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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-tRNA$_i^{Met}$ to the small (40S) ribosome to form the 43S preinitiation complex. The Met-tRNA$_i^{Met}$ 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 m^{7}G-capped 5′-end of the mRNA, and scans the mRNA leader for an initiation codon (AUG). On base pairing of the Met-tRNA$_i^{Met}$ with AUG, hydrolysis of the GTP bound to eIF2 is completed, releasing eIF2–GDP, and the 60S subunit joins to form the 80S initiation complex competent for protein synthesis. The eIF2–GDP is recycled to eIF2–GTP by the guanine nucleotide exchange factor eIF2B, enabling reassembly of the TC, and this reaction is inhibited by a family of

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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 eIF2(αP) is 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 rebind the TC in time to reinitiate translation at uORFs 3 or 4, and because of non-permissive sequences surrounding the stop 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 can 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

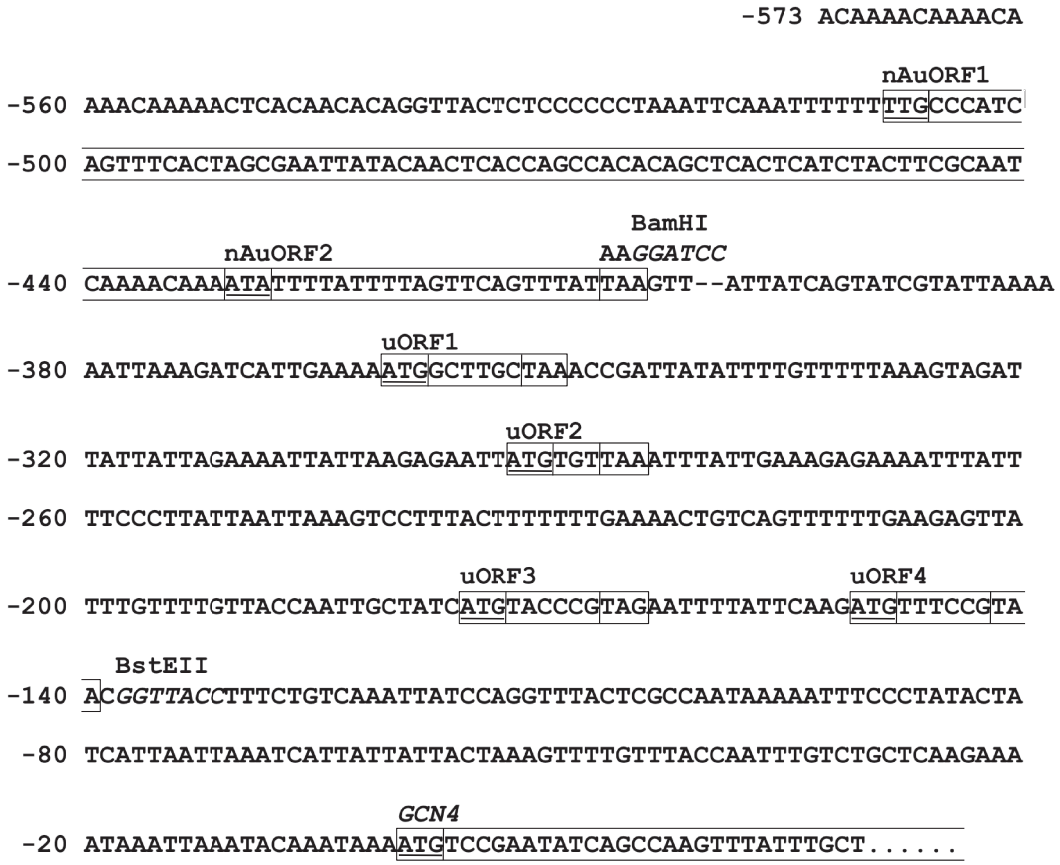

Figure 1. Locations of nAuORFs 1 and 2 and the four conventional uORFs in the *GCN4* mRNA leader. The sequence is shown numbered relative to the ATG (+1) of the Gcn4 coding sequence. Open reading frames are enclosed with rectangles, and both the ATG, or near-cognate start codons, and the stop codon are boxed within each rectangle and the start codons are also underlined. The nucleotide substitution and insertion mutations introduced to remove the nAuORF stop codon and introduce a BamHI site for constructing the *nAuORF-lacZ* fusion are indicated above the sequence, and the naturally occurring BstEII site exploited for cloning purposes is also indicated.

whether these elements participate in *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 *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 *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 *HIS3*. Interestingly, this Gcn$^-$ (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 Gcn$^-$ 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 *et al.* (17) have a role in *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 *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 2nd 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 *GCN4* translational control. By constructing a *lacZ* fusion to the overlapping nAuORFs 1 and 2, we have obtained evidence that nAuORF2 is translated *in vivo* dependent on its AUA near-cognate start codon, whereas translation of nAuORF1 was not detected. Then using several different assays to measure *GCN4* expression, we found that impairing 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 SalI–BstEII fragments bearing the appropriate mutations, using primers listed in Table 2, and p164 DNA as template. The SalI–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 SalI–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 used primers FZP353 and FZP346 as forward and reverse primers containing SalI and BstEII restriction sites. The following primer sets were employed in these constructions: FZP347/FZP348 (pLfz450 and pLfz460), FZP349/FZP350 (pLfz453 and pLfz463), FZP351/FZP352 (pLfz456 and pLfz466), FZP362/FZP363 (pLfz469 and pLfz473), FZP362/FZP363 (pLfz470 and pLfz474 with pLfz450 as template) and FZP366/FZP367 (pLfz482). The *nAuORF-lacZ* constructs pLfz489, pLfz491, pLfz493 and pLfz495 were made in two steps. First, SalI–BstEII DNA fragments containing the appropriate nAuORF start codon mutations and a BamHI site replacing the stop codon of the nAuORFs at position −405 (Figure 1) were produced by fusion PCR, employing primers listed in Table 2 (forward and reverse primers FZP353 and FZP346, and primer set FZP366/FZP367 harboring BamHI site), and used to replace the WT SalI–BstEII fragment of p180. The following plasmids with either the WT 5′ leader or containing nAuORF mutations were used as PCR templates for the plasmid constructions indicated in parenthesis: p180 (for pLfz489), pLfz473 (for pLfz491), pLfz460 (for pLfz493) and pLfz474 (for pLfz495). The resulting plasmids were digested with BamHI and the two largest of the three BamHI fragments were religated to generate the final constructs: pLfz489, pLfz491, pLfz493 and pLfz495.

Yeast strains used in this study are H2833 (*MATa leu2-Δ0 met15-Δ0 ura3-Δ0*), H2835 (*MATa gcn4Δ::kanMX4 leu2-Δ0 met15–Δ0 ura3-Δ0*) and H2931 (*MATa gcn2-Δ::hisG leu2–Δ0 met15-Δ0 ura3-Δ0*) (20,21).

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

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

Table 2. Primers used in this study

Primer Name	Primer Sequence 5′->3′ (with selected restriction sites italicized)
FZP346	TTTGACAGAAA*GGTAACC*GT
FZP347	GAAACTGATGGGGAGAAAAAATTTGAATT
FZP348	AATTCAAATTTTTTCTCCCCATCAGTTTC
FZP349	CTAAAATAAAAGAGTTTGTTTTGATTGCGA
FZP350	TCGCAATCAAAACAAACTCTTTTATTTTAG
FZP351	TGAGTGAGCTGTGTGGCTGGTGAGTTGTATAATTCGCTAGTGAAACTGATGGGGAGAAAAAATTTGAATT
FZP352	TTATACAACTCACCAGCCACACAGCTCACTCATCTACTTCGCAATCAAAACAAACTCTTTTATTTTAGTT
FZP353	CTCTCAAGGGCATCG*GTCGAC*
FZP355	ATTTAATTAATGATAGTATAGG
FZP362	TCGCAATCAAAACAAAAAATTTTATTTTAG
FZP363	CTAAAATAAAATTTTTTGTTTTGATTGCGA
FZP366	TATTTTAGTTCAGTTTATAA*GGATCC*ATTATCAGTATCGTATTA
FZP367	TAATACGATACTGATAAT*GGATCC*TTATAAACTGAACTAAAATA

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* fusions containing the wild-type leader or the 'uORF-less' version with point mutations in the ATG codons of all four uORFs, and a previously described *uORF1-lacZ* fusion (25) (Figure 2A, constructs 1–2 and 4).

In agreement with previous results (26), β-galactosidase activity expressed from the WT *GCN4-lacZ* reporter is induced ∼12-fold in cells treated with 3-AT, reflecting the known induction of *GCN4* translation by amino acid starvation (Figure 2B, columns 1–2). As expected, the uORF-less *GCN4-lacZ* reporter produces high, nearly constitutive β-galactosidase activities at levels ∼50-fold above the repressed level of the WT reporter (Figure 2C, columns 1–2). The *uORF1-lacZ* fusion also produces high levels of β-galactosidase activity under both growth conditions, displaying a <25% increase in response to histidine starvation (Figure 2C, columns 5–6). The high, constitutive expression of the uORF-less *GCN4-lacZ* and *uORF1-lacZ* fusions reflect efficient initiation at the 5′-proximal AUG start codons of these constructs under both starvation and non-starvation conditions.

Interestingly, β-galactosidase was expressed from the WT *nAuORF-lacZ* fusion under non-starvation conditions (Figure 2B, column 3) at a level that is 20- to 30-fold lower than that of the uORF-less *GCN4-lacZ* and *uORF1-lacZ* constructs just described under the same growth conditions: 16 U versus 630 U and 310 U, respectively (Figure 2C, columns 1 and 5). 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 increases 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 ($nAuORF1_{CUC}$-*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 ($nAuORF2_{AAA}$-*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,

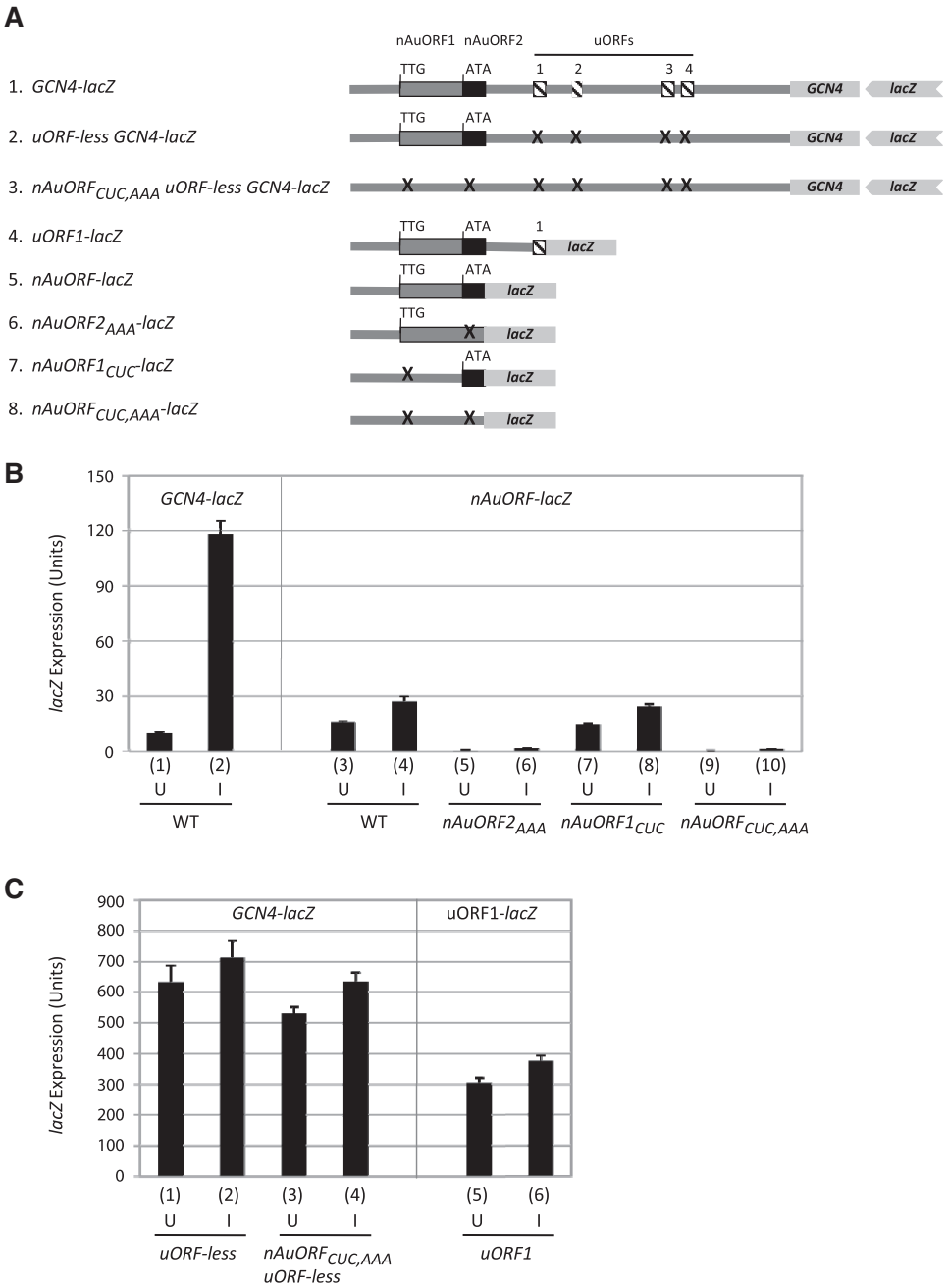

Figure 2. Evidence that nAuORF2 is translated *in vivo* from its AUA near-cognate start codon. (**A**) Schematic of *GCN4-lacZ* and *nAuORF-lacZ* constructs. (1) The nAuORFs 1 and 2 and uORFs 1–4 are depicted schematically in the leader of the wild-type *GCN4-lacZ* allele (on plasmid p180) drawn approximately to scale, indicating the TTG and ATA triplets that encode the UUG and AUA start codons of nAuORFs 1 and 2, respectively. (2–3) Variants of *GCN4-lacZ* containing point mutations in the AUG codons of the four uORFs (construct 2, on plasmid p227) and also in the non-AUG codons of the two nAuORFs (construct 3, on plasmid pLfz482), shown as 'X's that eliminate the cognate uORFs/nAuORFs. (4) The *uORF1-lacZ* construct (on plasmid p466). (5–8) Wild-type and mutant versions of the *nAuORF-lacZ* constructs (on plasmids pLfz489, pLfz491, pLfz493 and pLfz495, respectively) depicted as described above. (**B** and **C**) *nAuORF-lacZ* expression requires the AUA start codon of nAuORF2. Transformants of *GCN4* strain H2833 harboring the indicated *lacZ* constructs on single-copy plasmids described in panel A were cultured in SC-Ura to saturation, diluted into fresh SC-Ura and grown for 6 h to an A_{600} of ~1 (U, for uninduced), or for 2 h in SC-Ura-His and then an additional 6 h with 10 mM 3-AT (I, for induced). WCEs were prepared and assayed for β-galactosidase, measured in units of nmol of ONPG cleaved min^{-1} mg^{-1} of protein. The results obtained from three independent transformants were averaged and the mean and SEM values plotted.

translation of *nAuORF-lacZ* 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 *nAuORF1$_{CUC}$-lacZ* fusion (lacking the nAuORF1 UUG start codon) increased by ~1.7 in response to 3-AT, which might

indicate 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-AT^S) phenotype of a *gcn4Δ* 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 Gcn2. 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-AT^R) 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 Gcn2 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 *gcn4Δ* 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 *gcn4Δ* 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

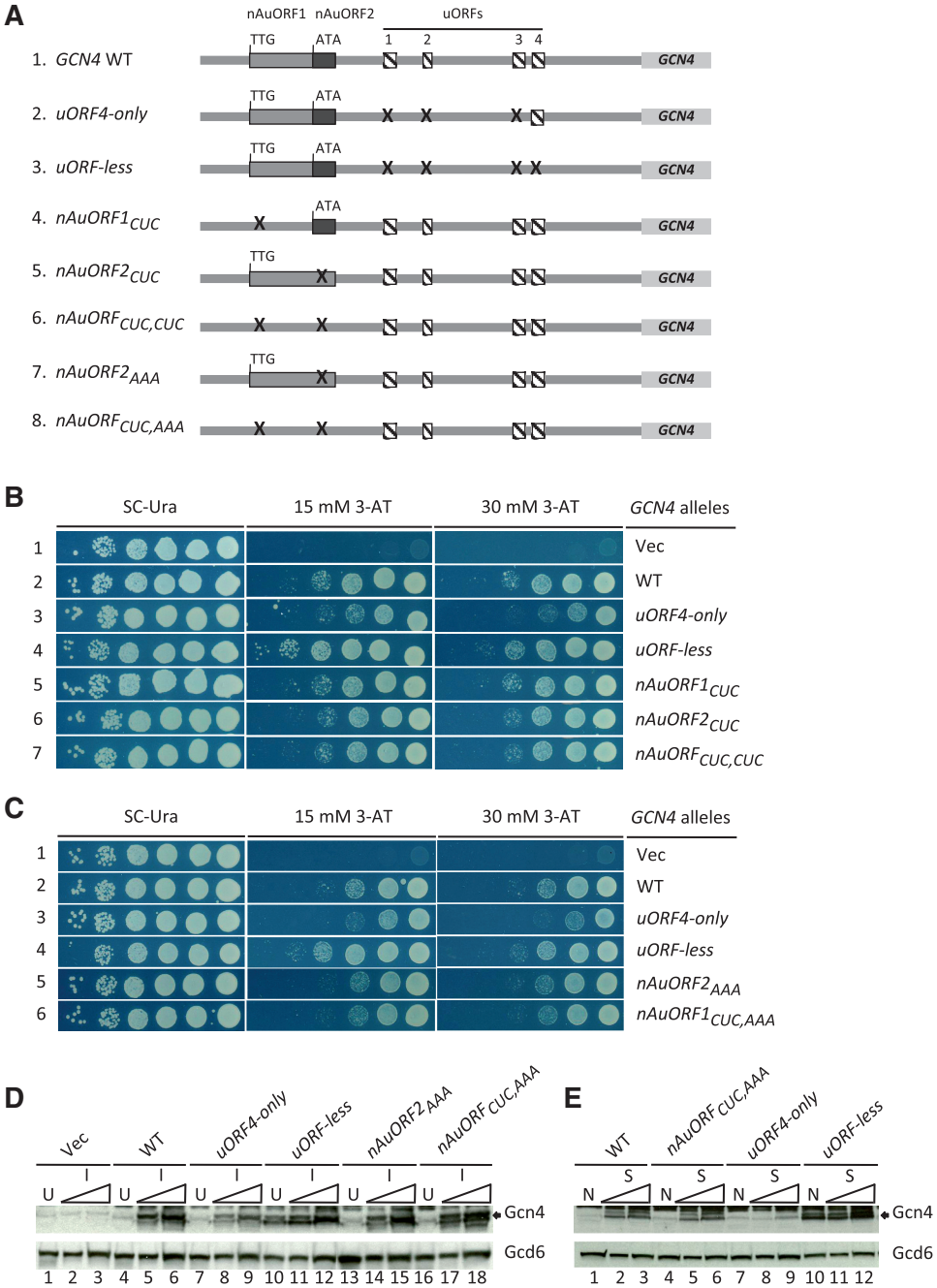

Figure 3. Eliminating the near-cognate start codons of nAuORFs 1 and 2 has no effect on regulation of Gcn4 protein levels in non-starved cells, histidine-limited cells, or during nutritional shift-down. (**A**) Schematics of *GCN4* alleles. The wild-type (1) or mutant (2–8) *GCN4* alleles under examination, 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 *gcn4Δ* by mutant *GCN4* alleles. Transformants of *gcn4Δ* 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^- 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 tricholoracetic 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 A_{600} 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.

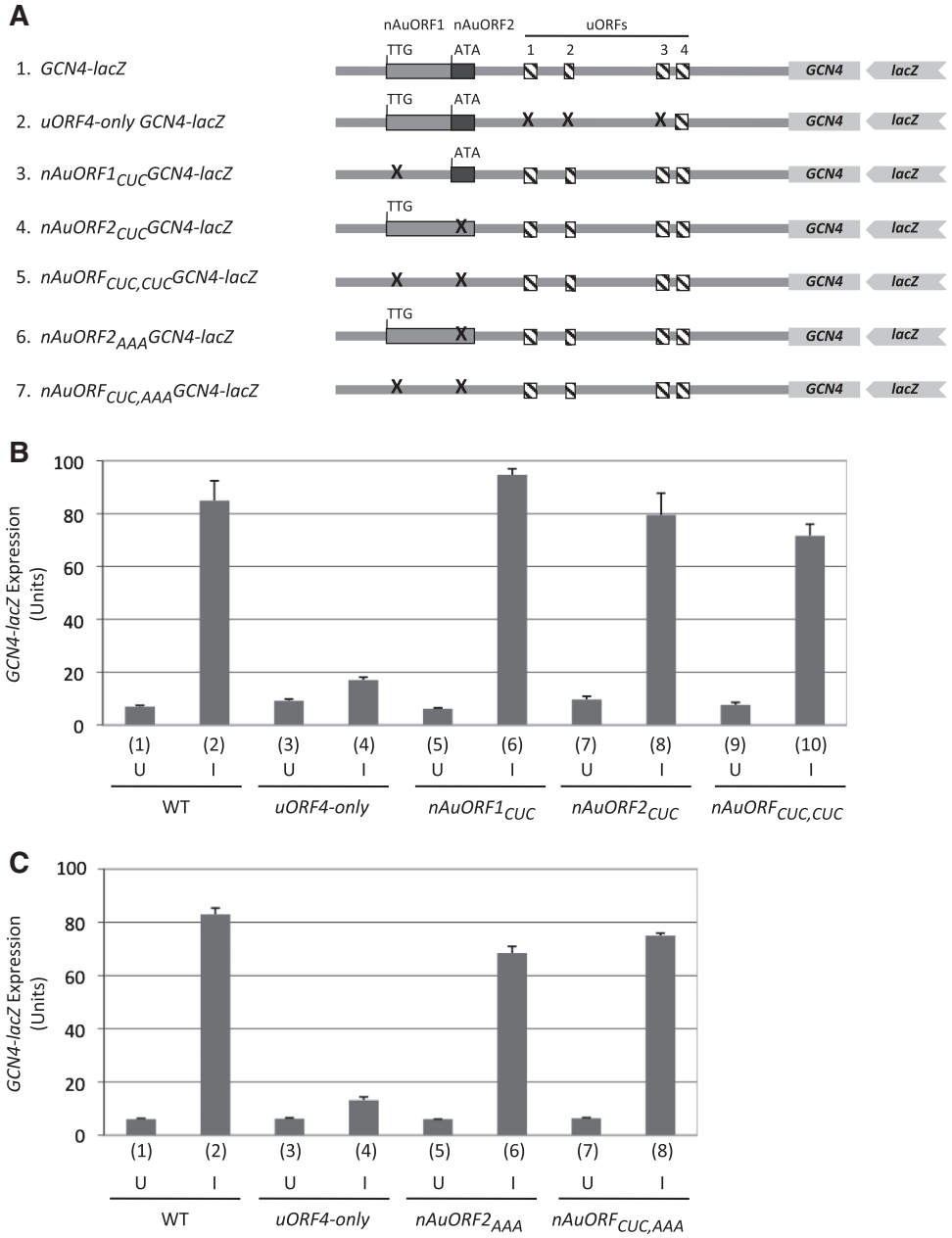

Figure 4. Eliminating the near-cognate start codons of nAuORFs 1 and 2 has no effect on regulated *GCN4-lacZ* expression in non-starved or histidine-limited cells. (**A**) Schematics of *GCN4-lacZ* alleles. The wild-type (1) or mutant (2–7) *GCN4-lacZ* alleles under examination, depicted as described in Figure 2A (contained on plasmids p180, p226, pLfz460, pLfz463, pLfz466, pLfz473 and pLfz474, 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 β-galactosidase, as described in Figure 2B and C.

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 *GCN4* during nutritional shift-down of an auxotroph.

Finally, we considered the possibility that the nAuORFs might be important for translational induction of *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 alkylating agent methyl methanesulfonate (32), all of which are known to induce *GCN4* translation in nutrient replete medium. As shown in Figure 5, these three treatments evoked derepression of the

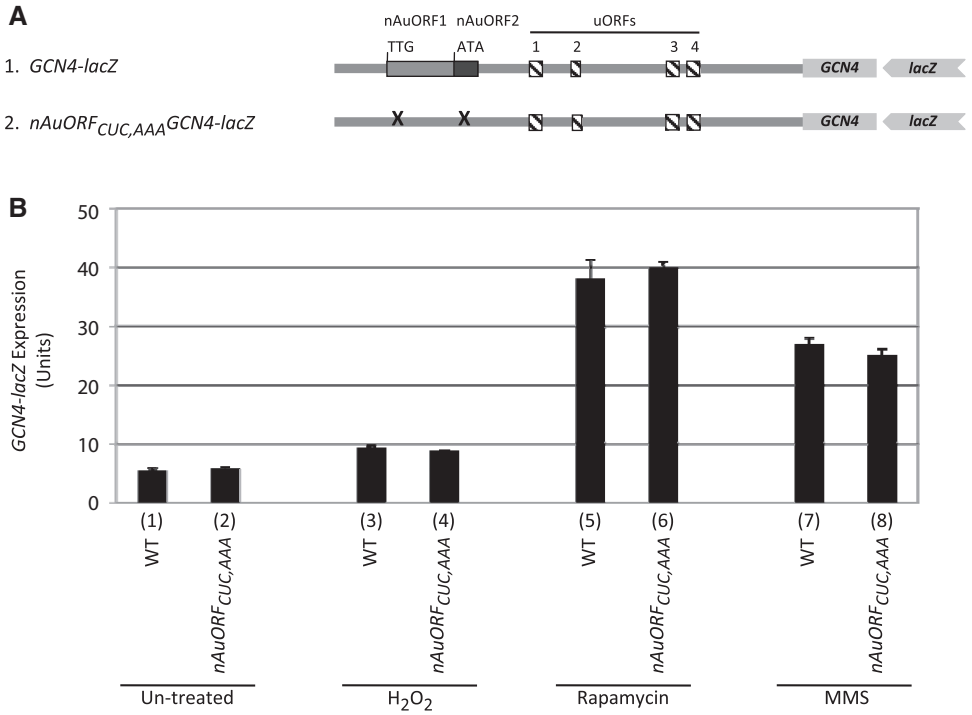

Figure 5. Mutating the near-cognate start codons of nAuORFs 1 and 2 to non-cognate triplicates has no effect on *GCN4-lacZ* expression under various stress conditions. (**A**) Schematics of the *GCN4-lacZ* alleles under examination, depicted as in Figure 2A, contained on plasmids p180 and pLfz474, respectively. (**B**) Transformants of yeast strain H2833 harboring *GCN4-lacZ* constructs described in (A), were cultured in SC-Ura (Un-treated) or in SC-Ura and treated with 2 mM hydrogen peroxide (H_2O_2) for 2 h, 200 ng/ml rapamycin for 4 h or 0.07% (v/v) methyl methanesulfonate (MMS) for 1 h. WCEs were assayed β-galactosidase activity 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 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, $A_{-3}A_{-2}A_{-1}\underline{AUA}U_{+4}$, conforms

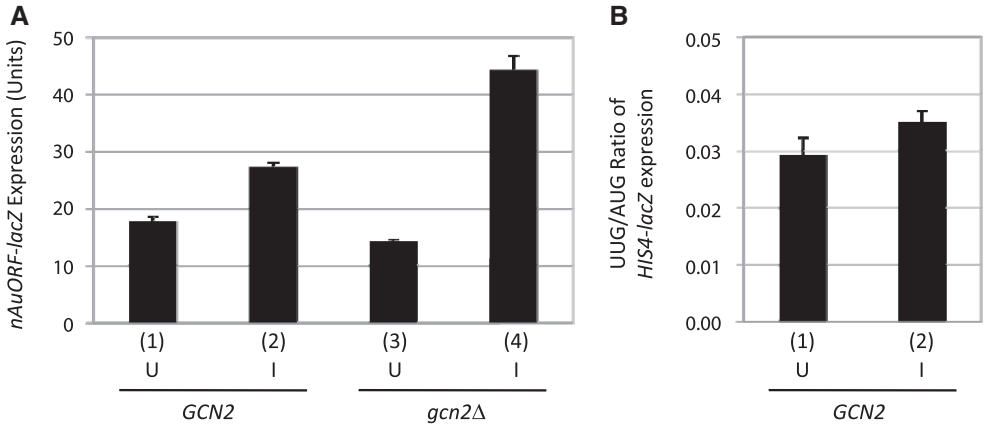

Figure 6. Histidine limitation imposed with 3-AT does not substantially induce initiation at near-cognate start codons. (**A**) nA*uORF-lacZ* fusion. Transformants of *GCN2* strain H2833 or isogenic *gcn2Δ* strain H2931 harboring the wild-type nA*uORF-lacZ* construct on plasmid pLfz489 were cultured 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* fusion containing an ATG start codon (p367) or TTG start codon (p391) were cultured and analyzed for β-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.

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 $A_{-3}A_{-2}(A/G)_{-1}\underline{UUG}A_{+4}$, 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, $U_{-3}U_{-2}U_{-1}\underline{UUG}C_{+4}$, 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 *nAuORF1$_{CUC}$-lacZ* reporter (lacking the nAuORF1 start codon) conferred 25 units of β-galactosidase in histidine-starved cells, then we might expect to have observed ∼5 units of β-galactosidase (25/5 units) expressed from the *nAuORF2$_{AAA}$-lacZ* reporter (lacking the nAuORF2 start codon) in 3-AT-treated cells, resulting from translation of the *nAuORF1-lacZ* fusion. However, <1 unit of activity could be attributed to initiation at the UUG start codon of nAuORF1, calculated as the difference in expression between the *nAuORF2$_{AAA}$-lacZ* and *nAuORF$_{CUC,AAA}$-lacZ* reporters (1.3−1.0 units). To explain our inability to detect translation of nAuORF1 in histidine-limited cells, it could be proposed that the fusion of *lacZ* coding sequences to the nAuORF altered the structure of the nAuORF1 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 *nAuORF1$_{CUC}$-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 *nAuORF1$_{CUC}$-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$_{CUC}$*-lacZ* reporter) in response to histidine starvation compared to the ~4.5-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 *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 *gcn4Δ* mutant, induction of native Gcn4 protein, or the regulated expression of a *GCN4-lacZ* reporter was detectably perturbed by the AUA-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 *GCN4* mRNA translation in non-starvation conditions, and for derepression of *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 *GCN4* expression. However, a comparison of the amount of β-galactosidase produced by the *nAuORF-lacZ* fusion (~25 U) to that given by the *uORF1-lacZ* (~400 U) or the uORF-less *GCN4-lacZ* construct (~700 U) in 3-AT-treated cells (Figure 2B and C) suggests that only a small fraction (~5%) 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 ~95% 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 *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 *GCN4* translation by only 5%, which might be difficult to detect by western analysis of 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 *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 *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 *et al*. (17), particularly the $A_{-3}A_{-2}A_{-1}\underline{AUU}A_{+4}$ and $A_{-3}A_{-2}A_{-1}\underline{AUC}A_{+4}UU$ 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 *GCN4* leader. The initiation at multiple near-cognate start codons in the 5′-UTRs of other yeast genes detected by Ingolia *et al*. might involve a similar mechanism.

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Conflict of interest statement. None declared.

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