Translational Efficiency of Redundant ACG Initiator Codons Is Enhanced by a Favorable Sequence Context and Remedial Initiation*

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Earlier studies showed that the redundancy of ACG initiation codons enhanced the efficiency of translation initiation by 3- to 6-fold. Evidence presented here shows that this “redundancy effect” can be attributed to a favorable sequence context and, to a lesser extent, remedial initiation. In the case of redundant ACG initiator codons, the second ACG not only acts as a remedial initiation site for scanning ribosomes that skip the first ACG but also enhances the activity of the preceding initiator by providing a preferable “A” at its relative +4 position. Hence, non-successive ACG codons can be as effective as successive ACG codons in initiation, if positioned within a similar context. In contrast, redundant GUG initiation codons (GUG/GUG) bear an unfavorable “G” nucleotide at both the +4 and −3 positions relative to the first and second GUGs, respectively, such that redundant GUG codons act more poorly as translation initiation sites than does a single GUG with a favorable “A” nucleotide in the +4 position (−2.5-fold). Thus, the sequence context plays a much more important role than remedial initiation in modulating the efficiency of translational initiation from redundant non-AUG codons.

Aminoacyl-tRNA synthetases are a group of primordial enzymes, each of which catalyzes the attachment of a specific amino acid to its cognate tRNAs. Aminoacyl-tRNAs are then delivered by elongation factor-1 to ribosomes for protein translation. Typically, there are 20 aminoacyl-tRNA synthetases in prokaryotes, one for each amino acid (1−4). In eukaryotes, protein synthesis occurs not only in the cytoplasm, but also in organelles, such as mitochondria and chloroplasts (5). Thus, eukaryotes, such as yeast, need two distinct sets of enzymes for each aminoacylation activity, one localized to the cytoplasm and the other to the mitochondria. Each set aminoacylates the isoaccepting tRNAs within its respective cell compartment and is sequenced from the isoacceptors confined in other compartments. In most cases, cytoplasmic and mitochondrial synthetase activities are encoded by two distinct nuclear genes, regardless of the cell compartments to which they are confined. However, two Saccharomyces cerevisiae genes, HTS1 (the gene encoding histidyl-tRNA synthetase) (6) and VAS1 (the gene encoding valyl-tRNA synthetase (ValRS)) (7), specify both the mitochondrial and cytosolic enzymes through alternative initiation from two in-frame AUG codons. Each of these genes encodes more than one mRNA, and the mRNA species produced differ only at their 5′-ends. The mitochondrial form of the enzyme is translated from the first AUG on “long” mRNAs, while the cytosolic form is translated from the second AUG on “short” mRNAs, the 5′-ends of which are located between the first and second AUG initiator codons. Hence, mitochondrial enzymes have the same polypeptide sequences as their cytosolic counterparts, except for a short N-terminal mitochondrial targeting sequence. The transit peptide is subsequently cleaved away upon being imported into mitochondria. Because the isozymes are targeted to different compartments, the two isoforms of ValRS, for example, cannot be substituted for each other in vivo (7, 8). A similar scenario has been observed for genes encoding the mitochondrial and cytoplasmic forms of Arabidopsis thaliana alanine-tRNA synthetase (AlaRS), threonyl-tRNA synthetase, and ValRS (9). Paradoxically, although two isoforms of AlaRS are generated in a similar manner in Candida albicans, the longer form per se appears to be dual-targeted and is thus functional in both compartments (10). Recently, two rare cases of one gene encoding both activities have been reported in yeast in which the protein isoforms are produced by alternative use of two in-frame initiation codons: an upstream non-AUG initiator and a downstream AUG initiator (11, 12).

Translation initiation in eukaryotes is a stringent process requiring not only initiator tRNA but also many protein factors, including eukaryotic initiation factors elf1, elf2, elf3, elf4F, and elf5. Upon binding to the cap structure, the 43 S preinitiation complex, composed of the 40 S ribosome and initiation factors, moves along the mRNA in a 3′ direction until it encounters the first AUG codon. At this point, GTP hydrolysis leads to dissociation of the elf2-GDP complex from the initiator tRNA in the preinitiation complex and subsequent Pi

The abbreviations used are: ValRS, valyl-tRNA synthetase; aaRS, aminoacyl-tRNA synthetase; ADH, alcohol dehydrogenase; AlaRS, alanyl-tRNA synthetase; GlyRS, glycyl-tRNA synthetase; YPG, yeast extract-peptone-glycerol.

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release signifies a 3-bp codon-anticodon interaction between Met-tRNA(i)(Met) and the start codon (13). It has recently been shown that eIF1 plays a critical role in start codon selection. Mutations of eIF1 may lead to reduced interaction of this initiation factor with 40 S subunits and increased initiation at UUG codons (14).

Previous studies on CYC1 (15) in S. cerevisiae suggested that AUG is the only codon recognized as a translational initiator, and that the AUG codon nearest the 5′-end of mRNA serves as the start site for translation. If the first AUG codon is mutated, then initiation can begin at the next available AUG from the 5′-end of the message. The same rules apply to all eukaryotes. However, there are many examples in higher eukaryotes, where cellular and viral mRNAs can initiate from “non-AUG” codons that differ from AUG by just one nucleotide (16). The relatively weak base pairing between a non-AUG codon and the anticodon of an initiator tRNA appears to be compensated for by interactions with nearby nucleotides, in particular a purine (A or G) at position −3 and a “G” at position +4 (17, 18). Similarly, mutations in the sequence region surrounding the first AUG can lead to its inefficient utilization as an initiator and subsequent use of AUG at a downstream location. In addition to the sequence context, a stable hairpin structure located 12–15 nucleotides downstream from the initiator can also facilitate recognition of a weak initiator by the 40 S ribosomal subunit (19).

Although some reports have suggested that sequences immediately preceding the initiation codon may also play a role in modulating the efficiency of AUG translation initiation in yeast, the magnitude of this context effect appears relatively insignificant (20–22). Perhaps for that reason, yeast cannot efficiently use non-AUG codons as the translation start site (23, 24). Nonetheless, three yeast genes, GRS1 (one of the two glycyl-tRNA synthetase (GlyRS) genes in S. cerevisiae) (11), ALA1 (the only alanyl-tRNA synthetase (AlaRS) gene in S. cerevisiae) (12), and CARP2A (the gene coding for acidic ribosomal protein P2A in Candida albicans) (25), have recently been reported to use naturally occurring non-AUG triplets as translation initiators. Moreover, a very recent study suggested that the translational efficiency of non-AUG initiation codons is significantly affected (up to 32-fold) by nucleotides at its relative −3 to −1 position, and, to a lesser extent, +4 (26). The nucleotide at position −3 is the most influential one. AARuugA (R represents A or G; uug represents a non-AUG initiation codon) appears to be the most favorable sequence context for a non-AUG initiation site (26).

In the case of CARP2A, a non-AUG codon, i.e. GUG, serves as the exclusive translation initiator, whereas in the cases of ALA1 and GRS1, non-AUG codons act as alternative translation initiators that are accompanied by a downstream in-frame AUG initiation codon. Although two homologous GlyRS genes have been identified in the yeast genome (GRS1 and GRS2), only GRS1 is functional by possessing both cytoplasmic and mitochondrial aminoaacylating activities, while GRS2 appears to be dispensable for growth (27). Further studies indicated that two functionally exclusive protein isoforms are alternatively generated from GRS1. A short form that is responsible for the cytoplasmic activity of GlyRS is translationally initiated from a classic AUG initiator, whereas a longer isoform that provides the mitochondrial activity is initiated from an upstream in-frame UUG codon (11). Expression of ALA1 essentially follows a similar mechanism. However, it is noteworthy that the mitochondrial form of AlaRS is initiated from two successive in-frame ACG codons 69 nucleotides upstream of the AUG initiator of the cytoplasmic form (12, 28). Further studies showed that redundant ACGs contain stronger initiation activity than does a single ACG and can functionally substitute for the alternative AUG initiator codons of VAS1 (coding for mitochondrial and cytoplasmic isoforms of ValRS) in vivo. This feature of redundancy of non-AUG initiator codons may in itself represent a novel mechanism to improve the overall efficiency of a poor initiation event (29). So far, it is not clear whether this mechanism is a general feature of all possible non-AUG initiation codons. Moreover, because the sequence context also plays a role in modulating the efficiency of translation initiation from a non-AUG codon (26), redundancy of non-AUG initiation codons may run into a situation in which the initiation codons are flanked by an unfavorable sequence context imposed by themselves. For example, redundant GUG codons (GUG/GUG) bear an unfavorable nucleotide G at both the +4 and −3 positions relative to the first and second GUGs, respectively. Hence, the redundancy of GUG codons might not improve the efficiency of translation at all, but instead may impair the initiating activity of the preceding GUG codon and the overall efficiency of translation. In the work described here, we tested this hypothesis by comparing the translational efficiencies of several pairs of redundant and single non-AUG initiation codons and determined the effect of the sequence context on the translational activity. Our results suggested that a redundancy of non-AUG initiation codons does not always significantly enhance the translational efficiency and does so only when the first nucleotide of the initiation codons is an “A.”

**Experimental Procedures**

**Construction of Plasmids—**Various ALA1-lexA fusions for the Western blot analysis were constructed as previously described (11, 29). Briefly, an initiator mutant of lexA was amplified by PCR as an Spel-XhoI fragment and cloned in appropriate sites of pADH (11). To construct various ALA1-lexA fusions, a wild-type or mutant ALA1 sequence containing base pairs −105 to −24 relative to ATG1 was amplified by PCR as a PstI-SpeI fragment and cloned in-frame into the 5′-end of the lexA mutant. The expression of these constructs was under the control of a constitutive ADH promoter (30). Note that, because the native initiator codon of lexA was rendered inactive, the only translational initiation sites for these fusion constructs are those which exist in the ALA1 portion. To generate mutations in the 5′ leader region of the ALA1 gene, a short ALA1 sequence containing bp −300 to +60 (relative to ATG1) was PCR-amplified from the full-length ALA1 clone as an EagI-XbaI fragment and cloned in appropriate sites of pBluescript II SK (Stratagene, La Jolla, CA). This construct was subsequently used as a template for mutagenesis. Mutagenesis was carried out following standard protocols provided by the manufacturer (Stratagene). After mutagenesis, the ALA1 fragment carrying the desired mutation was recovered from the
plasmid by EagI/XbaI digestion and then fused in-frame to the 5’-end of a truncated version of ALA1 lacking the EagI-XbaI segment, yielding a full-length ALA1 clone. Note that the XbaI site is a native restriction site between bp +55 and +60 of ALA1. To generate mutations in the 5’ leader region of the VASI gene, a similar approach was followed (29).

Complementation Assays for the Mitochondrial Function of VASI—The yeast VASI knockout strain, CW1, was previously described (8). To test the mitochondrial function of the wild-type and mutant VASI constructs, CW1 was first transformed with a test plasmid (carrying a LEU2 marker) and plated onto selection medium lacking uracil and leucine. Following 5’-flouroorotate selections, a single colony of transformants was selected and grown to the stationary phase in synthetic medium lacking leucine with 2% glucose and 100 μg/ml cycloheximide. The cells were washed twice and then grown in medium lacking leucine with 2% raffinose to a cell density of 5 × 10^8/ml. The cells were then washed twice and grown in medium containing 10% galactose for 1–2 h, and the proteins were transferred to a polyvinylidene difluoride membrane and immunoblotted with the appropriate antibody.

RT-PCR—To determine the relative levels of specific ALA1-lexA mRNAs derived from these fusion constructs, a semi-quantitative reverse-transcription (RT)-PCR experiment was carried out following the protocols provided by the manufacturer (Invitrogen). Total RNA was first isolated from the transformant, and aliquots (~1 μg) of the RNA were then reverse-transcribed into single-stranded complementary DNA (cDNA) using an oligo(dT) primer. After RNase H treatment, the single-stranded cDNA products were amplified by PCR using a pair of specific primers. The forward and reverse primers contained sequences complementary to nucleotides +90 to +70 of ALA1 (5’-TATGAAAGCAGTGTGGTTGAA-3’) and nucleotides +370 to +390 of lexA (5’-CAAGTCCATCCATAATGGCC-3’), respectively. To obtain more reliable data, two different cycle numbers of PCR amplification were carried out for each cDNA preparation as indicated in the figure. As a control, the relative levels of actin-specific mRNAs in each preparation were also determined using a set of primers complementary to nucleotides +537 to +560 (5’-ACCAACTGGGAGCATATGGAAAAGGAG-3’) and nucleotides +696 to +719 (5’-TTGATGAGAACTGAGGGCTTG-3’) of actin, respectively, and only the cDNA products of 21 cycles of PCR amplification are shown.

β-Galactosidase Assay—Yeast cells were pelleted by centrifugation at 12,000 × g for 30 s and resuspended in 100 μl of breaking buffer (100 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol, 10% glycerol, and 2 mM phenylmethanesulfonyl fluoride) and 100 μl of beads. Cells were then lysed at 4 °C using a bead beater, followed by centrifugation at 12,000 × g for 2 min. Aliquots of the supernatants (25 μg) were diluted to 0.8 ml with Z buffer (60 mM NaHPO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4, and 50 mM 2-mercaptoethanol). β-Galactosidase activity assays were initiated (at 37 °C) by adding 0.2 ml of o-nitrophenyl β-D-galactoside (4 mg/ml). The reaction mixtures were incubated with constant shaking at 37 °C for 20 min and then terminated by the addition of 0.4 ml of 1 M Na2CO3. The reaction mixtures were centrifuged at 12,000 × g for 2 min, and the absorbance (A420) of the supernatants was determined. Relative β-galactosidase activities were calculated from A420 readings normalized to protein concentrations.

RESULTS

Translational Efficiencies of Redundant versus Single Non-AUG Initiation Codons—Our previous study showed that two distinct protein isoforms are generated from the yeast ALA1 gene via alternative use of two in-frame initiation sites. The longer form that accounts for the mitochondrial activity is initiated from AUG1 (the numbers “25” and “24” in parenthesis refer to the 25th and 24th codon triplets that precede AUG1), whereas the short form that is responsible for the cytoplasmic activity is initiated from AUG1 (11). Further study suggested that the redundancy of ACG initiation codons may represent a novel mechanism to enhance the over-
Efficiencies of translation initiation from various redundant and single non-AUG initiation codons. A, schematic summary of ALA1 mutants. The ALA1 sequence shown includes nucleotides −12 to +15 relative to the first ACG initiation codon. For clarity, non-AUG initiation codons, ACG, GTG, TTG, and CTG, are shaded and nucleotides that were mutated are underlined. B, assay of protein expression by Western blotting. Upper panel, AlaRS-LexA fusion; lower panel, actin (as an internal control). C, RT-PCR. Relative levels of specific ALA1-lexA mRNAs generated from each construct were determined by RT-PCR. Upper panel, ALA1-lexA fusion; lower panel, actin (as an internal control). D, β-galactosidase assay. Efficiencies of translation from various redundant or single non-AUG initiator codons were determined by introducing the ALA1-LacZ constructs into yeast and testing the relative β-galactosidase activities in extracts prepared from the transformants. E, assay of protein expression by Western blotting. In B, C, and E, numbers 1–13 (circled) denote fusion constructs with their ALA1 portions derived from constructs 1–13 shown in A, respectively.

all efficiency of translation (29). To investigate whether this is a common feature of all possible non-AUG initiation codons (that differ from AUG by a single nucleotide), redundant ACG initiation codons were mutated to various redundant or single non-AUG initiation codons (Fig. 1A), and the translational efficiencies of the resultant constructs were tested. We used an initiator mutant of lexA as a reporter to monitor the relative initiating activities and thus levels of expression of various ALA1-lexA fusions (see “Experimental Procedures”) (12). The first nucleotide of all initiator candidates chosen for this assay differed (Fig. 1A), so that we could simultaneously examine “remedial initiation” and the “context effect” (particularly the −3 and +4 nucleotides relative to the second and first initiator codons, respectively). Consistent with previous observations (29), Fig. 1B shows that redundant ACG codons acted better than ACG/ACC and ACG/TCG as a translational initiation site by 2.5- and 6.0-fold, respectively (Fig. 1B, numbers 1–3). In contrast to the case scenario of AUG initiation, redundant GTG codons did not exhibit the expected “redundancy effect” at all. Instead, the translational efficiency of GTG/ACC was almost equivalent to that of a single GTG (GTG/GTC) with an unfavorable G nucleotide in its relative +4 position, and 2.5-fold lower than that of a single GTG (GTG/ACC) with a favorable “A” nucleotide in its relative +4 position (Fig. 1B, numbers 4 and 5). This result suggested that the second GTG in redundant GTG codons is essentially inactive as a remedial initiation site, probably due to the presence of an unfavorable G nucleotide in its relative −3 position (Fig. 1B, numbers 4 and 6). As a result, a single GTG codon with a favorable A nucleotide in its relative +4 position functioned better as a translational initiation site than did redundant GTG codons (Fig. 1B, numbers 4 and 5).
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Similar to the case of redundant GTG codons, redundancy of TTG initiation codons failed to enhance the protein yield (Fig. 1, A and B, numbers 7–9). The initiating activity of TTG/ACC was slightly higher than that of redundant TTG codons (~1.5-fold) (Fig. 1B, numbers 7 and 8). Strangely enough, the initiating activity of TTG/TTC was also slightly higher than that of redundant TTG codons (~1.5-fold) (Fig. 1B, numbers 7 and 9).

Also, TTG/TTC and TTG/TTT expressed similar levels of proteins (Fig. 1E, numbers 9 and 13). Note that both TTG and TTC code for Phe. Although this result suggests that the second amino acid may affect the stability of the protein product (31), the possibility that the nucleotide at position +6 plays a minor role in modulating the efficiency of translation initiation cannot be ruled out at the moment. As for CTG initiation, the initiating activity of redundant CTG codons was equivalent to that of a single CTG codon with a favorable +4 A nucleotide, and was 2-fold higher than that of a single CTG with an unfavorable +4 C nucleotide (Fig. 1, A and B, numbers 10–12). Therefore, it appears that a favorable nucleotide at position +4 is just as good as redundancy of initiator codons in this instance. To check whether the ALA1-lexA fusions shown in Fig. 1B expressed similar levels of mRNAs, a semiquantitative RT-PCR was conducted. Two different cycle numbers of PCR amplification were carried out for each construct and compared. Fig. 1C shows that similar levels of cDNA products were generated from these fusions, suggesting that these mutations did not impair the stability of the specific mRNAs in vivo.

To verify whether the data of Western blots shown in Fig. 1B truthfully reflect the translational efficiencies of the various initiation codons, we next used lacZ as a reporter gene. The lexA portion of constructs 1–6 used in Fig. 1B was replaced by a lacZ reporter gene where the native initiator codon ATG had been mutated to ACT. Therefore, the redundant ACG initiator codons (or their derivatives) in the ALA1 portion became the only initiation sites for the translation of the AlaRS-LacZ fusion proteins. To examine the initiating efficiencies of various initiation codons, aliquots of the soluble protein extracts were prepared from the transformant and assayed for their translation codons, aliquots of the soluble protein extracts were pre-

Translation Efficiency versus Turnover Rate—The steady-state levels of the AlaRS-LexA fusions are determined not only by their translational efficiencies but also by their turnover rates. Many mutations shown in Fig. 1 involved changes in the second amino acid residue. To investigate whether the second amino acid has a strong impact on the turnover or stability of the fusion proteins, we inserted AUG into the initiation site for some of the constructs to minimize the effect of sequence context (26) and then subcloned these fusions into a yeast shuttle vector with an inducible GAL1 promoter. Transformants carrying these constructs were first grown to a cell density of ~1.0 A600 in medium lacking leucine with 2% raffinose and then induced with 2% galactose for 1 h. Afterward, the cells were washed twice and then grown in medium lacking leucine with 2% glucose and 100 μg/ml cycloheximide. Aliquots of the cell cultures were harvested at 0, 2, 4, 8, 16, and 32 h postinduction and prepared for Western blotting. As shown in Fig. 1, these fusions expressed a similar level of protein after induction (see T0 in Fig. 2B), and the second residue, Ser, Val, Leu, Thr, or Phe, had little effect on the turnover of the fusion proteins. The proteins having Ser, Val, Leu, Thr, or Phe as their second amino acid retained an almost constant level throughout the time period tested. Thus, changes in protein levels shown in Fig. 1 are a valid readout of initiation efficiency. It should be noted that the amino acids tested in Fig. 2 encompass all the penultimate N-terminal residues that appeared in Fig. 1.

Efficiencies of Translation from Various Combinations of Non-AUG Initiation Codons—We have so far assayed the translational efficiencies of several pairs of redundant and single non-ATG initiation codons and shown that redundant non-ATG initiation codons are not always stronger than a single non-ATG initiation codon. In fact, ACG appears to be the only initiator codon among the four candidates that showed a strong redundancy effect. To provide a more in-depth picture on this characteristic, we next tested the translational efficiencies of various combinations of non-ATG initiator codons such as ACG/TTG, ACG/ATT, TTG/TTG, and TTG/ACG. These non-ATG initiation codons were individually introduced into the ALA1 sequence by mutagenesis to replace the native ACG/ACG initiator codons (Fig. 3A), and the protein yields of the resultant constructs were assayed (Fig. 3B). As shown in Fig. 3, ACG/ACG and ACG/ATT possessed higher initiating activity than did TTG/TTG (2- to 3-fold) (numbers 1–3). Similarly, TTG/ACG and TTG/ATT possessed higher initiating activity than did TTG/TTG (~1.5-fold) (numbers 4–6). Despite the fact that TTG per se is a stronger initiation site than ACG in a similar context (Fig. 1B, numbers 2 and 8), TTG was not preferred as a remedial initiation site over ACG (Fig. 3). In conjunction with the results shown in Fig. 1, this result strongly favored the notion that the nucleotide at position +4 relative to the preceding non-ATG initiator codon plays an important role in modulating the efficiency of redundant non-ATG initiation codons. In other words, the nature of the first nucleotide of the remedial initiator codon is far more important than the codon itself in modulating the efficiency of translation in these instances.

Effect of Nucleotide at Position +3 on Non-AUG Initiation—In Figs. 1 and 2, we demonstrate the effect of the nucleotide at position +4 on the translational activity of a non-AUG initiator codon. However, the effect of the nucleotide at position +3, presumably the most influential position, on the translational activity of a non-AUG initiator codon has not yet been fully illustrated in the ALA1 gene. To further address this issue, we next mutated the −3 nucleotide (Fig. 4A) and tested its effect on the translational efficiency (Fig. 4B). As shown in Fig. 4, mutation of −3 A to T, G, and C reduced the initiation activity by
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FIGURE 2. Effect of the penultimate N-terminal residue on the turnover of AlaRS-LexA fusions. A, schematic summary of ALA1 mutants. The ALA1 sequence shown includes nucleotides −12 to +15 relative to the first ACG initiation codon. For clarity, initiation codons are shaded, and nucleotides that were mutated are underlined. Amino acid residues encoded by the ALA1 fragment are shown under the respective codon sequence. B, degradation assay. These constructs were expressed under the control of an inducible GAL1 promoter. Upper panel, AlaRS-LexA fusion; lower panel, PGK (as a loading control). T0, T2, T4, T8, T16, and T32, mean 0, 2, 4, 8, 16, and 32 h postinduction, respectively. In B, numbers 1–5 (circled) denote constructs with their ALA1 portions derived from constructs 1–5 shown in A, respectively. Quantitative data for the relative initiating activities of the ALA1-lexA constructs are shown as a separate diagram below the blots.

FIGURE 3. Efficiencies of translation initiation from various combinations of non-AUG initiation codons. A, schematic summary of ALA1 mutants. The ALA1 sequence shown includes nucleotides −12 to +15 relative to the first ACG initiation codon. For clarity, non-AUG initiation codons are shaded, and nucleotides that were mutated are underlined. B, assay of protein expression by Western blotting. Upper panel, AlaRS-LexA fusion; lower panel, PGK (as a loading control). Quantitative data for the relative initiating activities of the ALA1-lexA constructs are shown as a separate diagram beside the blots. In B, numbers 1–6 (circled) denote constructs with their ALA1 portions derived from constructs 1–6 shown in A, respectively.

3.2-, 1.6-, and 2.2-fold, respectively (Fig. 4, numbers 1–4). This result suggested that the nucleotide at position −3 is important to the ACG initiator codons, and that A is the most favorable nucleotide at this position. Nevertheless, this effect was not as profound as anticipated for nucleotide changes at −3 (26). Perhaps that is because the overall initiation was mediated by redundant ACG initiator codons, and changes at the nucleotide position −3 affected mainly, if not only, the first initiator codon. To test this hypothesis and also extend our assay to other non-ATG initiator codons, we next tested the effect of the nucleotide at this position on a single GTG initiator codon. As expected, the effect of the nucleotide at position −3 on a single GTG initiator codon was much stronger than on redundant ACG initiator codons. Mutation of the −3 nucleotide from A to T, G, and C lowered the protein yields by 2.6-, 3.5-, and 12-fold, respectively (Fig. 4, numbers 5–8). Together, these results suggested that the nucleotide at position −3 plays a key role in modulating the efficiency of a non-AUG initiator. So, it is conceivable that in cases of redundant non-AUG initiator codons, the −3 and +1 nucleotides (relative to the first initiator codon) largely determine the efficiency of the preceding and remedial initiator codons, respectively.

Translational Efficiency of Three Successive ACG Initiation Codons—Because the translational efficiency of an ACG initiator codon can be enhanced by redundancy of the initiation

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| Construct | Nucleotides -12 +15 relative to ACG(-25) |
|-----------|--------------------------------------|
| pKJ258   | ATT TAA GAC AAG ACG ACG TCA ACT ACC   |
| pSJ118   | ATT TAA GAC TAG ACG ACG TCA ACT ACC   |
| pSJ110   | ATT TAA GAC GAG ACG ACG TCA ACT ACC   |
| pSJ109   | ATT TAA GAC CAG ACG ACG TCA ACT ACC   |
| pSJ22    | ATT TAA GAC GAG GTG GAC TCA ACT ACC   |
| pSJ25    | ATT TAA GAC CAG GTG GAC TCA ACT ACC   |
| pSJ27    | ATT TAA GAC AAG GTG GAC TCA ACT ACC   |
| pSJ26    | ATT TAA GAC GAG GTG GAC TCA ACT ACC   |

FIGURE 4. Effect of the nucleotide at position −3 on non-AUG initiation. A, schematic summary of ALA1 mutants. The ALA1 sequence shown includes nucleotides −12 to +15 relative to the first ACG initiation codon. Non-AUG initiation codons are shaded, and nucleotides that were mutated are underlined. B, assay of protein expression by Western blotting. Upper panel, AlaRS-LexA fusion; lower panel, PGK (as a loading control). Quantitative data for the relative initiating activities of the ALA1-lexA constructs are shown as a separate diagram beside the blots. In B, numbers 1–8 (circled) denote constructs with their ALA1 portions derived from constructs 1–8 shown in A, respectively.

codonts, we wondered whether this effect could be further extended by inserting more in-frame ACG codons. To investigate this possibility, the codons preceding and following the native ACG initiator codons were individually mutated to an ACG codon and the translational efficiencies of the resultant constructs were tested. As shown in Fig. 5, whereas mutation of TCA(−23) to ACG (resulting in pSJ350) enhanced the protein yield by ~1.3-fold, mutation of AAG(−26) to ACG (resulting in pSJ349) slightly reduced the protein yield by ~1.3-fold. At first glance, these two outcomes appeared to be contradictory to each other. However, further inspection of the nucleotide sequences surrounding the ACG initiation codons suggests that these outcomes were actually caused by different sequence contexts and are still consistent with other examples shown in Figs. 1 and 3. In the case of pSJ349, mutation of AAG(−26) to ACG created only a poor initiation codon (ACG(−26)) with an unfavorable G nucleotide at its relative position −3 and, more importantly, provided an unfavorable nucleotide C at position −2 relative to ACG(−25) (26), such that ACG/ACG/ACG (codons −26 to −24) did not function better than ACG/ACG (codons −25 to −24) in translational initiation (Fig. 5, numbers 1 and 2). Conceivably, context effect plays a predominant role in modulating the translational efficiency of triple ACG codons in this case. In contrast, in the case of pSJ350, mutation of TCA(−23) to ACG changed the nucleotide at position +4 relative to ACG(−24) from T to a preferable A and created a potential initiation site (ACG(−23)) with a favorable A nucleotide at both the relative −3 and +4 positions. Hence, ACG/ACG/ACG (codon −25 to −23) acted stronger than ACG/ACG (codon −25 to −24) as a translational initiation site (Fig. 5, numbers 1 and 3).

Translational Efficiency of Non-successive ACG Initiation Codons—In the case of redundant ACG initiator codons, the second ACG codon not only acts as a remedial initiation site, but also provides a preferable nucleotide A for the preceding ACG at its relative +4 position (Fig. 1) (29). This observation promoted us to ask whether an in-frame, non-successive ACG triplet can also serve as a remedial initiation site and enhance the overall efficiency of translation. To test this possibility, an ACG codon nine nucleotides downstream of ACG(−25) was introduced into the ALA1 sequence, and the translational efficiency of the resultant construct was tested. As shown in Fig. 6, mutation of ACG(−24) to ACC alone reduced the protein yield by 2.5-fold (Fig. 6, numbers 1 and 2), whereas a secondary mutation that changed ACC(−21) to ACG successfully restored the protein yield (Fig. 6, numbers 2 and 3). To ascertain that this compensatory effect actually resulted from the newly inserted non-successive ACG codon, the nucleotide at its relative −3 position was mutated from A to an unfavorable nucleotide C (resulting in pSJ352), and the initiating activity of the resultant construct was assayed. As expected, once the sequence context of ACG(−21) was compromised, the initiating activity of this non-successive initiation codon and the associated compensatory effect became negligible (Fig. 6, numbers 3 and 4). This result provides strong evidence that non-successive ACG codons can be as effective as successive ACG codons in initia-
Redundant Non-AUG Initiation Codons

FIGURE 6. Efficiency of translation initiation from non-successive ACG codons. A, schematic summary of ALA1 mutants. The ALA1 sequence shown includes nucleotides –6 to +21 relative to the first ACG initiation codon. Non-AUG initiation codons are shaded, and nucleotides that were mutated are underlined. B, assay of protein expression by Western blotting. Upper panel, AlaRS-LexA fusion; lower panel, PGK (as a loading control). Quantitative data for the relative initiating activities of the ALA1-lexA constructs are shown as a separate diagram beside the blots. C, RT-PCR. Relative levels of specific ALA1-lexA mRNAs generated from each construct were determined by RT-PCR. Upper panel, ALA1-lexA fusion; lower panel, actin (as an internal control). D, β-galactosidase assay. In B and C, numbers 1–4 (circled) denote constructs with their ALA1 portions derived from constructs 1–4 shown in A, respectively.

Substituting the ATG Initiator of the Mitochondrial Form of ValRS with Redundant GTG Codons—Our previous study showed that redundant ACG triplets can effectively substitute for the ATG initiator codon of the mitochondrial form of ValRS in vivo, while a single ACG triplet can hardly do so (29) (Fig. 7, numbers 3 and 4). To test whether redundant GTG codons can assume a similar function in VAS1 and whether redundant GTG codons act more strongly as an initiation site than does a single GTG codon in this gene, the ATG1 initiator codon of VAS1 was substituted with a single GTG or redundant GTG triplets, and the ability of the resultant constructs to translate the mitochondrial form of ValRS and rescue a vas/− yeast strain on YPG plates was tested. As shown in Fig. 7, redundant GTG codons could functionally substitute for the ATG1 initiator codon of VAS1; the resultant construct successfully rescued the growth defect of the knockout strain on a YPG plate (Fig. 7, number 6). In addition, redundant GTG codons had initiation activity equivalent to that of redundant ACG codons (Fig. 7, numbers 4 and 6). Paradoxically, a single GTG codon acted more strongly as an initiation site than did redundant GTG or ACG codons (Fig. 7, numbers 4–6). This finding is in agreement with the data of the Western blots shown in Fig. 1B (numbers 1, 4, and 5). Thus, the feature of a redundancy effect appears to be independent of the target genes used for analysis. We did not directly assay the relative levels of proteins initiated from these non-ATG initiator codons, because translational initiation from ATG1 (or these non-ATG codons) is likely to be blocked by an upstream, out-of-frame ATG triplet (nucleotides –5 to –3 relative to ATG1) (Fig. 7A), making the proteins practically undetectable by Western blot analysis.

DISCUSSION

In contrast to the scenario of ACG initiation, where redundancy of ACG initiation codons significantly enhances the translational efficiency, we present evidence herein that a redundancy of GTG initiation codons does not enhance the translational efficiency at all, but instead lowers the translational efficiency (Fig. 1). Thus, a redundancy of non-ATG initiation codons does not always improve the overall translational efficiency (Fig. 1). Thus, a redundancy of non-ATG codons is likely to be associated with its function in translation, providing they contain a similar sequence context. Fig. 6C shows that similar levels of cDNA products were generated from these fusions, suggesting that mutations at these sites did not compromise the stability of the specific mRNAs in vivo. To substantiate the inability to create a non-successive remedial initiation site, we next used LacZ as a reporter gene. The results obtained from the ALA1-LacZ fusion constructs were consistent with those obtained from the ALA1-lexA constructs (Fig. 6D).

### TABLE 1

| Construct | Nucleotides –6 +21 relative to ACG (-25) |
|-----------|------------------------------------------|
| pSJ352    | GAT GGT ACG ACC TCA ACT ACC GGA TTA     |
| pSJ355    | GAT GGT ACG ACC TCA ACT GGA TTA         |
| pKJ96     | GAT GGT ACG ACC TCA ACT ACC GGA TTA     |
| pKJ67     | GAT GGT ACG ACC TCA ACT ACC GGA TTA     |
| pSJ399    | GAT GGT ACG ACC TCA ACT ACC GGA TTA     |
| pSJ400    | GAT GGT ACG ACC TCA ACT ACC GGA TTA     |

FIGURE 7. Functional substitution of ATG1 of VAS1 with redundant GTG codons. A, schematic summary of VAS1 mutants. The VAS1 sequence shown includes nucleotides –6 to +16 relative to ATG1. Non-ATG initiator codons are shaded, and nucleotides that were mutated are underlined. B, complementation assays for the mitochondrial function on YPG plates. CW1 was transformed with the wild-type and mutant VAS1 constructs and then its growth phenotypes were tested. In B, numbers 1–6 (circled) denote the VAS1 constructs shown in A.
Redundant Non-AUG Initiation Codons

only a favorable nucleotide A at position −3 for the remedial
initiator codon, but also a favorable nucleotide A at position +4
for the preceding initiator codon (Figs. 1–3). Thus, the redundant
effect manifested by the ACG initiator codons is in effect attributed to a favorable sequence context and, to a lesser extent, remedial initiation (Figs. 1–6). In contrast, if the first nucleotide of the non-AUG initiator codon is a G, redundancy of the initiation codons provides both an unfavorable +4 nucle-
otide G for the preceding initiator codon and an unfavorable −3 nucleotide G for the remedial initiator codon. As a result, the remedial initiator codon is essentially non-functional, and redundant GTG codons act worse than a single GTG codon with a favorable +4 nucleotide A (Fig. 1). This is a unique and interesting scenario, where both “remedial initiation” and “sequence context” contribute to the translational efficiency of redundant non-AUG initiator codons. Conceivably, a portion of the scanning ribosome must be able to skip the first initiator codon and begin from the adjacent initiator codon under such circumstances. This feature is further supported by the observation that a non-successive ACG codon can also serve as an efficient remedial initiation site and enhance the overall translational efficiency (Fig. 6). On the contrary, if the first initiator codon is an ATG triplet, then leaky scanning or remedial initi-
ation is unlikely to take place anywhere downstream of this initia-
tion site. Therefore, no redundancy effect would normally be expected for an ATG initiator codon. In this sense, it is inter-
esting to mention that the expressions of two yeast genes, MODS (coding for isopentenyl pyrophosphate: tRNA isopenten-
yl transferase) (32) and CCAI (coding for ATP (CTP): tRNA nucleotidyltransferase) (33), also involve leaky scanning. How-
ever, leaky scanning occurs in these two genes not because the upstream initiator is a non-AUG codon or an AUG initiator with a suboptimal sequence context, but because the first AUG codon in both genes is positioned too close to the 5′-end of the mRNA. In contrast to the few cases of leaky scanning found in yeast, this mechanism has frequently been observed in mammals (34–37).

Many cellular and viral mRNAs have been shown to use non-
AUG codons, such as AUU, CUG, and GUG, as translation start sites (38). Occasionally, non-AUG codons act as exclusive translation initiators, such as in mRNAs derived from the Arabidopsis AGAMOUS gene (39) and the latently expressed kaposi
locus of Kaposi sarcoma-associated herpesvirus (40). How-
ever, in most cases, non-AUG codons serve as alternative translation start sites that are accompanied by a downstream in-frame AUG initiator (17). Recognition of the non-AUG initi-
tiators in these mRNAs by initiating ribosomes appears to be compensated for by interactions with nucleotide sequences flanking the initiators. A recent study suggested that compo-
nents of the 48 S translation initiation complex, in particular eIF2 and 18 S ribosomal RNA, might be involved in specific recognition of the context nucleotides at the −3 and +4 posi-
tions (18). So, an optimal sequence context is important to the efficient recognition of a non-AUG initiator. Likewise, recogni-
tion of a canonical AUG initiator can be severely compromised by a suboptimal sequence context. In contrast to the active par-
ticipation of the sequence context in translation initiation in higher eukaryotes, many reports have argued that the sequence context does not substantially affect the initiating activity of an AUG initiator in yeast (21, 22, 41). Mostly for this reason, yeast was previously thought to be unable to use non-AUG codons as translation start sites (24). Recently, two genes in S. cerevisiae (ALA1 and GRS1) and one gene in C. albicans (CARP2A) were reported to use naturally occurring non-AUG triplets as translation initiators. Further investigation of the GRS1 genes of 14 yeast species suggested that, except for GRS1 of Cryptococcus neofor-
mans, all of these genes contain an alternative non-AUG initiation site followed by an in-frame downstream AUG initia-
tor codon.4 Fascinatingly, two of these genes contain success-
ive non-AUG initiator codons: Schizosaccharomyces pombe contains 5′-ugcAUAAUucu-3′ (nucleotides −69 to −58 rel-
ative to AUG1) and Yarrowia lipolytica contains 5′-gccAUU-
GUCGucu-3′ (nucleotides −96 to −85 relative to AUG1). A functional assay further suggested that AUU and GUG are the primary initiator codons of the mitochondrial GlyRSs of S. pombe and Y. lipolytica, respectively, lending further support to our finding that the sequence context, in particular the −3 nucleotide, plays a key role in modulating the efficiency of non-
AUG initiators in yeast (Fig. 4) (26).

In higher eukaryotes, the most critical nucleotide positions are −3 and +4 relative to the initiator, and the best (or most favorable) context contains a purine (A or G) at position −3 and a G at position +4 (38). Although sequences immediately flank-
ing AUG initiation codons are somewhat preferred in yeast, the bias in the nucleotide distribution (5′-A(A/Y)A(A/Y)AaugUCY-
3′) is highly divergent from the higher eukaryotic consensus (5′-C(A/G)G-3′), with the exception of a preference for A at the −3 position (20). Consistent with this observation, many highly expressed yeast genes were found to have an A at the −3 position and an A-rich leader region (20). However, many reports have also argued that the sequence context plays only a minor role in translation of yeast mRNAs initiated from AUG. Therefore, the yeast consensus deduced solely from sequence analysis might not actually represent the best context for trans-
lational initiation in yeast. We recently discovered that the best context for the UUG initiator of the yeast GRS1 gene is AAR (R represents A or G) at nucleotide positions −3 to −1, and the most critical nucleotide position is −3. Mutation of −3 A to C alone reduced the initiating activity of the UUG initiator by up to 12-fold (26). Interestingly, the UUG initiator of GRS1 hap-
pens to have AAA at its relative positions −3 to −1, which may explain why only this particular UUG triplet, and not other non-AUG codons that also differ from AUG by a nucleotide elsewhere in the leader sequence, can efficiently function as a translation start site (11). In addition, the redundant ACG ini-
tiator codons of the yeast ALA1 gene also contain the optimal 5′ sequence context AAR (AAG in this case) (Fig. 1) (12), lending further support to our findings. In contrast to positions −3 to −1, the nucleotide at position +4 appears to have a relatively mild effect on the translational efficiency of the UUG initiator. Mutation of the nucleotide at this position from A to any other nucleotide affected the initiating activity at most by 2-fold under the conditions used (Figs. 1 and 3) (26).

4 S.-J. Chen and C.-C. Wang, unpublished data.
Interestingly, several studies suggested that the nature of the penultimate N-terminal residue may influence the substrate preferences of the methionine aminopeptidase and N-\(\alpha\)-acetyltransferase and in turn affect the stability of the protein produced (31, 42). It should be noted that the destabilization by the N-end rule only affects proteins processed by endoproteases and does not influence proteins processed by methionine aminopeptidase alone (43). The penultimate N-terminal residues that favor N-terminal Met removal (e.g. Val, Ser, and Thr) are also considered stabilizing N-terminal residues (44). Thus, any protein that has its N-terminal Met removed by methionine aminopeptidase will still retain a stabilizing N-terminal residue. In contrast, N-terminal residues that are considered destabilizing (e.g. Leu, Phe, and Tyr) are not good substrates for methionine aminopeptidase. Therefore, these proteins will not be processed by methionine aminopeptidase and will retain the stabilizing N-terminal Met. Perhaps for these reasons, our fusions with a different penultimate N-terminal residue showed a similar stability (Fig. 2).

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