Redundancy of Non-AUG Initiators

A CLEVER MECHANISM TO ENHANCE THE EFFICIENCY OF TRANSLATION IN YEAST*

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It was recently shown that ALA1, the only alanyl-tRNA synthetase gene in Saccharomyces cerevisiae, uses two successive ACG triplets as the translation initiators for its mitochondrial form. Evidence presented here argues that the second ACG triplet 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 position +4. Therefore, ALA1 constructs with redundant ACG initiators exhibit stronger complementing activity and express a higher level of protein than do those with a single ACG initiator. A similar scenario is seen when a single or redundant ACG triplets are placed in the positions of the first and second AUG initiators of VAS1, which serve as the start sites of the mitochondrial and cytoplasmic forms of valyl-tRNA synthetase, respectively. Cumulatively, the results suggest that this feature of redundancy of non-AUG initiators in a single mRNA per se may represent a novel paradigm for improving the efficiency of a poor or otherwise nonproductive initiation event.

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 distinct sets of proteins 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 sequestered from isoacceptors confined in other compartments. In most cases, the cytoplasmic and mitochondrial aminoacyl-tRNA 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 forms through alternative initiation from two in-frame AUG codons. Each of these genes encodes mRNAs with distinct 5′-ends. Some of these mRNAs have their 5′-ends located upstream of the first AUG codon, whereas others have their 5′-ends located between the first and second AUG codons. The mitochondrial form of the enzyme is translated from the second in-frame AUG on the “short” messages, whereas the cytosolic form is translated from the second in-frame AUG on the “short” messages. Hence, the mitochondrial enzymes have the same polypeptide sequences as their cytosolic counterparts, except for a short amino-terminal mitochondrial targeting sequence. The transit peptide is subsequently cleaved away upon import into the mitochondria. Because the two isoforms are targeted to different compartments, the two isoforms of ValRS, for example, cannot substitute for each other in vivo (7). A similar scenario has been observed for the genes that encode the mitochondrial and cytoplasmic forms of Arabidopsis thaliana alanyl-tRNA synthetase (AlaRS), threonyl-tRNA synthetase, and ValRS (8). Recently, two rare cases of a single gene encoding both activities have also been discovered 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 (9, 10).

Translation initiation in eukaryotes is a stringent process requiring not only initiator tRNA but also many protein factors. 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 that leads to dissociation of the eIF2-GDP complex from the initiator tRNA in the preinitiation complex signifies 3′-bp codon/anticodon interaction between Met-tRNAiMet and the start codon (11). Recognition of a non-AUG initiator is probably mediated by a similar mechanism but with a lower level of GTP hydrolysis, due to poor interaction between the start codon and initiator tRNA. Previous studies on CYC1 (12) in S. cerevisiae suggested that AUG is the only codon recognized as the translational initiator and that the AUG codon nearest the 5′-end of an 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 (13). 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) in position −3 and a “G” in position +4 (14). Thus, mutations in the sequence region surrounding the first AUG can lead to its inefficient utilization as initiator and subsequent use of an AUG at a downstream location. In addition to sequence context, a stable hairpin structure located 12–15 nucleotides downstream from the initiator can also facilitate recognition of a non-AUG codon by the 40 S ribosomal subunit (15).

In contrast, there seems to be little effect from sequence context in yeast (16), and perhaps for that reason, yeast cannot efficiently use non-AUG codons as the translation start site (17). So far, only three yeast genes, GRS1 (one of the two glycyl-tRNA synthetase genes in S. cerevisiae) (9), ALA1 (the only AlaRS gene in S. cerevisiae) (10), and CARP2A (the gene coding for acidic ribosomal protein 2A in Candida albicans) (18) have been reported to use naturally occurring non-AUG triplets as translation initiation sites.

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. Although there are two homologous glycyl-tRNA synthetase genes, namely GRS1 and GRS2, in the yeast genome, only one (i.e. GRS1) is functional and provides both cytoplasmic and mitochondrial aminoacylation activities, whereas the other (i.e. GRS2) appears to be dispensable for growth (19). Further studies by site-specific mutagenesis indicated that these two activities are provided by two functionally exclusive protein isoforms alternatively generated from a single message of GRS1. A short form that is responsible for the cytoplasmic activity is translationally initiated from a classical AUG initiator, whereas a longer isoform that provides the mitochondrial activity is initiated from an upstream in-frame UUG codon (9). Expression of ALA1 follows a similar scenario. However, unlike all previous examples of non-AUG initiation, the mitochondrial form of AlaRS is initiated from two successive in-frame ACG triplets 69 nucleotides upstream of the putative AUG initiator of the cytoplasmic form (i.e. ACG(−25) and ACG(−24)) (10). (The numbers −25 and −24 in parenthesis refer to the 25- and 24-codon triplets that precede AUG1, respectively.) These two ACG triplets function redundantly in initiation of protein translation. In this report, we investigated the unique feature of redundancy of non-AUG initiators in protein synthesis and the effect of sequence context on initiation from these noncanonical start sites. In particular, we wondered whether redundant ACG triplets could function in a gene that normally uses an AUG initiator. Quantitative analyses by growth curve assays as well as Western blots showed that redundant ACG triplets are more efficient in initiation of protein translation than a single ACG triplet. Sequence context, in particular the nucleotide at position +4 relative to the non-AUG initiator, also considerably affected the efficiency of translation initiation. Remarkably, redundant ACG triplets could not only substitute for the AUG initiator of the mitochondrial form but also the AUG initiator of the cytoplasmic isoform of VAS1. A single ACG triplet, on the other hand, could barely do so. But most surprising of all, redundant ACG triplets could still function as initiators when relocated to a position 5 codons downstream of the native initiation site. It is therefore likely that the efficiency of a relatively weak initiation event can be enhanced by successive recognition of two or more poor initiators by leaky ribosomes. Considering the fact that sequence context provides only limited help for recognition of the start site in yeast, this elegant feature of redundancy of non-AUG initiators may be important for expression of certain genes in this microorganism.

EXPERIMENTAL PROCEDURES

Construction of Mutants and Plasmids—The wild-type ALA1 gene (designated as wt ALA1) (20), extending from 250-bp upstream of its open reading frame to 300-bp downstream of the stop codon, was first amplified by PCR, using yeast genomic DNA as the template, and then cloned into the low copy number yeast shuttle vector pRS315 (21) for expression. Various point mutations, such as ACG(−25) to AAT and ACG(−24) to TCG, were subsequently introduced into the wild-type clone following standard protocols. Construction of the wild-type VAS1 gene and its mutants in the high copy number yeast shuttle vector pRS425 followed a similar strategy (22).

Construction of the lexA gene and various ALA1-lexA fusions for Western blot analysis was as previously described (10). The open reading frame of lexA was amplified by PCR as an NdeI-XhoI fragment and cloned in the high copy number yeast shuttle vector pADH to give wild-type lexA, where the initiator ATG is part of the NdeI site. The NdeI site at the 5’-end of lexA was subsequently mutated to a SpeI site, resulting in lexA*, where the asterisk denotes a mutation in the ATG initiator of lexA. 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 PstI/SpeI sites 5’ to lexA*, resulting in ALA1-lexA* fusions. These ALA1-lexA* constructs were expressed under the control of a constitutive ADH promoter (23). Construction of various VAS1-lexA* fusions followed a similar strategy.

Complementation Assays for the Cytoplasmic Function of ALA1—The yeast ALA1 knock-out strain TRY11 (MATa, his3Δ200, leu2Δ1, lys2-801, trp1Δ101, ura3-52, ala1Δ::TRP1) is maintained by a plasmid carrying the wild-type ALA1 gene and a URA3 marker (20). Complementation assays for the cytoplasmic function of plasmid-borne ALA1 and its derivatives were carried out by introducing a test plasmid (with a LEU2 marker) into TRY11 and determining the ability of transformants to grow in the presence of 5-fluoroorotic acid (5-FOA). The cultures were incubated at 30 °C for 3–5 days or until colonies appeared. Photos for the complementation assays were taken at day 3 following incubation. The transformants evicted the maintenance plasmid that carries a URA3 marker in the presence of 5-FOA. Thus, only an enzyme with the cytoplasmic AlaRS activity encoded by the test plasmid could rescue the growth defect.

Complementation Assays for the Mitochondrial Function of ALA1—Briefly, TRY11 was co-transformed with a test plasmid (carrying a LEU2 marker) and the second maintenance plasmid. In the presence of 5-FOA, the first maintenance plasmid (carrying a URA3 marker) was evicted from the co-transformants, whereas the second maintenance plasmid (carrying a HIS3 marker) was retained. Thus, all co-transformants survived 5-FOA selections, due to the presence of the cytoplasmic AlaRS derived from the second maintenance plasmid. The co-transformants were further tested on YPG plates for their mitochondrial phenotypes at 30 °C, with results documented at day 3 following plating. Because a yeast cell cannot survive on glycerol without functional mitochondria, the co-transformants do not grow on YPG plates unless a functional mitochondrial AlaRS is generated from the test plasmid. The second maintenance plasmid (with a HIS3 marker) contained ALA1(1−1)stop cloned in pRS313 (20). Complementation assays for the cytoplasmic and mitochondrial functions of VAS1 and its mutants were as previously described (22).

Western Blot—The protein expression patterns of the lexA fusions were determined by a chemiluminescence-based Western blot analysis following standard protocols. The lexA fusion constructs were first transformed into INVSc1 (Novagen), and the transformants were subsequently grown in a selection medium lacking leucine. Total protein extracts were prepared from each of the transformants with a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% SDS, 0.5% Triton X-100, 20 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. Aliquots of the protein extracts (~40 μg) were loaded onto a minigel (size 8 × 10 cm) containing 12% polyacrylamide and electrophoresed at 100 V for ~2 h. Following electrophoresis, the resolved proteins were transferred to a nitrocellulose membrane using a semidry transfer device. The membrane was probed with a horseradish peroxidase-conjugated anti-LexA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then exposed to an x-ray film following the addition of the appropriate substrates. The Western blot data were obtained from at least three independent experiments (only one blot was shown in each figure), and the relative intensity of the protein bands shown was presented as (mean ± 2 × S.D.) using the mean value of the intensity of the protein band initiated from the native initiator as a reference.

Growth Curve Assay—Growth curve assays for the plasmid-borne cytoplasmic and mitochondrial VaRS activities were carried out in 5-FOA and YPG broth, respectively. For determining the cytoplasmic activity, one colony of the CW1 (a vasI− strain) transformants grown on an
A

| **ALAI construct** | **Codons -26~21 in ALAI** | **Complementing activity (Mit)** |
|--------------------|---------------------------|---------------------------------|
| pCW413             | AAG ACG ACG TCA ACT ACC   | +                               |
| pKJ216             | AAG ACG **ACC** TCA ACT ACC | +                              |
| pHLT35             | AAG ACG **TCG** TCA ACT ACC | +                              |
| pKJ217             | AAG ACG **TTG** TCA ACT ACC | +                              |
| pHLT31             | AAG **AAT** ACG TCA ACT ACC | ±                              |
| pIVY86             | AAG **AAT** ACG **ACA** ACT ACC | +                              |
| pKJ180             | AAG **AAT** ACG **ACG** ACT ACC | +                              |
| pHLT27             | AAG **AAT** TCG TCA ACT ACC   | -                               |
| pIVY85             | AAG **AAT** ACG **AGA** **TCT** ACT ACC | +                              |

SD/-Ura-Leu plate was inoculated in 3 ml of SD broth lacking uracil and leucine and grown to stationary phase. Appropriate amounts of the cell culture were transferred to a flask containing 10 ml of 5-FOA broth to a final cell density of $A_{600} = 0.1$. The cell culture was shaken in a 30 °C incubator, and the cell density of the culture was checked every 16 h for a period of 96 h. For determining the mitochondrial activity, CW1 co-transformants grown on an SD/-Ura-His-Leu plate were first selected on a 5-FOA plate, and then one colony of the survivors was inoculated in 3 ml of SD broth lacking histidine and leucine and grown to stationary phase. Appropriate amounts of the cell culture were transferred to a flask containing 10 ml of YPG broth to a final cell density of $A_{600} = 0.1$. The cell culture was shaken in a 30 °C incubator, and the cell density of the culture was checked every 4 h for a period of 28 h.

B

C

FIGURE 1. Translation initiation of ALA1 from a single or redundant ACG triplets. The $\text{alal}^+$ strain, TRY11, was transformed with wild-type and mutant ALA1 constructs and then tested for its growth phenotypes. Complementation of the mitochondrial phenotype of the $\text{alal}^+$ strain was shown by its ability to lose the maintenance plasmid following 5-FOA selection and grow on a YPG plate. A, summary of the ALA1 constructs and their complementation activities. Nucleotide sequences shown include nucleotides -78 to -61 (i.e., codons -26 to -21 relative to ATG1 of ALA1), into which individual mutations were introduced. For clarity, codons that were mutated are shaded and boxed. +, ±, and −, positive, weakly positive, and negative complementation, respectively. Mit, mitochondrial. B, complementation assays for the mitochondrial AlaRS activity on a YPG plate. The numbers 1–9 denote pCW413–pIVY85 (from top to bottom), respectively. C, growth curve of the TRY11 transformants containing various plasmid-borne ALA1 constructs in YPG broth. The numbers (circled) 1, 3, 5, and 8 represent the growth curves of transformants harboring pCW413, pHLT35, pHLT31, and pHLT27, respectively.

RESULTS

Redundant ACG Triplets Acting as a Translation Start Site—Unlike all known genes that use a single non-AUG codon as an alternative or exclusive start site, ALA1, the only gene coding for AlaRS in the yeast genome (20), uses redundant ACG codons as the start sites for the synthesis of its mitochondrial form (10). These two ACG triplets function redundantly. Either triplet can initiate translation of the mitochondrial form in the absence of the other. Nevertheless, ACG(-25) appeared to play a predominant role in initiation, as demonstrated by a qualitative complementation test (10). We wondered whether this feature of redundancy is just a fluke or represents a novel mechanism of translational control of ALA1 gene expression. To gain further insight,
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Assaying mitochondrial AlaRS activity for constructs that express a functional cytoplasmic AlaRS could be carried out by plating the transformants on YPG (yeast extract-peptone-glycerol) following selection on 5-FOA. However, assaying mitochondrial AlaRS activity for constructs that do not encode a functional cytoplasmic AlaRS required an additional maintenance plasmid to provide the essential, cytoplasmic AlaRS activity. Mitochondria that expresses a functional cytoplasmic AlaRS but is defective in the mitochondrial AlaRS activity. We have previously shown that the efficiency of translation initiation from the redundant ACG triplets is around 30% compared with an AUG codon placed in the same position.

To look directly at the protein levels initiated from these various triplets, we turned to Western blot analysis. A wild-type or mutant *ALA1* sequence containing base pairs −105 to −24 was amplified by PCR from the above mentioned mutants (Fig. 1A) and fused in frame to the open reading frame of *lexA* (where the native ATG initiator had been inactivated by mutation to ACT), resulting in various *ALA1-lexA* constructs (exemplified in Fig. 2A). These fusions were expressed under the control of a constitutive *ADH1* promoter. As shown in Fig. 2B, more protein was produced from the redundant ACG triplets than from either of the ACG triplets (compare pKJ258, pKJ279, and pKJ281), and ACG(−25) is more robust than ACG(−24) (compare pKJ279 and pKJ281), consistent with the observations made in functional assays. When the redundant ACG triplets were both mutated to codons unsuitable for initiation, no protein band was observed (see pKJ284). When ACG(−24) was mutated to ACC (resulting in pKJ278), protein synthesis was also significantly reduced as compared with the redundant ACG triplets (compare pKJ258 and pKJ278), but was much higher than that from pKJ279, which has a T at nucleotide +4 relative to ACG(−25) (compare pKJ278 and pKJ279). This result suggested that the nature of the nucleotide at position +4 relative to ACG(−25) also affects the initiating potential of the poor initiator, with A being a more favorable nucleotide than T. Remarkably, when TCG(−24) in pKJ279 was further mutated to TTG (resulting in pKJ280), the overall production of protein increased to a level comparable with that from pKJ278 (compare pKJ278, pKJ279, and pKJ280), despite the fact that both pKJ279 and pKJ280 have a T at nucleotide +4 relative to ACG(−25). Thus, it appears that redundant non-AUG initiators, such as ACG/ACC (in pKJ258) and ACC/TTG (in pKJ280), act as superior start sites than a single non-AUG initiator, such as ACG/TTG (in pKJ279). In addition, sequence context also appears to play a significant role in initiation, as evidenced by the higher protein expression level from ACG/ACC than from ACG/TTG (in these two instances, only ACG(−25) actually initiates the protein translation, whereas the codon at position −24 modulates its initiating efficiency and is not by itself base-paired with initiator tRNA for initiation). A similar scenario was seen in the initiation event of ACG(−24), where ACG(−25) had been inactivated by mutation to AAT. When TCA(−23) in pKJ281 was mutated to ACA (resulting in pKJ282), the initiating activity of ACG(−24) was significantly enhanced (compare pKJ281 and pKJ282). When ACA(−23) in pKJ282 was further mutated to ACG (resulting in pKJ283), the overall production of protein...
increased even more (compare pKJ282 and pKJ283). Thus, redundant ACG triplets (ACG(25)/ACG(24) or ACG(24)/ACG(23)) function as a better start site than a single ACG triplet, irrespective of their locations. Since ACG(25) appears to be more robust than ACG(24) within a similar context, we wondered whether we could enhance the initiating activity of ACG(24) simply by inserting an extra codon downstream of this site, thereby changing the relative position of this ACG to codon position 25 relative to ATG1. As shown in Fig. 2B, inserting an extra codon behind ACG(24) (by changing TCA(23) to AGATCT) (resulting in pKJ285) did enhance its initiating activity (compare pKJ281 and pKJ285), but this effect appears to result from changes in sequence context (i.e. the nucleotide at position +4 relative to ACG(24) changed from T to A) rather than changes in position relative to ATG1 (compare pKJ278, pKJ282, and pKJ285). As a result, ACG/ACG appears to be the best initiation site among these variants, perhaps because it combines the beneficiary effects of both sequence context and redundancy of non-AUG initiators.

**FIGURE 3. Substituting the AUG initiator of the mitochondrial form of ValRS with a single or redundant ACG triplets.** The vas1 strain, CW1, was transformed with wild-type and mutant VAS1 constructs and then tested for its growth phenotypes. Complementation of the mitochondrial phenotype of the vas1 strain was shown by its ability to lose the maintenance plasmid following 5-FOA selection and grow on a YPG plate. A, summary of the VAS1 constructs and their complementation activities. Nucleotide sequences shown include nucleotides -6 to +24 (i.e. codons -2 to +8 relative to ATG1 of VAS1), into which individual mutations were introduced. For clarity, codons that were mutated are shaded and boxed. +, ±, and −, positive, weakly positive, and negative complementation, respectively. Mit, mitochondrial. B, complementation assays for the mitochondrial ValRS activity on a YPG plate. C, growth curve of the CW1 transformants containing various plasmid-borne VAS1 constructs in YPG broth. The numbers 1–6 denote pCW355–pKJ110 (from top to bottom), respectively.

Substituting the AUG Initiator of the Mitochondrial Form of ValRS with Redundant ACG Triplets—Our previous study has shown that redundant ACG triplets are only ~30% as strong as an AUG initiator located within a similar sequence context (10). We wondered whether redundant ACG triplets or even a single ACG triplet could serve as the start site for a gene that normally initiates from an ATG triplet. We chose VAS1, the yeast gene that codes for both cytoplasmic and mitochondrial forms of ValRS through alternative transcription and translation, as the target for its well characterized translation patterns and complementation activities (22). To start with, ATG1 of VAS1 (i.e. the ATG initiator of the mitochondrial form of ValRS) was substituted with a single ACG or redundant ACG triplets and tested for its effect on protein synthesis and thus function of VAS1. As demonstrated previously (10), mutation of ATG1 to GCG (resulting in pCW327) deprived the construct of its mitochondrial activity (Fig. 3), suggesting that ATG1 was the only translation initiator of the mitochondrial form. Whereas redundant ACG triplets could effectively substitute for ATG1 in initia-
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The relative ratio of the intensity of each protein expression profiles of various *VAS1*-lexA fusion protein generated from each construct. The indicated on the top is the migrating position of the VAS1-lexA construct used for expression (pIVY46 to pKJ239). The relative ratio of the intensity of each protein band was shown below the blot, using pIVY46 as a reference.

| VAS1-lexA* construct | Codons -2 ~ +8 in VAS1 |
|----------------------|------------------------|
| 1. pIVY46            | GAT GGT ATG AAT AAG TGG TTA AAC ACA TTA |
| 2. pIVY70            | GAT GGT GCG AAT AAG TGG TTA AAC ACA TTA |
| 3. pIVY71            | GAT GGT ACG AAT AAG TGG TTA AAC ACA TTA |
| 4. pIVY72            | GAT GGT ACG ACG AAT AAG TGG TTA AAC ACA TTA |
| 5. pKJ224            | GAT CAT ATG AAT AAG TGG TTA AAC ACA TTA |
| 6. pKJ400            | GAT CAT GCG AAT AAG TGG TTA AAC ACA TTA |
| 7. pKJ237            | GAT CAT ACG AAT AAG TGG TTA AAC ACA TTA |
| 8. pKJ239            | GAT CAT ACG ACG AAT AAG TGG TTA AAC ACA TTA |

A

B

FIGURE 4. Western blot analysis of VAS1-lexA fusions initiated from a single or redundant ACG triplets. The initiating activity of a single or redundant ACG triplets was determined by their efficiency to start the synthesis of a LexA fusion protein from various *VAS1*-lexA constructs. A, summary of the VAS1-lexA* constructs. A wild-type or mutant VAS1 sequence (containing base pairs 15 to 110 relative to ATG1) was fused in frame to the 5'-end of the lexA* sequence, and the resultant VAS1-lexA* constructs were transformed into INVSc1 for expression. Nucleotide sequences shown include nucleotides 6 to +24 (i.e. codons -2 to +8 relative to ATG1 of VAS1), into which individual mutations were introduced. For clarity, codons that were mutated are shaded and boxed. B, Western blot analysis of the expression profiles of various VAS1-lexA* fusions. Indicated on the left is the migrating position of the fusion protein generated from each construct. The VAS1-lexA* constructs were used for expression (pIVY46 to pKJ239) are indicated as 1–8 at the top of each lane. The relative ratio of the intensity of each protein band was shown below the blot, using pIVY46 as a reference.

The initiating activity of a single or redundant ACG triplet could hardly do so (see pKJ96) (Fig. 3B). Transformants carrying pKJ67 (with redundant ACG initiators) grew almost as well as those carrying pCW355 (with a canonical ATG initiator) in a growth curve assay, whereas transformants carrying pKJ96 (with a single ACG initiator) could hardly grow in YPG broth (Fig. 3C). Because the redundant ACG triplets were placed in the position of the native initiation site in this case, we wondered whether redundant ACG triplets worked well at this position simply because they were placed in an optimal sequence context or helped by some unidentified RNA structure at a specific distance. To verify our hypothesis, we then relocated redundant ACG triplets 15 nucleotides downstream to a position with a suboptimal context (with a T at nucleotide positions -3 and +4 relative to the non-ATG initiators) and checked their ability to function as a start site. Amazingly, redundant ACG triplets also functioned well at this position of the gene. As shown in Fig. 3B, the introduction of redundant ACG triplets to codon positions 6 and 7 of pCW327 (which had a mutation of ATG1 to GCG) (resulting in pKJ110) efficiently rescued its mitochondrial defect, whereas introduction of a single ACG to a similar position (resulting in pKJ110) efficiently rescued its mitochondrial defect, whereas introduction of a single ACG triplet to codon position 1 of the VAS1 portion. In addition, it was noticed that much less protein was produced from the VAS1-lexA* constructs than from analogous ALA1-lexA* fusions under similar conditions (compare Figs. 2 and 4). Comparison of the primary sequences flanking the upstream initiator of VAS1 and ALA1 led to the discovery of an out-of-frame ATG triplet located between nucleotide positions -5 to -3 relative to ATG1 of VAS1, which might contribute to the lower level of protein expression in VAS1-lexA*. Indeed, mutation of the out-of-frame ATG triplet radically enhanced production of the fusion proteins from the putative initiator and its derivatives (compare lanes 1–4 with lanes 5–8). Even under these conditions, redundant ACG triplets still function more efficiently than a single ACG triplet in the initiation of protein translation (compare lanes 7 and 8). It is therefore likely that under normal conditions, the expression of the mitochondrial form of ValRS is delicately modulated at the translational level through an upstream out-of-frame AUG triplet. Likewise, when the out-of-frame AUG triplet in pKJ96 was removed, the resulting mutant exhibited a higher complementing activity (data not shown).

Substituting the AUG Initiator of the Cytoplasmic Form of ValRS with Redundant ACG Triplets—Our results so far have shown that redundant ACG triplets or even a single ACG triplet could functionally substitute for ATG1 of VAS1 (i.e. the ATG initiator of the mitochondrial form of ValRS). We wondered whether redundant ACG initiators are strong enough to substitute for ATG47 of VAS1 (i.e. the ATG initiator of the cytoplasmic form of ValRS). Therefore, ATG47 of VAS1 was changed to GCG, ACG, or ACG/ACG by mutagenesis, and the resulting constructs were tested for their ability to synthesize the cytoplasmic enzyme. Because high expression of the mitochondrial form (from
VAS1 constructs cloned in the high copy number vector pRS425) could partially rescue the growth defect of a yeast vas1 Δ strain, CW1, was shown by its ability to lose the maintenance plasmid and grow on a 5-FOA plate. A summary of the VAS1 constructs and their complementation activities. Nucleotide sequences shown include nucleotides 136–150 (i.e. codons 46–50 relative to ATG1 of VAS1), into which individual mutations were introduced. For clarity, codons that were mutated are shaded and boxed. Note that ATG1 was mutated to GCG in all these constructs. + and −, positive and negative complementation, respectively. Cyt, cytoplasmic. B, complementation assays for the cytoplasmic ValRS activity on a 5-FOA plate. C, growth curve of the CW1 transformants containing various plasmid-borne VAS1 constructs in 5-FOA broth. In B and C, the numbers 1–4 denote pCW327, pCW338, pKJ193, and pKJ63, respectively. D, Western blot analysis of the expression profiles of various VAS1-lexA* fusions. The VAS1 portion in these constructs (numbered 1–4) was amplified in order from the constructs shown in A, indicated on the left is the migrating position of the fusion protein generated from each construct.

**FIGURE 5.** Substituting the AUG initiator of the cytoplasmic form of ValRS with a single or redundant ACG triplets. Complementation of the cytoplasmic phenotype of the vas1 Δ strain. CW1, was shown by its ability to lose the maintenance plasmid and grow on a 5-FOA plate. A, summary of the VAS1 constructs and their complementation activities. Nucleotide sequences shown include nucleotides 136–150 (i.e. codons 46–50 relative to ATG1 of VAS1), into which individual mutations were introduced. For clarity, codons that were mutated are shaded and boxed. Note that ATG1 was mutated to GCG in all these constructs. + and −, positive and negative complementation, respectively. Cyt, cytoplasmic. B, complementation assays for the cytoplasmic ValRS activity on a 5-FOA plate. C, growth curve of the CW1 transformants containing various plasmid-borne VAS1 constructs in 5-FOA broth. In B and C, the numbers 1–4 denote pCW327, pCW338, pKJ193, and pKJ63, respectively. D, Western blot analysis of the expression profiles of various VAS1-lexA* fusions. The VAS1 portion in these constructs (numbered 1–4) was amplified in order from the constructs shown in A, indicated on the left is the migrating position of the fusion protein generated from each construct.

VAS1 constructs cloned in the high copy number vector pRS425) could partially rescue the growth defect of a yeast vas1 Δ strain on a 5-FOA plate (22), ATG1 of VAS1 was also mutated (to GCG) in all of these constructs to diminish the background level of growth in 5-FOA. The yeast VAS1 knock-out strain CW1 is maintained by a plasmid carrying the wild-type VAS1 gene and a URA3 marker (22). Complementation assays for the cytoplasmic function of plasmid-borne VAS1 and its derivatives were carried out by introducing a test plasmid (with a LEU2 marker) into CW1 and determining the ability of transformants to grow in the presence of 5-FOA. The transformants evicted the maintenance plasmid that carries a URA3 marker in the presence of 5-FOA. Thus, only an enzyme with the cytoplasmic ValRS activity encoded by the test plasmid could rescue the growth defect. As shown in Fig. 5, A and B, whereas mutation of ATG47 to GCG completely abolished the cytoplasmic function of the construct (compare pCW327 and pCW338), mutation of the same codon to ACG or mutation of ATG47/AGC48 to ACG/ACG did not impair its cytoplasmic function (compare pCW327, pKJ193, and pKJ63) (i.e. transformants formed colonies after 2–3 days of incubation on a 5-FOA plate). Further quantitative analysis by a growth curve assay showed that redundant ACG triplets are noticeably more efficient in translation initiation than a single ACG triplet (Fig. 5 C), albeit less efficient than an ATG triplet. We next used Western blot to assay the efficiency of translation initiated from ATG47 or its derivatives. A wild-type or mutant VAS1 sequence containing base pairs 96–180 was PCR-amplified and fused in frame to an initiator mutant of lexA, resulting in various VAS1-lexA* fusions. As shown in Fig. 5 D, much more protein was produced from the construct with an AUG initiator (lane 1; pGL79) than from those with a GCG (lane 2; pGL78), a single ACG (lane 3; pGL81), or redundant ACG initiators (lane 4; pGL80). Even so, it was still evident that redundant ACG triplets served as a better start site than a single ACG (compare lanes 3 and 4), and no protein was produced from pGL78 (lane 2). These results also indicate
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that protein levels can be reduced significantly (Fig. 5D), but complementation still functions at fairly high efficiency (Fig. 5, B and C).

DISCUSSION

The results presented here provided evidence that redundant ACG triplets function more efficiently as a translation start site than a single ACG triplet, irrespective of their sequence context, as determined by Western blots as well as quantitative growth curve assays (Figs. 1–5). In particular, our functional assays showed that redundant ACG triplets could efficiently serve as a translation start site in a gene that normally starts from a classical AUG initiator (Figs. 3 and 5). A similar observation has been made in the yeast NFS1 gene (coding for a mitochondrial cysteine desulfurase), where the AUG start codon can be substituted for by UUG (24). However, two major differences are worthy of further discussion. First, redundant ACG triplets could substitute not only for the AUG initiator of the mitochondrial form of ValRS but also for the AUG initiator of its cytoplasmic isoform. This is quite unexpected in light of the common perception that a cytoplasmic enzyme actively involved in the translation process is in general expressed at considerably high levels to cope with the needs of a fast growing yeast cell. As a result, protein levels of major enzymes derived from substitution of the mitochondrion of this gene appears to also be translated from a single transcript: one initiated at the non-AUG codon and the other at a downstream AUG. Although this mechanism has been observed frequently in mammals (29–32), it has not been reported previously in yeast. In this regard, ALA1 is to our knowledge the first example in yeast wherein two functionally exclusive protein isoforms can be translated from a single transcript, with redundant ACG triplets being the initiator of the mitochondrial form and a downstream in-frame AUG triplet being the initiator of the cytoplasmic isoform. Because redundant ACG triplets in the sequence context of ALA1 are only ~30% as efficient in initiation of protein translation as a classical AUG initiator placed at the same position (10), it is feasible that a large portion of the scanning ribosomes might skip the relatively weak initiation site and continue scanning downward until they encounter the first AUG initiator. Despite being weaker than canonical AUG initiators, the level of protein produced from redundant ACG triplets is still substantial compared with other examples of non-AUG initiation (16). Therefore, we reckoned that the second ACG triplet in the redundant ACG initiators serves as a remedial initiation site for the "leaky" ribosomes that skip the first ACG triplet, thereby improving the overall efficiency of protein translation. By the same token, we speculate that changing TCA(−23) to ACG would further enhance the efficiency of translation; however, due to possible effects of codon position (Fig. 1), the increase in protein production should be limited to a lesser extent. It is worth mentioning that a different mode of leaky scanning has previously been reported in yeast MOD5 (coding for isopentenyl pyrophosphatetRNA isopenyltransferase) (33) and CCA1 (coding for ATP (CTP):tRNA nucleotidyltransferase) (34), where leaky scanning occurs because the first AUG codon is positioned too close to the 5′-end of the mRNA.

In higher eukaryotes, both sequence context and secondary structure can significantly modulate the efficiency of translation initiation. For example, an optimal sequence context, particularly an A/G in nucleotide position −3 and a G in position +4, can appreciably enhance the efficiency of translation initiation (13). Also, a stem-loop structure located at a specific distance downstream from the start site has been shown to slow scanning by the 40 S ribosome, thereby providing more time for recognition of an AUG initiator in a suboptimal context or a non-AUG initiator (15). In contrast, in low eukaryotes, sequence context plays a significant role in initiation of translation (35), and the effect of secondary structure on initiation has not been well documented. For example, Cigan et al. (16) have demonstrated that sequence context changes both 5′ and 3′ to the yeast HIS4 AUG initiator resulted in no more than a 2-fold decrease of expression. However, in the case of ALA1, the nucleotide at position +4 relative to the ACG initiators appears to have a stronger effect on initiation than expected, as determined by Western blots (Fig. 2). Comparison between ACG(−25)/ACC(−24) and ACG(−25)/TCA(−24) (compare pKJ278 and pKJ279) shows that the +4 nucleotide can modulate the efficiency of the ACG initiator by more than doubling the amount of protein produced, with A being more favorable than T (Fig. 2). A similar observation is made in the comparison between ACG(−24)/ACA(−23) and ACG(−24)/TCA(−23) (compare pKJ282 and pKJ281). Perhaps for this reason, ACG(−25)/ACG(−24) works better than ACG(−25)/TTC(−24) (compare pKJ258 and pKJ280 in Fig. 2) despite the fact that both are composed of successive non-AUG initiators. Although a segment of transcript starting 17 nucleotides downstream of ACG(−25) was predicted to form a pseudoknot structure, in which AAGGAA (nucleotides −55 to −50) paired with UUUUCU (nucleotides −41 to −36) and CUUGA (nucleotides −49 to −45) paired with UCAAG (nucleotides −35 to −31), multiple mutations aimed at disrupting the tentative stem or knot structure affected neither the complementation activity nor protein expression under the conditions used.

3 K.-J. Chang, G. Lin, L.-C. Men, and C.-C. Wang, unpublished results.
not shown). Thus, if RNA secondary structure is important, then the structural features must be subtle and distinct from what we have expected. However, regardless of the detailed interpretation, the most striking finding of the results reported here is the unique feature of redundancy of non-AUG initiators in a single mRNA, which may in itself represent a novel paradigm for augmenting translation initiation from the poor initiators and compensate for poor recognition of the non-AUG initiators by yeast ribosomes.

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