved paradigm of translational control, and an important validation of the scanning mechanism of translation initiation in eukaryotes (1). GCN4 encodes a bZIP transcriptional activator that induces the expression of the majority of amino acid biosynthetic enzymes in response to starvation for any amino acid, a cross-pathway regulatory response dubbed general amino acid control. Amino acid starvation increases the level of Gcn4 protein, in large part, by stimulating the translation of GCN4 mRNA. The induction of Gcn4 is also augmented by a ~2-fold increase in GCN4 mRNA abundance and by stabilization of Gcn4 protein in response to prolonged or severe starvation. The trans-acting factors that regulate GCN4 translation have general functions in protein synthesis initiation, or regulate the activities of such factors, and the molecular events that induce GCN4 translation reduce the rate of general translation initiation. This dual regulatory response enables cells to limit consumption of amino acids in general protein synthesis, while increasing their amino acid biosynthetic capacity by induction of Gcn4 and its target genes under conditions of amino acid limitation. Remarkably, mammalian cells use the same strategy to downregulate protein synthesis and induce transcriptional activators under various stress conditions, including amino acid starvation [reviewed in (2,3)].

The translation initiation pathway begins with the binding of Met-tRNAiMet to the small (40S) ribosome to form the 43S preinitiation complex. The Met-tRNAiMet is transferred to the 40S subunit in a ternary complex (TC) with initiation factor 2 (eIF2) in its active, guanosine triphosphate (GTP)-bound form. The 43S complex binds to the m7G-capped 5′-end of the mRNA, and scans the mRNA leader for an initiation codon (AUG). On base pairing of the Met-tRNAiMet kinase of yeast, in histidine-deprived cells. Thus, although nAuORF2 is translated in vivo, it appears that this event is not stimulated by eIF2α phosphorylation nor significantly influences GCN4 translational induction under various starvation or stress conditions.

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stress-activated protein kinases, including Gcn2 in both yeast and mammals, that phosphorylate Ser-51 in the α-subunit of eIF2. As eIF2(αP) is a competitive inhibitor of eIF2B, phosphorylation of only a fraction of eIF2 reduces the rate of protein synthesis, but simultaneously stimulates translation of GCN4 mRNA (4,5).

The paradoxical induction of GCN4 translation by eIF2α phosphorylation mediated by the four short open reading frames (uORFs) in the leader of GCN4 mRNA [(reviewed in (2))]. In nutrient-replete cells, essentially all of the ribosomes that scan from the cap, will translate uORF1, and after terminating at the uORF1 stop codon, ~50% of the 40S subunits resume scanning downstream, owing to a permissive sequence context at uORF1 and a function of eIF3 in retaining post-termination 40S subunits (6). Virtually all of these ‘re-scanning’ 40S subunits (uORF3 in retaining post-termination 40S subunits (6). Virtually all of these ‘re-scanning’ 40S subunits rebind the TC in time to reinitiate translation at uORFs 3 or 4, and because of non-mergence, surrounding stopping codons at these downstream uORFs, they subsequently dissociate from the mRNA and fail to translate GCN4. When the TC level is reduced in starvation conditions by Gcn2 phosphorylation of eIF2α, a fraction (≤50%) of the 40S subunits that resume scanning after terminating at uORF1 do not rebind TC until after bypassing uORFs 3–4, enabling them to reinitiate at GCN4 instead. Thus, a reduction in the TC level shifts the probability of reinitiation by the post-termination 40S subunits generated at uORF1 from the inhibitory uORFs 3–4 to the GCN4 start codon (2).

The dual response to amino acid starvation by eIF2α phosphorylation elucidated in yeast also operates in mammalian cells, as the mRNAs encoding transcription factors Atf4 (7,8) and Atf5 (9) are translationally regulated according to the GCN4 paradigm. Interestingly, translational control by mammalian Gcn2 is important for lipid homeostasis under starvation conditions (10), in behavioral aversion to amino acid-deficient diets (11), and in learning and memory (12). Mammals also contain three other eIF2α-Ser51 kinases, which exhibit extensive sequence similarities in their kinase domains but are activated by different stresses via distinct regulatory regions: PKR (virus infection), PERK (ER stress), and HRI (hemin starvation) (13).

Most of the experimental evidence supporting the model for GCN4 translational control is genetic, involving the effects of mutations in the uORFs or surrounding sequences, or insertions or deletions that alter the spacing between uORFs and the GCN4 ORF, on the translational efficiencies of GCN4 or GCN4-lacZ reporter mRNAs [(reviewed in (2)]. Moreover, key predictions of the model are supported by biochemical data obtained using a cell-free translation system in which the positions of 80S initiation complexes (stalled with the elongation inhibitor cycloheximide) were mapped by primer extension inhibition (toeprint) analysis. These data confirmed (i) that ribosomes scan linearly from the 5'-end of GCN4 mRNA and form initiation complexes at each of the four uAUGs, with a strong preference for the (5′ proximal) uAUG-1, (ii) that uORF1 allows greater reinitiation at the GCN4 AUG than does uORF4, (iii) that uORF1 enables scanning ribosomes to bypass uORF4 and initiate at GCN4 instead, and (iv) that functional eIF2 promotes reinitiation at uORF4 at the expense of the GCN4 start codon (14).

Supporting evidence for the proposed mechanism of GCN4 translational control has also come from biochemical analysis of GCN4 mRNA translation in vivo. Measuring the average size of cycloheximide-arrested polysomes formed on GCN4 mRNA demonstrated that this transcript is largely non-polysomal in nutrient rich, repressing conditions and a proportion of the mRNA enters the polysome pool during steady-state starvation for histidine in a manner requiring Gcn2. Moreover, uORFs 2–4 were found to be necessary and sufficient to restrict GCN4 mRNA to the non-polysomal pool in repressing conditions (15). Using ‘ribosome density mapping’, in which the numbers of ribosomes associated with specific segments of an mRNA can be assessed, it was confirmed that ribosomes occupy GCN4 mRNA leader segments containing either uORFs 1–2 or uORFs 3–4, under both repressing and derepressing conditions, but they occupy the GCN4 coding sequences only under derepressing conditions (16).

More recently, a high-resolution ribosome mapping technique was employed, involving deep sequencing of ribosome-protected mRNA fragments, in which the occupancies of cycloheximide-stalled 80S ribosomes were quantified at sub-codon resolution on mRNAs genome wide (17). This study revealed high-level ribosome occupancy of uORF1, modest but significant occupancies of uORFs 2–4, and low-level occupancies of the GCN4 ORF in non-starved cells growing in rich medium. Consistent with the idea that uORF1 translation enables reinitiating ribosomes to bypass uORFs 2–4 to translate GCN4, withdrawal of amino acids from an auxotrophic strain reduced the 80S occupancies of uORFs 2–4 but substantially increased occupancy of the GCN4 ORF, with little effect at uORF1.

Surprisingly, relatively high 80S occupancies were also observed at two regions upstream of the GCN4 uORF1, which coincide with coding sequences of 8 and 34 codons beginning with the near-cognate start codons AUA and UUG, respectively. The 8-codon uORF beginning with AUA is located at the 3′-end of the longer, 34-codon uORF initiating with UUG, and they share the same reading frame and stop codon (Figure 1). [Henceforth, we refer to upstream ORFs initiated with non-AUGs as nAuORFs (for non-AUG uORFs) and reserve the term uORF only for upstream ORFs with AUG start codons]. The fact that nAuORF2 exhibits a much higher ribosome density compared to the non-shared codons of nAuORF1, provides evidence for independent initiation events at these nAuORFs. Interestingly, the ribosome occupancies of both nAuORFs 1 and 2 increased considerably in response to amino acid starvation. In fact, this study provided evidence for the translation of 143 different nAuORFs, which accounted for 20% of the ribosome footprints detected in mRNA leader sequences genome wide. Furthermore, the nAuORFs as a group showed marked increases in ribosome occupancy during amino acid starvation (17).

The occurrence of high-level ribosome occupancy at nAuORFs 1 and 2 raises the obvious question of
whether these elements participate in \textit{GCN4} translational control. Because ribosomes must be in the reinitiation mode to efficiently bypass uORFs 2-4 when TC levels decline, the prior translation of uORF1 is a prerequisite for efficient translational induction of \textit{GCN4} in starved cells. Accordingly, mutations that remove the uORF1 start codon (uAUG-1), or disrupt its surrounding sequence elements necessary for efficient reinitiation, all impair the induction of \textit{GCN4} translation. As such, they confer sensitivity to inhibitors of amino acid biosynthesis, such as 3-aminotriazole (3-AT), which impairs the histidine biosynthetic enzyme encoded by \textit{HIS3}. Interestingly, this \textit{Gcn}^\textit{C0} (general control non-inducible) phenotype was described previously for a 40-nt deletion that removes sequences between 22 and 61 nt upstream of uAUG-1. This finding, combined with the \textit{Gcn}^\textit{C0} phenotypes observed for various other mutations that altered sequences upstream of uORF1, led to the conclusion that sequences between 22 and 61 nt 5' of uORF1 contribute to the unusually high frequency of reinitiation following translation of uORF1 (18). Interestingly, this interval includes the stop codon and penultimate 5 codons of the two nAuORFs described above.

We set out to determine whether the nAuORFs detected by Ingolia \textit{et al.} (17) have a role in \textit{GCN4} translational control by mutating their near-cognate initiation codons, UUG and AUA, to the non-cognate start codons CUC or AAA. It was shown previously that UUG, AUA and five other near-cognate triplets, which differ at only one position from AUG, exhibit significant levels of translation initiation of a reporter mRNA \textit{in vivo}, at levels between 1% and 7% of that observed for AUG. In contrast, a CUC start codon, which differs from AUG at two positions, or the near-cognates containing purines at the second position of the codon, allowed no detectable translation initiation (19). Hence, it is expected that replacing the UUG and AUA start codons of the nAuORFs with CUC or AAA should eliminate initiation at these sequences and abolish any possible effect on \textit{GCN4} translational control. By constructing a \textit{lacZ} fusion to the overlapping nAuORFs 1 and 2, we have obtained evidence that nAuORF2 translation by...
substituting its AUA start codon had no significant effect on repression of GCN4 in non-starvation conditions, nor its derepression during histidine-limited growth, in a nutritional shift-down from amino acid-rich to minimal medium, or in response to various other stresses that activate GCN4 translation. These results rule out an important function for the two nAuORFs in the derepression of GCN4 translation mediated by the conventional uORFs under the starvation and stress conditions examined here.

MATERIALS AND METHODS

Strains and plasmids

The plasmids employed in this work are listed in Table 1. Plasmids pLfz450, pLfz453, pLfz456, pLfz469, pLfz470, pLfz460, pLfz463, pLfz466, pLfz473 and pLfz474, harboring point mutations at nAuORF start codons, were constructed as follows. Fusion polymerase chain reaction (PCR) was employed to generate SacII–BstEII fragments bearing the appropriate mutations, using primers listed in Table 2, and p164 DNA as template. The SacII–BstEII interval encompasses the GCN4 promoter, start site of transcription and mRNA leader sequences extending from −573 to −138. The resulting fragments were used to replace the cognate SacII–BstEII fragments of p164 or p180 to create the final GCN4 or GCN4-lacZ constructs, respectively. Plasmid pLfz482 was constructed as just described except using p227 DNA as template, harboring point mutations in the AUG codons of uORFs 1–4. All PCR fusions except using p227 DNA as template, harboring point mutations at nAuORF start codons, were constructed as described previously (22). Expression of GCN4-lacZ, uORF-lacZ and nAuORF-lacZ fusions was measured by assaying β-galactosidase in whole-cell extracts (WCEs) as described previously (23). Expression of native Gcn4 was measured by western analysis of WCEs using

| Plasmid Name | Gene | Allele Description | Reference |
|--------------|------|--------------------|-----------|
| p164         | GCN4 | WT                 | (37)      |
| p237         | GCN4 | uORF4-only         | (26)      |
| p238         | GCN4 | uORF-less          | (26)      |
| p180         | GCN4-lacZ | WT               | (37)      |
| p226         | GCN4-lacZ | uORF4-only     | (26)      |
| p227         | GCN4-lacZ | uORF-less       | (26)      |
| p466         | uORF1-lacZ | uORF1-lacZ, "FG" construct | (25) |
| p367         | HIS4-lacZ | ATG start codon | (38) |
| p391         | HIS4-lacZ | TTG start codon | (38) |
| pLfz450      | GCN4 | nAuORF1 UUG to CUC | This study |
| pLfz453      | GCN4 | nAuORF2 AUA to CUC | This study |
| pLfz456      | GCN4 | nAuORF1 UUG to CUC, nAuORF2 AUA to CUC | This study |
| pLfz469      | GCN4 | nAuORF2 AUA to AAA | This study |
| pLfz470      | GCN4 | nAuORF1 UUG to CUC, nAuORF2 AUA to AAA | This study |
| pLfz460      | GCN4-lacZ | nAuORF1 UUG to CUC | This study |
| pLfz463      | GCN4-lacZ | nAuORF2 AUA to CUC | This study |
| pLfz466      | GCN4-lacZ | nAuORF1 UUG to CUC, nAuORF2 AUA to CUC | This study |
| pLfz473      | GCN4-lacZ | nAuORF2 AUA to AAA | This study |
| pLfz474      | GCN4-lacZ | nAuORF1 UUG to CUC, nAuORF2 AUA to AAA | This study |
| pLfz482      | GCN4-lacZ | nAuORF1 UUG to CUC, nAuORF2 AUA to AAA in uORF-less backbone | This study |
| pLfz489      | nAuORF-lacZ | WT              | This study |
| pLfz491      | nAuORF-lacZ | nAuORF2 AUA to AAA | This study |
| pLfz493      | nAuORF-lacZ | nAuORF1 UUG to CUC | This study |
| pLfz495      | nAuORF-lacZ | nAuORF1 UUG to CUC, nAuORF2 AUA to AAA | This study |

Analyses of GCN4 expression

Sensitivity to 3-aminotriazole (3-AT) was analyzed as described previously (22). Expression of GCN4-lacZ, uORF-lacZ and nAuORF-lacZ fusions was measured by assaying β-galactosidase in whole-cell extracts (WCEs) as previously described (23). Expression of native Gcn4 was measured by western analysis of WCEs using

Table 1. Plasmids used in this study
affinity-purified antibodies against Gcn4, as described previously (24).

RESULTS

nAuORF2 is translated in vivo from its AUA start codon

To investigate whether the nAuORFs are translated in vivo, we mutated their shared TAA stop codon (at position −407 relative to the GCN4 AUG, Figure 1) and fused lacZ coding sequences at this position in-frame with both nAuORFs, as they occur in the same reading frame. We also generated variants of this nAuORF-lacZ construct in which the UUG start codon of nAuORF1 was replaced with the non-cognate triplet CUC, and the AUA start codon of nAuORF2 was replaced by non-cognate AAA, either singly or in combination (schematized in Figure 2A, constructs 5–8). As noted above, replacing AUG with CUC, or with triplets containing A or G at the second nucleotide of the codon, completely abolished translation of a luciferase reporter mRNA in yeast cells (19). Hence, we reasoned that either CUC or AAA replacements of the near-cognate start codons of the nAuORFs would abolish their recognition in vivo. β-Galactosidase production in strains containing the mutant or WT nAuORF-lacZ constructs was assayed after growth in non-starvation conditions or in medium containing 3-AT to provoke histidine starvation. As controls, we assayed expression of GCN4-lacZ and uORF1-lacZ fusions, respectively (Figure 2C, columns 1–2 and 5–6). The relatively low level of expression from nAuORF-lacZ is consistent with the previous finding that luciferase reporter genes with UUG or AUA start codons are expressed at ~4–5% of the level observed with an AUG start codon (19). The nAuORF-lacZ fusion displayed a somewhat greater induction by 3-AT, a ~1.7-fold increase (Figure 2B, columns 3–4), compared to the ~1.1-fold and ~1.2-fold induction observed for the uORF-less GCN4-lacZ and uORF1-lacZ fusions, respectively (Figure 2C, columns 1–2 and 5–6).

Expression of the nAuORF-lacZ variant with the UUG start codon of nAuORF1 replaced with CUC (nAuORF1CUC-lacZ) was only ~10% lower than that of WT nAuORF-lacZ, and this difference was not statistically significant (Figure 2B, columns 7–8 versus 3–4). Importantly, however, replacing the AUA start codon of nAuORF2 with AAA (nAuORF2AAA-lacZ) nearly abolished nAuORF-lacZ expression, reducing it by 98% and 95% under non-starvation and starvation conditions, respectively (Figure 2B, columns 5–6 versus 3–4). A similar strong reduction in β-galactosidase expression was observed for the nAuORF_CUC,AAA-lacZ variant containing the substitutions in both UUG and AUA start codons of the nAuORFs (Figure 2B, columns 9–10 versus 3–4). In contrast, these mutations had no significant effect on β-galactosidase production when introduced into the uORF-less GCN4-lacZ construct (Figure 2C, columns 3–4 versus columns 1–2), arguing against a non-translational mechanism for their deleterious effect on nAuORF-lacZ expression. Taken together, the results obtained from substituting the UUG and AUA start codons of the nAuORFs indicate that most, if not all,
translation of nAuORF2 initiates at the AUA start codon of nAuORF2. This conclusion is in general agreement with the findings of Ingolia et al. (17) that revealed considerably higher occupancies of 80S ribosomes in the nAuORF2 portion of these overlapping coding sequences. Finally, we noted that expression of the nAuORF1CUC-lacZ fusion (lacking the nAuORF1 UUG start codon) increased by ~1.7 in response to 3-AT, which might...
indicating a modest increase in initiation at the (remaining) nAuORF2 AUA start codon during amino acid starvation. This last issue is considered further below.

**Substituting the nAuORF start codons with non-cognate triplets has little effect on GCN4 translational control**

Having obtained evidence that nAuORF2 is translated in vivo, we examined the effects of substituting its AUA start codon with non-cognate triplets on translational induction of GCN4. We first tested the effects of such mutations on the ability of plasmid-borne GCN4 to complement the 3-AT-sensitive (3-ATS) phenotype of a gcna4A mutant. As noted above, the absence of uORF1 increases sensitivity to 3-AT, owing to a marked reduction in the proportion of reinitiating 40S subunits able to bypass uORFs 2–4 when eIF2α is phosphorylated by Gen2. Hence, the GCN4 allele containing uORF4 only (with point mutations in uAUGs 1–3, construct 2 in Figure 3A) confers a reduced level of 3-AT resistance compared to the WT GCN4 allele, which is most evident at 30 mM 3-AT (Figure 3B, cf. rows 2–3). The GCN4 construct lacking all four uORFs (uORF-less construct 3 in Figure 3A) confers even stronger 3-AT resistance (3-ATR) than does WT GCN4 (Figure 3B, 15 mM 3-AT, cf. rows 2 and 4), which is expected from previous findings that the inhibitory effects of uORFs 2–4 on GCN4 translation are not fully overcome by the derepression mechanism provided by uORF1 and Gen2 in histidine-starved cells (26).

GCN4 alleles containing the non-cognate CUC triplet substituting either the UUG start codon of nAuORF1, the AUA start codon of nAuORF2, or both near-cognate start codons simultaneously, complemented the gcna4A mutant indistinguishably from WT GCN4 (Figure 3B, 30 mM 3-AT, row 2 versus 5–7). Importantly, GCN4 alleles with AAA substituting the AUA start codon of nAuORF2, and one containing both CUC and AAA substitutions in nAuORF1 and nAuORF2, respectively, i.e. the mutations shown above to abolish translation of the nAuORF-lacZ fusion, also provided WT complementation of the gcna4A mutant (Figure 3C, cf. rows 2 and 5–6). These findings suggest that inactivating translation of nAuORF2 by replacing its near-cognate AUA with a non-cognate CUC or AAA triplet does not perturb induction of GCN4 translation in response to histidine starvation.

To examine more directly the effects of these mutations on GCN4 induction, and also to investigate their effects on maintaining the repressed state of GCN4 translation in non-starvation conditions, we conducted western analysis of Gcn4 in WCEs after growing the strains just described in non-starvation conditions or for 2 h in the presence of 3-AT. As expected, Gcn4 was strongly induced by 3-AT in the WT GCN4 strain but showed a reduced level of induction in the strain containing the uORF4-only GCN4 allele (Figure 3D, lanes 4–6 versus 7–9). As discussed below, much of the residual induction of Gcn4 given by the uORF4-only construct likely reflects the non-translational components of Gcn4 induction that compensate for diminished translational activation in the absence of uORF1. The GCN4 alleles containing an AAA substitution in the start codon of uAuORF2, or with CUC and AAA substitutions in the start codons of both nAuORFs, conferred 3-AT-induced levels of Gcn4 that were indistinguishable from that conferred by WT GCN4 (Figure 3D, lanes 13–18 versus 4–6). Furthermore, there was no evidence of derepression of Gcn4 production in non-starvation conditions for these two mutant constructs (Figure 3D, cf. lanes 4, 13 and 16) in the manner observed for uORF-less GCN4 (Figure 3D, lane 10 versus 4). Thus, substituting the near-cognate start codons of the nAuORFs with non-cognate triplets had no discernible effect on the induction of Gcn4 in histidine-starved cells or its repression in non-starvation conditions.

To assay more explicitly the effects of the nAuORF mutations on translation of GCN4, we examined their effects on expression of the GCN4-lacZ reporter described above, by assaying the constructs depicted schematically in Figure 4A. Although the WT GCN4-lacZ reporter (construct 1) displays the ~2-fold increase in mRNA expression in amino acid-starved WT cells (26) exhibited by native GCN4 mRNA (27), it lacks the determinants of regulated protein stability in Gcn4 (28) and, hence, excludes the component of GCN4 control operating at the level of protein degradation. As shown previously (26), the absence of uORFs 1–3 in the uORF4-only version of GCN4-lacZ (construct 2) evokes a marked reduction in β-galactosidase expression under starvation conditions, decreasing the induction ratio to only a factor of ~2 (Figure 4B, cf. columns 3–4 and 1–2), which is comparable to the 2-fold increase in GCN4-lacZ reporter mRNA measured previously for this construct (26). Importantly, the GCN4-lacZ alleles containing a CUC substitution in the start codons of one or both of the nAuORFs, or with an AAA substitution in the start codon of nAuORF2, produced β-galactosidase at levels that were not significantly different from that given by the WT fusion, in both non-starved and histidine-starved cells (Figure 4B, columns 5–10 versus 1–2; Figure 4C columns 5–8 versus 1–2). These findings are in accordance with the conclusions reached from western analysis of Gcn4 expression that both nAuORFs are dispensable for efficient repression of GCN4 translation in non-starvation conditions, and that neither is required for efficient induction of GCN4 translation in histidine-starved cells.

In the ribosomal profiling experiments of Ingolia et al. (17), amino acid limitation was imposed by shifting a his3Δ leu2Δ met15Δ auxotrophic strain from amino acid-rich medium to minimal medium lacking all amino acids for 20 min, rather than using 3-AT treatment to provoke sustained histidine limitation. Hence, we considered the possibility that the nAuORFs might affect translational control of GCN4 during the nutritional shift-down conditions employed by Ingolia et al. (17). To examine this possibility, we compared the induction of Gcn4 protein from WT GCN4 versus the mutant allele containing CUC and AAA substitutions in nAuORF1 and nAuORF2 in a leu2Δ met15Δ auxotroph after shifting cells from amino acid-complete to minimal
Figure 3. Eliminating the near-cognate start codons of nAuORFs 1 and 2 has no effect on regulation of Gcn4 protein levels in non-starved cells, histidine-limited cells, or during nutritional shift-down. (A) Schematics of GCN4 alleles. The wild-type (1) or mutant (2–8) GCN4 alleles under examination, depicted as described in Figure 2A (contained on plasmids p164, p237, p238, pLfz450, pLfz453, pLfz456, pLfz469 and pLfz470, respectively). (B and C) Substituting the start codons of nAuORF1 and 2 has no effect on complementation of gcn4D by mutant GCN4 alleles. Transformants of gcn4D strain H2835 harboring the indicated GCN4 alleles described in (A) were cultured in SC-Ura to saturation and serial 10-fold dilutions were spotted on SC-Ura plates or SC-Ura,-His plates supplemented with 15 mM or 30 mM 3-AT (and excess leucine to exacerbate the Gcn/C0 phenotype) and incubated at 30°C for 2–3 days. Essentially identical results were obtained for an independent set of transformants for these groups of constructs (data not shown). (D and E) Substituting the start codons of nAuORF1 and 2 has no effect on regulated expression of Gcn4 protein in response to histidine starvation or nutritional shift-down. (D) Histidine starvation. Strains described in (B–C) were cultured as described in Figure 2 for assaying β-galactosidase, except that they were induced with 3-AT for only 2 h. WCEs were prepared under denaturing conditions by extraction with trichloroacetic acid and aliquots representing equal proportions of total WCE (or 2X of this amount) were resolved by SDS–PAGE and subjected to western analysis using antibodies against Gcn4 or, to provide a loading control, the eIF2B subunit Gcd6, which is not under GCN4 control. Triangles depict loading of 1X and 2X amounts of the same WCE in successive lanes. (E) Nutritional shift-down. Strains described in (B–C) were cultured in SC-Ura to A600 of 0.8–1.0 and aliquots were collected by centrifugation, resuspended in SD and incubated for 20 min prior to harvesting. WCEs were prepared and subjected to western analysis as in (D). U, uninduced; I, induced by 3-AT; N, non-starved; S, starved by nutritional shift-down.
medium lacking all amino acids. There was no observable difference in the induction of Gcn4 from these two alleles (Figure 3E, lanes 1–3 versus 4–6). As expected, the induction of Gcn4 under these conditions was not observed for the uORF4-only allele, and the high-level of Gcn4 protein produced by the uORF-less construct was not augmented by nutritional shift-down (Figure 3E, lanes 7–12). We conclude that nAuORFs 1 and 2 are dispensible for translational induction of \textit{GCN4} during nutritional shift-down of an auxotroph.

Finally, we considered the possibility that the nAuORFs might be important for translational induction of \textit{GCN4} in response to stresses besides nutrient deprivation, including oxidative stress imposed with hydrogen peroxide (29), inhibition of the TORC1 complex containing protein kinases Tor1 or Tor2 with the drug rapamycin (30,31), and the alkylation agent methyl methanesulfonate (32), all of which are known to induce \textit{GCN4} translation in nutrient replete medium. As shown in Figure 5, these three treatments evoked derepression of the

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**Figure 4.** Eliminating the near-cognate start codons of nAuORFs 1 and 2 has no effect on regulated GCN4-lacZ expression in non-starved or histidine-limited cells. (A) Schematics of GCN4-lacZ alleles. The wild-type (1) or mutant (2–7) GCN4-lacZ alleles under examination, depicted as described in Figure 2A (contained on plasmids p180, p226, pLiz460, pLiz463, pLiz466, pLiz473 and pLiz474, respectively). (B and C) Regulation of GCN4-lacZ expression is not altered by mutations in the nAuORF start codons. Transformants of GCN4 strain H2833 harboring the indicated lacZ constructs described in (A) were cultured in the absence (U) or presence (I) of 3-AT and WCEs were assayed for units of \(\beta\)-galactosidase, as described in Figure 2B and C.
GCN4-lacZ fusion to different extents, but in all cases there was no significant reduction in expression conferred by substituting both near-cognate start codons of nAuORFs 1 and 2 with non-cognate triplets. Thus, the nAuORFs are not required for wild-type induction of GCN4-lacZ expression in response to these stress conditions.

Initiation at near-cognate start codons at GCN4 and HIS4 is not substantially increased by histidine starvation or dependent on Gcn2

As noted above, ribosome profiling analysis indicated that the 80S occupancies of the GCN4 nAuORFs 1 and 2, and of many nAuORFs across the genome, increased markedly during amino acid starvation, and it was proposed that initiation at non-AUG codons might represent a previously undetected response to increased eIF2α phosphorylation (17). As shown above, the nAuORF-lacZ fusion, which reports on initiation at the AUA codon of nAuORF2, exhibits a 1.7-fold increase in β-galactosidase production in response to 3-AT treatment (Figure 2B, columns 3–4). Examination of an isogenic gcn2Δ mutant revealed that eliminating Gcn2 produced a somewhat higher, rather than lower, level of nAuORF-lacZ expression under starvation conditions (Figure 6A, cf. columns 2 and 4). Thus, the moderate increase in nAuORF-lacZ expression observed in histidine-starved cells occurs independently of Gcn2. We also examined the effect of histidine starvation on initiation at a UUG start codon at HIS4. As observed previously [e.g. (33)], β-galactosidase production from a HIS4-lacZ fusion containing UUG in place of the normal AUG start codon occurs at ~3% of the level observed for the matching wild-type HIS4-lacZ fusion with an AUG codon under non-starvation conditions. The ratio of UUG:AUG initiation measured with these fusions was not significantly increased by 3-AT treatment (P-value = 0.10) (Figure 6B). Thus, the translation rates of the two different lacZ reporters containing near-cognate start codons described in Figure 6A and B show relatively little or no increase under conditions of histidine starvation shown previously to induce eIF2α phosphorylation and depression of GCN4 translation (34), and the moderate increase observed for nAuORF-lacZ is independent of Gcn2 (Figure 6A).

DISCUSSION

Our finding that replacing the AUA start codon of nAuORF2 with the non-cognate triplet AAA abolished β-galactosidase production from the nAuORF-lacZ construct supports the conclusion reached from ribosome profiling (17) that the AUA start codon of GCN4 nAuORF2 is recognized in vivo. At the same time, it suggests that the UUG start codon of nAuORF1 is utilized very poorly, if at all, as a start codon under the conditions of our experiments. These conclusions are consistent with the fact that the sequence context of the nAuORF2 start codon, A_{-3}A_{-2}A_{-1}AUAU_{+4}, conforms...
well to the preferred sequence context defined recently by Chen et al. for a naturally occurring UUG initiation codon at the yeast GRS1 gene, of A−3A−2(A/G)−1UUGA+4, with the A at −3 exerting the greatest effect and the A at +4 the least effect on initiation frequency (35). In contrast, the sequence context of the GCN4 nAuORF1 start codon, U−3U−2U−1UUGC+4, diverges at all four positions flanking the UUG from the consensus sequence proposed by Chen et al.

Using the ribosome occupancy data of Ingolia et al. (17), we estimated that the average ribosome density in nAuORF2 is ~5-fold higher than that of nAuORF1 under the starvation conditions employed in their study. If we equate average ribosome density with translation rate, and noting that the nAuORF1CUC-lacZ reporter (lacking the nAuORF1 start codon) conferred 25 units of lacZ-galactosidase expression as described in Figure 2B and C, and the ratio of enzyme activities observed for the TTG to ATG fusions was determined and plotted.

Figure 6. Histidine limitation imposed with 3-AT does not substantially induce initiation at near-cognate start codons. (A) nAuORF-lacZ fusion. Transformants of GCN2 strain H2833 or isogenic gcn2Δ strain H2931 harboring the wild-type nAuORF-lacZ construct on plasmid pLPz409 were cultured and analyzed for β-galactosidase expression as described in Figure 2B and C. (B) HIS4-lacZ fusion. Transformants of GCN2 strain H2833 harboring a plasmid-borne HIS4-lacZ reporter (lacking the nAuORF2 start codon) in 3-AT-treated cells, it could be proposed that the fusion of lacZ coding sequences to the nAuORF altered the structure of the nAuORF initiation region in a manner that impairs recognition of the UUG start codon without similarly reducing recognition of the AUA initiation site at nAuORF2. This seems unlikely considering that the fusion junction is ~100 nt downstream of the nAuORF1 start codon and only ~25 nt 3′ of the nAuORF2 initiation site. Alternatively, it is possible that the fusion of lacZ sequences activates recognition of the nAuORF2 start codon in a manner that does not occur at the nAuORF1 start site further upstream. This might occur if the 5′-end of lacZ sequences form a structure that evokes ribosome pausing specifically in the initiation region of nAuORF2. This mechanism also seems unlikely, however, as Kozak demonstrated that the distance between the start codon and the base of a secondary structure able to compensate for a poor initiation sequence context must be ≤14 nt—the approximate distance between the leading edge of the ribosome and the start codon positioned in the ribosomal P-site (36). Thus, the junction with lacZ sequences in our nAuORF-lacZ fusion is probably located too far downstream (~25 nt) from the AUA start codon to activate nAuORF2 translation by this pausing mechanism; although we cannot rule out the possibility that lacZ sequences base pair with GCN4 sequences located just downstream of the AUA start codon to form the requisite structure.

Another discrepancy between our results using lacZ reporters and the ribosome profiling data of Ingolia et al. (17) concerns the relative translational rates of nAuORF2 and uORF1. Estimating the average ribosome densities of nAuORF2 and uORF1 from their profiling data suggests that the uORF1-lacZ fusion should be translated at a rate only ~3.8-fold higher than that of the nAuORF1CUC-lacZ reporter (lacking the nAuORF1 start codon) under starvation conditions, whereas the actual difference measured here for 3-AT treated cells is 15-fold (Figure 2B and C). The ribosome occupancy of nAuORF2 measured by Ingolia et al. is about 4.5-fold lower under non-starvation versus starvation conditions, whereas the occupancy of uORF1 is relatively higher in non-starved cells, leading to the prediction that the uORF1-lacZ fusion should be translated at a rate ~20-fold higher than that of nAuORF1CUC-lacZ in non-starved cells, which actually agrees well with our measurements under these conditions (Figure 2B and C). Thus, the main discrepancy between our data and that of Ingolia et al. regarding the relative translation rates of uORF1 versus nAuORF2 is that we observed only a small (~1.7-fold) increase in translation initiation from the AUA start codon of nAuORF2 (the
nAuORF1\textsubscript{CUC-lacZ} reporter) in response to histidine starvation compared to the \(\sim4.5\)-fold increase observed in starved cells by ribosomal profiling. We also did not observe increased initiation at a UUG versus AUG start codon for a HIS4-lacZ fusion in response to histidine limitation by 3-AT. Thus, the prediction made from ribosomal profiling data that the rate of initiation at non-AUG codons is considerably higher in starved versus non-starved cells probably should be treated with caution.

Although our results on the nAuORF-lacZ construct support the conclusion that nAuORF2 is translated \textit{in vivo}, we did not observe any consequence of eliminating translation of this element by replacing its AUA start codon with the non-cognate AAA triplet. Neither complementation of the amino acid analog sensitivity of a \textit{gcn4A} mutant, induction of native \textit{Gcn4} protein, or the regulated expression of a GCN4-lacZ reporter was detectably perturbed by the AUG-to-AAA replacement in nAuORF2, by the UUG-to-CUC replacement in the start codon of nAuORF1, or by the double mutation. Thus, it seems clear that nAuORFs 1 and 2 are both dispensable for wild-type repression of \textit{GCN4} mRNA translation in non-starvation conditions, and for derepression of \textit{GCN4} translation in response to histidine limitation imposed with 3-AT, nutritional shift-down of an amino acid auxotroph, or treatment with rapamycin, methyl methanesulfonate or hydrogen peroxide.

Considering the evidence presented here that nAuORF2 is translated under starvation conditions, it might seem surprising that eliminating its AUA start codon would have no detectable impact on \textit{GCN4} expression. However, a comparison of the amount of \(\beta\)-galactosidase produced by the nAuORF-lacZ fusion \((\sim25\text{U})\) to that given by the uORF1-lacZ \((\sim400\text{U})\) or the UORF-less GCN4-lacZ construct \((\sim700\text{U})\) in 3-AT-treated cells (Figure 2B and C) suggests that only a small fraction \((\sim5\%)\) of the 43S complexes that can scan from the cap and initiate at the AUG of uORF1 or the GCN4 ORF, when present as the 5'-proximal AUG, are able to initiate at the AUA of nAuORF2. This implies, in turn, that \(\sim95\%\) of the 43S complexes scanning from the cap will leaky-scan past the nAuORF2 AUA and continues downstream to uORF1, where they can engage in the regulated reinitiation process responsible for \textit{GCN4} translational control. Thus, even if the entire 5\% of the scanning 43S complexes that translate nAuORF2 fail to resume scanning downstream, this would reduce the level of \textit{GCN4} translation by only 5\%, which might be difficult to detect by western analysis of \textit{Gcn4} or assaying the GCN4-lacZ reporter.

A final interesting point to consider is that, besides the UUG and AUA start codons of nAuORFs 1 and 2, the \textit{GCN4} mRNA leader contains 7 other potential near-cognate start codons with a perfect consensus at the \(-1\) to \(-3\) positions as defined by Chen \textit{et al.} (35). It is thus unclear why 80S ribosome occupancies comparable to those seen for nAuORF2 were not observed at any of these other locations by Ingolia \textit{et al.} (17), particularly the A\textsubscript{3}A\textsubscript{2}A\textsubscript{1}AUAA\textsubscript{4} and A\textsubscript{3}A\textsubscript{2}A\textsubscript{1}AUCA\textsubscript{4}UU sequences present just upstream from uORF1 (Figure 1, \(-382\) to \(-376\), and \(-375\) to \(-369\)). Perhaps the sequences immediately downstream from the AUA start codon of nAuORF2 produces a secondary structure that pauses the 43S complex with the AUA in the P-site, enhancing recognition of this particular near-cognate start codon in the \textit{GCN4} leader. The initiation at multiple near-cognate start codons in the 5'-UTRs of other yeast genes detected by Ingolia \textit{et al.} might involve a similar mechanism.

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REFERENCES

1. Kozak, M. (1989) The scanning model for translation: an update. \textit{J. Cell Biol.}, \textbf{108}, 229–241.
2. Hinnebusch, A.G. (2005) Translational regulation of \textit{GCN4} and the general amino acid control of yeast. \textit{Annu. Rev. Microbiol.}, \textbf{59}, 407–450.
3. Ron, D. and Harding, H.P. (2007) eIF2a phosphorylation in cellular stress responses and disease. In Mathews, M., Sonenberg, N. and Hershey, J.W.B. (eds), \textit{Translational Control in Biology and Medicine}. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 345–368.
4. Pestova, T.V., Lorsch, J.R. and Hellen, C.U.T. (2007) The mechanism of translation initiation in eukaryotes. In Mathews, M., Sonenberg, N. and Hershey, J.W.B. (eds), \textit{Translational Control in Biology and Medicine}. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 87–128.
5. Hinnebusch, A.G., Dever, T.E. and Asano, K. (2007) Mechanism of translation initiation in the Yeast \textit{Saccharomyces cerevisiae}. In Mathews, M., Sonenberg, N. and Hershey, J.W.B. (eds), \textit{Translational Control in Biology and Medicine}. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 225–268.
6. Szmaczek, B., Rutkai, E., Cuchalova, L., Munzarova, V., Herrmannova, A., Nielsen, K.H., Burela, L., Hinnebusch, A.G. and Valasek, L. (2008) eIF3a cooperates with sequences 5' of uORF1 to promote resumption of scanning by post-termination ribosomes for reinitiation on \textit{GCN4} mRNA. \textit{Genes Dev.}, \textbf{22}, 2414–2425.
7. Harding, H.P., Novoa, L., Zhang, Y., Zeng, H., Wek, R., Chapin, M. and Ron, D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. \textit{Mol. Cell}, \textbf{6}, 1099–1108.
8. Vattem, K.M. and Wek, R.C. (2004) Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. \textit{Proc. Natl Acad. Sci. USA}, \textbf{101}, 11269–11274.
9. Zhou, D., Palam, L.R., Jiang, L., Narasimhan, J., Staschke, K.A. and Wek, R.C. (2008) Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. \textit{J. Biol. Chem.}, \textbf{283}, 7064–7073.
10. Guo, F. and Cavener, D.R. (2007) The \textit{GCN2} eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. \textit{Cell Metab.}, \textbf{5}, 103–114.
11. Hao, S., Sharp, J.W., Ross-Inta, C.M., McDaniell, B.J., Anthony, T.G., Wek, R.C., Cavener, D.R., McGrath, B.C., Rudell, J.B., Koehnle, T.J. \textit{et al.} (2005) Uncharged tRNA and sensing of amino acid deficiency in mammalian pinform cortex. \textit{Science}, \textbf{307}, 1776–1778.
12. Costa-Mattioli, M., Gobern, D., Harding, H., Herdy, B., Azzi, M., Bruno, M., Bidinosti, M., Ben Mamou, C., Marcinkiewicz, E.,
Yoshida,M. et al. (2005) Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature*, 436, 1166–1173.

Dever,T.E., Dar,A.C. and Sihcher,F. (2007) The eIF2z kinases. In Mathews,M., Sonenberg,N. and Hershey,J.W.B. (eds), *Translational Control in Biology and Medicine*. Cold Spring Harbor Laboratory Press, pp. 319–344.

Gaba,A., Wang,Z., Krishnamoorthy,T., Hinnenbusch,A.G. and Sachs,M.S. (2001) Physical evidence for distinct mechanisms of translational control by upstream open reading frames. *EMBO J.*, 20, 6453–6463.

Tzamarias,D., Roussou,I. and Thireos,G. (1989) The first and fourth upstream open reading frames in *Saccharomyces cerevisiae* impairs general amino acid control when overexpressed. *J. Biol. Chem.*, 264, 3980–3988.

Ingolia,N.T., Ghaemmaghami,S., Newman,J.R. and Weissman,J.S. (2005) Dissecting eukaryotic translation and its control by ribosome density mapping. *Nucleic Acids Res.*, 33, 2421–2432.

Dever,T.E., Dar,A.C. and Sicheri,F. (2007) The eIF2 alpha kinase GCN2. *Genes Dev.*, 21, 1217–1230.

Kolitz,S.E., Takacs,J.E. and Lorsch,J.R. (2009) Kinetic and thermodynamic analysis of the role of start codon/anticodon base pairing during eukaryotic translation initiation. *RNA*, 15, 138–152.

Sattlegger,E. and Hinnebusch,A.G. (2005) Polyribosome binding by GCN4 is required for full activation of eukaryotic translation initiation factor 2a [alpha] kinase GCN2 during amino acid starvation. *J. Biol. Chem.*, 280, 16514–16521.

Kolitz,S.E., Swanson,M.J., Asheraf,E.A., Jennings,J.L., Fekete,R.A., Link,A.J. and Hinnebusch,A.G. (2004) YIH1 is an actin-binding protein that inhibits protein kinase GCN2 and impairs general amino acid control when overexpressed. *J. Biol. Chem.*, 279, 29952–29962.

Hinnenbusch,A.G. and Fink,G.R. (1983) Positive regulation in the endosome attenuates transcriptional activation by Gcn4. *Mol. Cell. Biol.*, 3, 2331–2342.

Sattlegger,E. and Hinnebusch,A.G. (2008) Disrupting vesicular trafficking at the endosome attenuates transcriptional activation of Gcn4. *Mol. Cell. Biol.*, 28, 6796–6818.

Mueller,P.P., Jackson,B.M., Miller,P.F. and Hinnenbusch,A.G. (1988) The first and fourth upstream open reading frames in GCN4 mRNA have similar initiation efficiencies but respond differently in translational control to changes in length and sequence. *Mol. Cell. Biol.*, 8, 5439–5447.

Mueller,P.P. and Hinnebusch,A.G. (1986) Multiple upstream AUG codons mediate translational control of GCN4. *Cell*, 45, 201–207.

Albrecht,G., Mosch,H.U., Hoffmann,B., Reuser,U. and Braus,G.H. (1998) Monitoring the Gcn4 protein-mediated response in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, 273, 12696–12702.

Albrecht,G., Mosch,H.U., Hoffmann,B., Reuser,U. and Braus,G.H. (1998) Monitoring the Gcn4 protein-mediated response in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, 273, 12696–12702.

Kornitzer,D., Raboy,B., Kulk,R.G. and Fink,G.R. (1994) Regulated degradation of the transcription factor Gcn4. *EMBO J.*, 13, 6021–6030.

Shenton,D., Smirnova,J.B., Selley,J.N., Carroll,K., Hubbard,S.J., Pavitt,G.D., Ashe,M.P. and Grant,C.M. (2006) Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. *J. Biol. Chem.*, 281, 29011–29021.

Valenzuela,L., Aranda,C. and Gonzalez,A. (2001) TOR modulates GCN4-dependent expression of genes turned on by nitrogen limitation. *J. Bacteriol.*, 183, 2331–2334.

Cherkasova,V.A. and Hinnebusch,A.G. (2003) Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev.*, 17, 859–872.

Natarajan,K., Meyer,M.R., Jackson,B.M., Slade,D., Roberts,C., Hinnenbusch,A.G. and Marton,M.J. (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol. Cell. Biol.*, 21, 4347–4368.

Cheung,Y.N., Maag,D., Mitchell,S.F., Fekete,C.A., Algire,M.A., Takacs,J.E., Shirokikh,N., Pestova,T., Lorsch,J.R. and Hinnenbusch,A.G. (2007) Dissociation of eIF1 from the 4OS ribosomal subunit is a key step in start codon selection in vivo. *Genes Dev.*, 21, 1217–1230.

Dever,T.E., Feng,L., Wec,R.C., Cigan,A.M., Donahue,T.D. and Hinnenbusch,A.G. (1992) Phosphorylation of initiation factor 2a by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell*, 68, 585–596.

Chen,S.J., Lin,G., Chang,K.J., Yeh,L.S. and Wang,C.C. (2008) Translational efficiency of a non-AUG initiation codon is significantly affected by its sequence context in yeast. *J. Biol. Chem.*, 283, 3173–3180.

Kozak,M. (1990) Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. *Proc. Natl Acad. Sci. USA*, 87, 8301–8305.

Hinnenbusch,A.G. (1985) A hierarchy of trans-acting factors modulate translation of an activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, 5, 2349–2360.

Donahue,T.F. and Cigan,A.M. (1988) Genetic selection for mutations that reduce or abolish ribosomal recognition of the HIS4 translational initiator region. *Mol. Cell. Biol.*, 8, 2955–2963.