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Efficiency of Translation Initiation by Non-AUG Codons in *Saccharomyces cerevisiae*

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The quantitative levels of initiation of protein synthesis at codons other than AUG were determined with a *CYC7-lacZ* fused gene in the yeast *Saccharomyces cerevisiae*. AUG was the only codon which efficiently initiated translation, although some non-AUG codons allowed initiation at very low efficiency, below 1% of the normal level. Since translation initiates at codons other than AUG in at least two wild-type genes from eucaryotes, other factors presumably play a role in enhancing the activity of non-AUG codons.

The codon AUG is used to initiate translation of most proteins in procaryotes, eucaryotes, and organelles. Other codons, GUG, UUG, and AUU, have been found to initiate translation of a limited number of wild-type proteins in *Escherichia coli*, and there are examples of mutant genes using AUA as an initiation codon (10). When GUG (12) or UUG (19) initiator codons of *E. coli* were mutated to an AUG codon, there was a marked increase in expression of the respective gene, and conversely, mutating the initiator AUG codon to GUG and UUG resulted in decreased expression (16, 25). These observations have suggested that the use of non-AUG initiation codons in *E. coli* may be a mechanism to limit the level of translation of genes whose products are required at low levels, although mRNAs for some abundant proteins, such as the product of the *tufA* gene, use a non-AUG initiation codon (26).

The sequences of hundreds of wild-type genes from eucaryotes reveal that AUG is the initiator codon for all but two genes. The adeno-associated virus capsid protein, AAV-B, initiates at an ACG codon (3), whereas the product of the c-*myc-1* proto-oncogene probably initiates at a CUG codon (7). In addition, the potential use of non-AUG codons for initiating protein synthesis in eucaryotes has been investigated with mutants having altered AUG initiator codons. The analysis of iso-1-cytochrome *c* from *cyc1* revertants and the deduced DNA sequences of certain *cyc1* mutations (20) of the eucaryotic yeast *Saccharomyces cerevisiae* led to the conclusion that AUG was the only codon that could initiate translation at normal or near normal levels. However, Zitomer et al. (27) have reported that a UUG codon can initiate synthesis of a *CYC1-galK* fused protein in *S. cerevisiae* with an efficiency of 6.9%, whereas an AUA codon was used with an efficiency of 0.5%. Other studies have confirmed that an ACG codon can initiate translation; using a cell-free wheat germ translation system programmed with mRNA from a mutant bacteriophage T7 gene (0.3) in which the AUG initiator was altered to ACG, Anderson and Buzash-Pollert (1) showed by N-terminal analysis that approximately 15% of the mutant 0.3 protein initiated at the ACG codon, whereas the remainder initiated at a proximal AUG codon. Furthermore, experiments in vivo and in vitro with cultured monkey cells showed that translation of a mouse dihydrofolate reductase mRNA initiated from a mutant ACG codon with an efficiency as high as 5% when the ACG codon was in an optimal context (18). Thus, eucaryotes can initiate translation from non-AUG codons, sometimes at surprisingly high levels.

The sole use of the AUG codon for efficient initiation of translation in eucaryotes and the use of AUG, GUG, and UUG codons for initiation in procaryotes have been attributed to the presence of a hypermodified base, t^6A, immediately adjacent to the 3′ side of the anticodon of eucaryotic initiator tRNA and its absence in procaryotic initiator tRNA (6, 13, 20, 21, 24). It is thought that the presence of the hypermodified base prevents wobble base pairing with the first nucleotide of the initiation codon. Initiation at AUU and AUA codons in *E. coli* and at other codons in eucaryotes cannot therefore occur by this mechanism.

Since ACG and CUG initiator codons have been found in two wild-type genes from higher eucaryotes and because translation can initiate at codons mutated to ACG and possibly other codons, we devised a study to quantitatively determine the efficiency of translation initiation at all nine codons that differ from AUG by one base. We examined a series of *S. cerevisiae* strains, each having a single copy of a gene with an altered AUG initiator codon. The *CYC7-L* series was constructed from the yeast *CYC7* and *CYC7-H3* genes and the *E. coli lacZ* gene. In contrast to the *CYC1* gene, which encodes iso-1-cytochrome *c*, the *CYC7* gene, which encodes iso-2-cytochrome *c*, has a single major transcriptional start site approximately 90 nucleotides from the AUG initiator codon (5, 8). Thus, the potential contribution of the 5′ noncoding region of the transcript to translation can be more easily assessed in *CYC7*, since it produces only a single species of mRNA. The *CYC7-H3* promoter constitutively overproduces the *CYC7* transcript approximately 20-fold (14) and maintains the normal *CYC7* transcription start site (unpublished results). In order to conveniently quantitate the levels of expression, the *CYC7-H3* promoter region and *CYC7* 5′ region, including the first five codons, were fused to the *E. coli lacZ* gene, creating the allele *CYC7-L69* (Fig. 1 and 2). The fusion was transferred into a yeast integrative plasmid vector with the 3′ noncoding region of the *CYC7* gene placed downstream of *lacZ* to ensure efficient transcription termination of *CYC7-L* transcripts (Fig. 1). Expression of the *CYC7-L* series could thus be easily monitored by β-galactosidase assays.

By oligonucleotide-directed mutagenesis (11), the AUG

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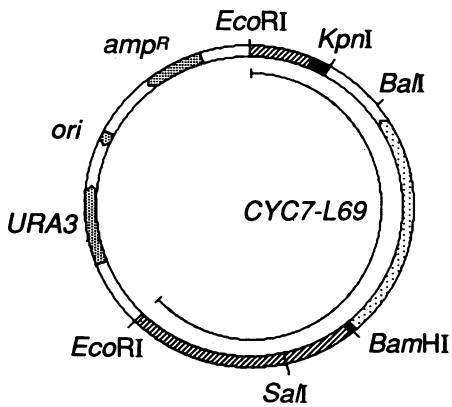

FIG. 1. Yeast integrative plasmid pAB304, with the *CYC7-L69* allele. The 5′ untranslated region of the *CYC7-H3* gene and the 3′ untranslated region of the *CYC7* gene are denoted by hatched boxes; the 5′ and 3′ translated regions of the *CYC7* gene are denoted by closed boxes; and the *E. coli lacZ* gene, derived from plasmid pMC1790 (4), is denoted by a lightly stippled box. The sequence of the junction of the *CYC7* and *lacZ* genes at the *Bam*HI site is shown in Fig. 2. Also shown are certain key restriction sties, the *CYC7-L69* gene, the yeast *URA3* gene, and the *E. coli* Amp^r and *ori* genes (directions of transcription are indicated by pointed ends). The construction of this plasmid is described in detail by Laz (T. Laz, Ph.D. thesis, University of Rochester, Rochester, N.Y., 1988). The plasmid and related derivatives were cut at the *Sal*I site and used to transform the yeast strain B-6442 (*MATa cyc1-363 cyc7::CYH2$^+$ ura3-52 cyh2 his3-Δ1 leu2-3 leu2-112 trp1-289 can1-100*) (9).

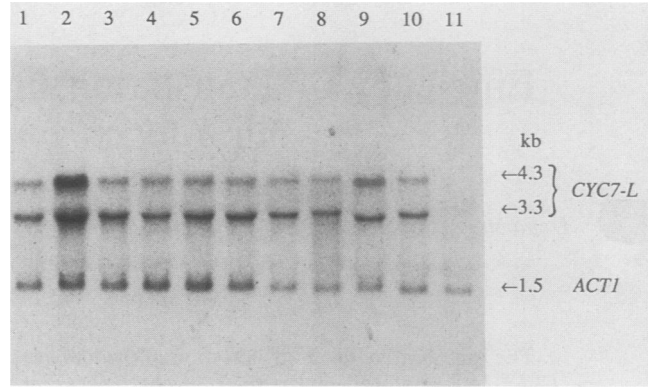

FIG. 3. Northern blot (RNA blot) analysis of non-AUG initiator mutants. Total RNA was isolated from yeast cultures grown in YPD (1% yeast extract, 2% peptone, 2% glucose); 20-μg portions were separated by electrophoresis in a 1.5% agarose gel containing formaldehyde and transferred to nitrocellulose (23). The filter was hybridized to [α-^{32}P]CTP-labeled RNA transcripts complementary to actin or *CYC7-L* mRNA, which were generated by T7 RNA polymerase from an *E. coli lacZ* 1.9-kilobase (kb) *Eco*RI-*Hpa*I fragment cloned in pGEM1 (Promega Biotec) and by SP6 RNA polymerase transcribed from a yeast *ACT1* 1.4-kb *Bam*HI-*Hin*dIII fragment (17) cloned in pGEM1. Hybridization conditions were as described by Thomas (23) except that the hybridization temperature was 55°C and the final wash temperature was 65°C. The lanes contained RNA derived from the following *CYC7* alleles: 1, *CYC7-L77*; 2, *CYC7-L76*; 3, *CYC7-L75*; 4, *CYC7-L74*; 5, *CYC7-L73*; 6, *CYC7-L72*; 7, *CYC7-L71*; 8, *CYC7-L70*; 9, *CYC7-L78*; 10, *CYC7-L69*; 11, *cyc7::CYH2$^+$*. The relative intensities of the bands corresponding to the *CYC7-L* and actin transcripts were compared by densitometry.

initiator codon of the *CYC7-L69* allele was mutated to the nine possible triplets that differ by one base pair from AUG, thus producing the *CYC7-L70* to *CYC7-L78* alleles. The resultant plasmids were linearized at the *Sal*I site (Fig. 1) and used to transform the yeast strain B-6442 (*MATa cyc1-363 cyc7::CYH2$^+$ ura3-52 his3-Δ1 leu2-3 leu2-112 trp1-289 can1-100 cyh2*); strains containing a single stable integrated copy of the plasmid at the *CYC7-H3* locus were identified by genomic blot analysis (data not shown). To further confirm the fidelity of the procedure, all nine alleles were recovered from their respective yeast strains and the nucleotide sequence surrounding the mutation was determined and found to contain the original mutation.

Total RNA from each mutant was separated in an agarose gel, transferred to nitrocellulose, and hybridized with radioactive RNA probes complimentary to *lacZ* and actin mRNAs, the latter being used as an internal control. The resultant blot (Fig. 3) demonstrates that the steady-state mRNA levels from the nine non-AUG initiator mutants did not significantly differ from those of the control strain *CYC7-L69*. Two transcripts of approximately 3.3 and 4.3 kilobases were observed for *CYC7-L* mRNA, which occurred due to transcription termination at two sites, one within the *E. coli* sequences immediately 3′ to the *lacZ* coding sequence and the other at the *CYC7* terminator.

The ability of each mutant to initiate translation was determined by measuring β-galactosidase activity (Table 1), using a modification of the standard assay (15), allowing low levels of activity to be measured. Yeast cells were harvested at an A_{600} of approximately 3.0. For each reaction, 300 μl of yeast culture, 200 μl of Z buffer (15), 30 μl of chloroform, and 20 μl of 0.1% sodium dodecyl sulfate were vortexed for 30 s and the reaction was started by the addition 200 μl of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (4 mg/ml). The reaction mixtures were incubated at 28°C for 300 min, and the reactions were terminated by the addition of 500 μl of 1 M Na_2CO_3. The cellular debris was removed by centrifugation, and the A_{420} was measured. The control reaction was determined by taking 10 μl of a *CYC7-L69* culture plus 290 μl of the parental strain B-6442 and performing the assay as described above. The assay was shown to be proportional

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-90          -80          -70          -60          -50          -40
 .            .            .            .            .            .
CAA AGA CUU AUA CAA GAA GAU CAC AUA CAA GCA UUU AUU CAC AUU ACU UUA AGU AAA

  -30          -20          -10           +1          +10
   .            .            .            .            .          BamHI
CUU CAG UAA ACU ACA UUA CAU CAU AAA CAA AAC AUG GCU AAA GAA AGU GGG GAT CCC
                                           Met Ala Lys Glu Ser Gly Asp Pro
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FIG. 2. Nucleotide sequence of the 5′ mRNA of *CYC7-L69*. The positions of the triplets that differ by one base from AUG and are in frame with the *CYC7-L69* coding sequence are underlined, as are the in-frame termination codon at position −27 and the AUG initiation codon at +1. The position of the *Bam*HI restriction site is shown above the sequence and represents the junction between the *CYC7* and *lacZ* genes.

TABLE 1. β-Galactosidase activity of non-AUG initiator mutants

CYC7 allele	Initiation codon	Relative sp act (% of control)[a]	SD
L69	AUG	100.00	
L76	GUG	0.50	0.060
L78	ACG	0.39	0.040
L75	AUU	0.38	0.065
L74	UUG	0.37	0.061
L73	AUA	0.29	0.062
L71	CUG	0.22	0.024
L72	AUC	0.05	0.011
L77	AGG	0.04	0.011
L70	AAG	0.02	0.009

[a] The results are from three separate experiments; triplicate assays were performed in each experiment.

to protein concentration and linear with time. The total cellular protein content of the yeast cells was determined by the boiling method of Strickland (22). A standard curve of protein content against the A_{600} of yeast cells was used to calculate the protein concentration in each reaction mixture. The activity of β-galactosidase per total cellular protein was determined and compared with the control values.

The GUG codon produced the highest level of initiation, 0.5% of the control level; ACG, AUU, UUG, AUA, and CUG gave intermediate values from 0.39 to 0.22% of the control activity; and AUC, AGG, and AAG showed 0.05% or less of the control activity. Thus there was a 25-fold range in the level of translation between the GUG and AAG codons. It should be noted that previous work has indicated that the bases surrounding the AUG codon can slightly affect translation in *S. cerevisiae* (2; T. Laz, J. M. Clements, and F. Sherman, manuscript in preparation) and that the most favorable context requires an A at the −3 position, which is the case in *CYC7-L69* and related genes.

It could be argued that the translation levels we observed were due to initiation from codons other than those mutated. An examination of the noncoding and downstream coding regions of the *CYC7-L* alleles reveals the presence of four codons at positions −81, −66, −54, and −48 which differ by only one base from AUG and are in frame with the *CYC7-L* coding sequence (Fig. 2). Since these codons all occur upstream of an in-frame translation termination codon at position −27, they would not be expected to contribute to the translation of β-galactosidase (Laz et al., in preparation). The next potential in-frame non-AUG codon downstream of the normal translation initiation site is at position +151, and initiation from this codon would not be expected to produce a functional protein. The lowest value obtained, 0.02% for AAG, may represent the level of initiation from other unidentified codons in the surrounding region. The observation that single base substitutions at the +1 position caused up to a 25-fold increase of translation over the lowest level strongly suggests that these increases were due to initiation of translation from this site.

Our results are in contrast with the results obtained with the *CYC1-galK* system, in which initiation presumably occurred with an efficiency of 6.9% at an UUG codon (27). Also, our results do not reveal particularly active initiation with ACG or CUG, the initiator codons used by certain mammalian genes (3, 7). Thus, the relative activity of non-AUG codons compared with that of the AUG codon does not appear to be a property solely of the non-AUG codons, suggesting that other factors play a role in enhancing the relative activity of non-AUG codons in initiating translation of proteins.

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