The Inhibitory Upstream Open Reading Frame from Mammalian S-Adenosylmethionine Decarboxylase mRNA Has a Strict Sequence Specificity in Critical Positions*

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The upstream open reading frame (uORF) in the 5′ leader of the mammalian mRNA encoding S-adenosylmethionine decarboxylase (AdoMetDC) serves as a negative regulatory element by suppressing translation of the associated downstream cistron. Certain changes in the amino acid sequence of the hexapeptide (sequence MAGDIS) encoded by the uORF destroy suppressive activity, implying specific interaction with a cellular target. In this paper, we examine the extent of alterations that can be tolerated in this uORF. The mammalian AdoMetDC uORF inhibits downstream translation when placed into the 5′ leader of a yeast mRNA with characteristics resembling those in mammalian cells, suggesting that the encoded peptide has a similar target across species. Using yeast for the initial screen, we tested the specificity of the critical three codons at the 3′ end of the uORF by saturation mutagenesis. Altered uORFs selected from the primary yeast screen were then retested in mammalian cells. The requirements at codons 4 and 5 were quite stringent; only aspartic acid at codon 4 yielded a fully suppressive peptide, and only valine could substitute productively for isoleucine at codon 5. The specificity at codon 6 was much looser, with many substitutions retaining suppressive activity in both yeast and mammalian cells.

The occurrence of open reading frames in the 5′ leaders of eukaryotic mRNA molecules is quite rare. These upstream open reading frames (uORFs) are found in less than 10% of cloned mammalian mRNAs; however, the small subset of genes that do contain uORFs is strongly biased toward those encoding growth-related products such as growth factors, growth factor receptors, tumor suppressors, and regulated transcription factors (1–4). It is striking that over two-thirds of the uORFs in eukaryotic cells, the RNA sequence flanking the termination codon and the amino acid sequence of the encoded peptide (1–3). In the yeast GCN4 gene, there are two suppressive uORFs in the 5′ leader (6). The suppressiveness of the GCN4 uORFs is not determined by the sequence of the encoded peptides but by the G + C content of the RNA sequence surrounding the termination codon (7). This led to the hypothesis that stable RNA-RNA interactions in the vicinity of the stop codon may result in suppression of downstream translation. In a second class of suppressive uORFs, referred to as “sequence-dependent uORFs,” suppressive activity depends on the amino acid sequence of the encoded peptide (1). Examples from this class are limited, but the best studied are in the mammalian AdoMetDC gene (8, 9) and the human cytomegalovirus gpUL40 (gp48) gene (10, 11). In both instances, particular missense mutations of the codons at the 3′ end of the uORF abolish inhibitory activity, whereas modifications that retain the wild type amino acid coding information (synonymous mutations) uniformly preserve the suppressive effect of these uORFs.

Because the sequence-dependent uORFs seem to act in cis on the ribosome that translated them (9, 11), a likely model is that the nascent peptide translated from the uORF interacts specifically with a component of the translation apparatus, such as peptidyltransferase, the peptide channel of the ribosome, or one of the termination factors, to stall the ribosome in the vicinity of the termination codon. The stalled ribosome thereby creates a blockade to further translation of the mRNA (1). Consistent with this model, Cao and Geballe (12) have located a ribosome arrested over the termination codon of the wild type, but not mutant, uORF of the gpUL40(gp48) gene.

The nature of the interaction between the encoded peptide and its target seems to be of paramount importance in defining the mechanism of inhibition by the sequence-dependent uORFs. The specificity of this interaction has not yet been examined systematically and in detail for any of the sequence-dependent uORFs. It is interesting to note that there is no similarity between the peptides encoded by the uORFs from the AdoMetDC and gpUL40(gp48) genes, implying that there are multiple intracellular targets for these regulatory molecules. In the studies described in this paper, we find that the specificity of this peptide-target interaction is widely conserved in nature, because the sequence-dependent inhibitory activity of the mammalian AdoMetDC uORF is retained when placed in a yeast 5′ leader. Using a yeast expression system as the primary screen, we examine by saturation mutagenesis the range of amino acids that can be substituted in critical positions of the AdoMetDC uORF with retention of suppressive activity. Substitutions of interest were retested in mammalian cells. The results show an exquisite dependence on amino acid sequence...
Oligonucleotides used in generating chimeric constructs

| uORF   | Sequence                                                                 |
|--------|--------------------------------------------------------------------------|
| MAGD1S | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| AUG → GUG | 5′-agcttagtGGCCGCCGACATTAGCTA-3′                                       |
| A → E   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| G → A   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| D → E   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| D → M   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| D → R   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| D → S   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| I → A   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| I → L   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| I → N   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| I → V   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| S → A   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| S → D   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| S → I   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| S → K   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| S → P   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| S → T   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |
| S → V   | 5′-agcttagtATGCCGCCGACATTAGCTA-3′                                       |

* Wild type amino acid sequence.

In that only aspartic acid is functional at position 4 in the peptide, and only valine will substitute for isoleucine at position 5. These results support the hypothesis of a highly specific interaction between the peptide encoded by the AdoMetDC uORF and its intracellular target.

EXPERIMENTAL PROCEDURES

Chimeric Expression Constructs—Synthetic oligonucleotides were used to insert wild type and modified uORFs into the 5′ leaders of transfection constructs between the AdoMetDC and GCN4 genes and reporter genes. Oligonucleotides (Table I) were designed to create 5′- HindIII and 3′-BglII cohesive ends for insertion into the leaders. All chimeric constructs were sequenced to verify the position and sequence of the uORF.

To generate the chimeric constructs between AdoMetDC and human growth hormone (hGH), unique HindIII and BglII sites flanking the uORF were engineered into the 5′ leader of pRS326, which has the AdoMetDC leader, with 47 nucleotides between the 5′ cap and the uORF, upstream of the hGH coding region (13). The wild type uORF was removed by digestion with HindIII and BglIII, and double-stranded oligonucleotides corresponding to the desired uORFs were cloned into the HindIII/BglII site.

Two chimeric constructs were made by inserting the double-stranded oligonucleotides between the HindIII and BglII sites of pM128 (14). pM128 is an Escherichia coli shuttle vector containing the 5′ leader of Saccharomyces cerevisiae GCN4 gene linked to the E. coli β-galactosidase reporter gene. Digestion of pM128 with HindIII and BglII removes the four wild type uORFs of GCN4.

Mammalian Cell Culture and Transfections—HeLa cells were cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% calf serum and 100 units of penicillin and 50 μg of streptomycin/ml. Stable transfectants were selected using HeLa medium supplemented with 400 μg of G-418 (Geneticin; Calbiochem) and 25 μg of puromycin/ml. After selection, stable transfectants were maintained in the presence of 200 μg of G-418/ml.

Transient transfection of HeLa cells was performed as described previously (8). Transfected cells were harvested after 48 h in culture.

Assays of hGH accumulation were performed on samples of culture medium as described previously (8). hGH produced by the cells was normalized to the amount of hGH mRNA in the cells. Total RNA was isolated and analyzed by Northern blots, using a probe for chimeric mRNA (15).

Stable transfection of HeLa cells was performed by using 150-mm dishes with 1 × 10⁶ cells, culturing overnight, adding fresh culture medium (adjusted to pH 7.2 by the addition of 1 M Heps, pH 7.2), and culturing an additional 4 h. Calcium phosphate precipitates were prepared (16) and layered onto the monolayers of HeLa cells. After culturing overnight, the cells were exposed for 4 min to medium containing 15% glycerol, and fresh medium was added for beginning G-418 selection.

For analysis of hGH expression, cultures of pooled clones were grown without G-418 for 48 h. The cells were washed twice with phosphate-buffered saline (2 mM KCl, 1.5 mM KH₂PO₄, 140 mM NaCl, 8 mM Na₂HPO₄) supplemented with 10% calf serum and replaced with warm preconditioned culture medium. Preconditioned medium was obtained from transfected HeLa cells transfected at low density for 6 h. Samples of the medium were removed immediately (T₀) and after culturing for 6 h (T₆). Also at 6 h, the cells were lysed for extraction of RNA. Measurement of hGH protein accumulation (T₆−T₀) and Northern blot analysis were performed as outlined above.

Measurements of Gene Expression in Yeast—GCN4/lacZ chimeric constructs were transfected into S. cerevisiae strain CRY1 (ade2-1″; can1-100; his3-11, 15; leu2-3, 112; trp1-1; ura3-1) (17) using lithium acetate (16). Colonies isolated from uracil-selective synthetic dextrose media were analyzed for β-galactosidase activity, which was normalized to the level of lacZ mRNA (16). Levels of lacZ mRNA were determined by RNAse protection (18) as follows. Total RNA was hybridized to a 200-nucleotide, 32P-labeled antisense transcript, which yielded a 130-nucleotide protected fragment after digestion with RNase A. The digested fragments were electrophoresed on 6% polyacrylamide gels in the presence of 8 M urea. The dried gels were subjected to PhosphorImager analysis. To normalize for mRNA recovery, Northern blots were hybridized to a 560-base pair pyruvate kinase fragment cloned from CRY1 mRNA by reverse transcription and polymerase chain reaction amplification and labeled with 32P by random priming (19). The results were reported as the ratio of lacZ mRNA to pyruvate kinase mRNA.

Random Mutagenesis—Random mutagenesis of the AdoMetDC uORF at codon 4 was performed by annealing a primer 5′- CGTATCAGGAAAGATCTA-3′ to 5′- ATCGTATTTAAGCTTTAGCTAAGC-3′ and complementing the complementary strand using the Klenow fragment of DNA polymerase I. The double-stranded fragments containing HindIII and BglII sites were ligated into pM128 digested with HindIII and BglII. E. coli transformants were sequenced using the polymerase chain reaction product sequencing kit (Amersham Pharmacia Biotech). DNA was transformed into S. cerevisiae strain CRY1, and β-galactosidase assays were performed as described above. Random mutagenesis at codons 4 and 5 were performed as described above, using the same primers with the following oligonucleotides: 5′- ATCGTATTTAAGCTTTAGCTAAGC-3′ and 5′- ATCGTATTATTAGCTAAGC-3′.

Analysis of Yeast Polysomes—Fractionation of yeast polysomes and isolation of RNA contained in the fractions was carried out using a Beckman ultracentrifuge rotor SW50.10 (20). Briefly, (425–600-μm) acid-washed glass beads (425–600 μm; Sigma) by eight cycles of vortexing for 15 s followed by incubation on ice for 45 s. The lysate was centrifuged from the beads and centrifuged for 5 min at 12,000 × g. The lysate was layered on top of a 7–47% (w/v) sucrose gradient and centrifuged at 39,000 rpm in a Beckman SW-40 rotor for 1 h at 4 °C. Gradients were separated into 12 equal fractions using a density gradient fractionator model 185 (Iseo, Lincoln, NE) while monitoring absorbance at 254 nm. Each fraction was precipitated by adding 3 volumes of 100% ethanol, and the resulting pellets were resuspended in 45 μl of RNA isolation buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 30 mM MgCl₂, 0.5 μg of heparin/ml, and 0.2 μl of diethyl pyrocarbonate/ml). The pellets were dissolved in water saturated phenol, once with phenol-chloroform (1:1), and once with chloroform. RNA was precipitated with ethanol and analyzed for β-galactosidase mRNA by RNAse protection as described above.

Radioactive Labeling and Immunoprecipitation of β-Galactosidase—Yeast cultures containing the indicated constructs were grown to mid-log phase and seven A₅₀₀ units were washed once with fresh medium and resuspended in fresh medium at A₅₀₀ = 1.0. The cells were labeled by the addition of 33 μCi [³H]Methionine (NE 2500 mCi/ml, New England Nuclear) and an equal volume of 425–600-μm acid-washed glass beads (Sigma) was added. After vortexing for 8 cycles as described above, the lysates were collected by centrifugation at 4 °C.


were cleared by centrifuging for 5 min at 12,000 × g, and the supernatant solutions were heated at 100 °C for 5 min. Aliquots from each sample were precipitated with trichloroacetic acid and counted in a liquid scintillation counter. Samples containing equal precipitable cpm were diluted in 7 volumes of 1% Triton X-100 in phosphate-buffered saline. To each sample 4.4 μg of anti-β-galactosidase antibody (Promega, Madison, WI) was added, and the samples were incubated with rocking for 16 h at 4 °C. After continued incubation with 25 μl of Pansorbin™ cells (Calbiochem) for 1 h, immunoprecipitates were collected by centrifugation and washed twice in phosphate-buffered saline containing 0.1% Triton X-100 and 1% SDS and twice in 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl. The immunoprecipitates were resuspended in 50 μl of sample buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.02% bromphenol blue), boiled for 5 min, and loaded onto a 7.5% SDS-PAGE gel. The gel was fixed in 100 mM methanol for about 20 min and then treated for about 20 min with 20% 2,5-diphenyloxazole in glacial acetic acid. After soaking in water for 2 min, the gel was dried and exposed to x-ray film.

RESULTS

Activity of the AdoMetDC uORF in HeLa Cells and Yeast—The sequence-dependent AdoMetDC uORF encodes a hexapeptide with the amino acid sequence MAGDIS (8). When the uORF was present in its natural context, 14 nucleotides from the 5' cap, it suppressed translation of the associated cistron in T lymphocytes but not in nonlymphoid cell types (8). When the uORF was placed in a position 47 nucleotides from the cap, it suppressed downstream translation irrespective of cell type (13). To test the influence of sequence on constitutive suppression of translation in the absence of cell-type regulation, the uORF of the AdoMetDC gene was placed 47 nucleotides from the cap using the hGH gene as a reporter (Fig. 1). Constructs with wild type and modified uORFs were transiently transfected into HeLa cells. Rate of hGH production and mRNA levels were measured (8). The wild type uORF reduced translational efficiency to approximately 1% that of the construct in which the uORF had been destroyed by modifying the initiator AUG codon to GUG. Consistent with previous results obtained with the AdoMetDC uORF located 14 nucleotides from the cap (9), alterations in codons 4, 5, and 6 all relieved a major proportion of suppression, leaving a residual 14–40% inhibition compared with the 99% inhibition produced by the wild type uORF. This residual inhibition probably reflects the efficiency of translational reinitiation on an mRNA after termination in HeLa cells.

To examine the coding requirements of a sequence-dependent uORF, it would be convenient to employ a microbial system to initially screen constructs generated by saturation mutagenesis of critical codons. To test whether the mammalian AdoMetDC uORF showed similar sequence-dependent activity in yeast, wild type and modified uORFs were placed into a derivative of the 5' leader of the GCN4 mRNA in which all of the natural uORFs had been removed (14). The translational efficiencies of the mRNAs, based on β-galactosidase expression from these constructs, are given in Table II. A pattern similar, but not identical, to that seen in mammalian cells was observed. The wild type uORF encoding the peptide MAGDIS suppressed translation to a level 5% that of the construct in which the initiator AUG (codon 1) had been altered. This result, based on β-galactosidase activity, was qualitatively confirmed by labeling cells with [35S]methionine and immunoprecipitating the labeled β-galactosidase (Fig. 2). Alteration of codons 2 and 3 produced no significant change in expression from the wild type uORF (Table II), consistent with results with mammalian cells (9), whereas changes in codons 4 and 5 relieved translational suppression by 4– to 6-fold. However, in contrast to what we observed in mammalian cells, changing the sixth codon from serine to alanine only minimally influenced suppression, increasing expression by only about 2-fold over wild type. This suggested that the requirements at the 3'-terminal codon might be less demanding than originally suspected (9), a conclusion that was confirmed in later experiments (described below). It should be noted that none of the constructs tested in Table II nor any examined elsewhere in this study (e.g. Fig. 4) exhibited substantial alteration in mRNA level, indicating the absence of nonsense-mediated decay promoted by these uORFs in the context of the 5' leader of GCN4.

One unique feature of sequence-specific translational suppression by the AdoMetDC uORF in mammalian cells is that the suppressed mRNA is associated primarily with one or two ribosomes instead of the potential 15 ribosomes if the mRNA were fully loaded (8, 9). To investigate further the behavior of the mammalian uORF in yeast, sucrose gradient centrifugation was performed on extracts prepared from yeast strains containing selected β-galactosidase reporter constructs with wild type and modified AdoMetDC uORFs. As was shown in Table II, mutation of the initiator AUG codon of the uORF abolished translational suppression, and as expected, this mRNA was well loaded with ribosomes (Fig. 3). In yeast, as well as in mammalian cells, the presence of the wild type uORF caused the associated mRNA to be associated strongly with monosomes, disomes, and trisomes (Fig. 3). The mRNA from a construct with codon 5 changed from isoleucine to valine, which retained suppressive activity of the uORF (see Fig. 4), showed...
before precipitation with Pansorbin™. II). The sample in initiator AUG, respectively (designated pMHY1 and pMHY3 in Table 2).

 Sequences that fell into the “weak suppression” category. Even alterations of these codons resulted in a 5- to 10-fold elevation of translation of the downstream reporter gene, yielding con-

Succinct and independent of reinitiation at the initiation codon, this residual suppression by the mutant uORFs probably is because of inefficient translational reinitiation at the initiation codon of the β-galactosidase reporter gene, which is sequence-independent and could be brought about by any uORF placed in a polycistronic mRNA. This is illustrated well by the construct with a termination codon at position 4, generating a uORF encoding just a tripeptide (sequence MAG). Although translation of this construct is enhanced 6-fold over the wild type construct, it is still suppressed to 29% of the control.

The requirements at codons 4 and 5 for maximum suppressive activity were quite stringent. At codon 4, no substitution provided the level of suppression obtained with the naturally occurring aspartic acid in that position. Substitution of three codons at this position, those encoding glutamic acid, methio-

Table II

| Altered codon | uORF derivative (plasmid designation) | β-Galactosidase | mRNA level | β-Gal/mRNA | Translational efficiency |
|---------------|--------------------------------------|----------------|------------|-----------|--------------------------|
| -             | MAGDIS (wild type, pMHY1)            | 13.6           | 2.22       | 6.12      | 0.05                     |
| 1             | AUG → GUG (pMHY3)                    | 348            | 3.12       | 112       | 1.0                      |
| 2             | A → E (pGM308)                       | 13.6           | 3.36       | 4.04      | 0.04                     |
| 3             | G → A (pGM309)                       | 13.2           | 3.70       | 3.56      | 0.03                     |
| 4             | C → R (pGM115)                       | 52.4           | 2.08       | 25.0      | 0.22                     |
| 5             | I → A (pGM145)                       | 87.0           | 2.86       | 30.4      | 0.27                     |
| 6             | S → A (pGM146)                       | 32.3           | 2.53       | 12.8      | 0.11                     |

* The indicated derivatives of the AdoMetDC uORF were ligated into the yeast GCN4 leader, lacking its own uORFs, and fused to the E. coli β-galactosidase-coding region as described under “Experimental Procedures.”

β-galactosidase activity of the strains was determined under “Experimental Procedures.”

β-Galactosidase mRNA level was measured as described under “Experimental Procedures.”

Ratio of β-galactosidase activity to mRNA, normalized to the construct with the initiator AUG mutated to GUG (pMHY3).
nine, and histidine, provided intermediate activities of 12, 11, and 15% that of control, respectively. At codon 5, only isoleucine, which occurs naturally in the AdoMetDC uORF, and its homologue valine, were suppressive. No other substitution yielded a construct that was more suppressed than one with a termination codon at that position. Interestingly, a structurally similar hydrophobic amino acid, leucine, was inactive at position 5.

At codon 6, it seems that any amino acid will suffice to retain suppression of translation of the downstream reporter gene. All coding substitutions at this position resulted in efficient suppression. The only exceptions were termination codons, which abolished all suppressive activity, and proline, which gave an intermediate phenotype (15% of control). Substitution of termination codons at position 6 rendered constructs with activities 31–33% that of control, thus abolishing sequence-dependent activity of the uORF.

As indicated in Fig. 4, multiple codons were recovered for many of the substitutions at all three codon positions. In all these cases, redundant substitutions showed identical suppressive activity.

For the purpose of exploring in detail the specificity in mammalian cells, uORFs with particularly interesting activities in yeast were chosen from those recovered in Fig. 4 and placed 47 nucleotides from the cap of the AdoMetDC leader. The constructs were stably transfected into HeLa cells, and the results are presented in Table III. At codons 4 and 5, the results of substitutions, including termination codons, were closely parallel to those found in yeast. None of the substitutions tested at codon 4 (aspartic acid) supported wild type suppression. Of the three substitutions that were tested at position 5, none (including leucine) yielded a uORF that approached wild type level of suppression, except valine, which was approximately 4-fold less active than the wild type isoleucine. The specificity at codon 6 (serine) differed significantly between yeast and mammalian cells. Three substitutions that retained wild type level of suppression in yeast (isoleucine, lysine, and threonine) also gave significant suppression in HeLa cells. However, four other substitutions that yielded suppressed constructs in yeast, were only marginally inhibitory in mammalian cells (alanine, valine, proline, aspartic acid). Introduction of a termination codon at position 6 abolished inhibition in HeLa cells, as it did in yeast. Also, an additional alanine codon (producing MAGDISA) eliminated suppression in both yeast and HeLa cells (data not shown). These results were seen in transient transfections of HeLa cells as well (not shown).

### Table III

Sequence dependence of translational suppression by the AdoMetDC uORF in HeLa cells

| uORF derivative | Activitya |
|----------------|----------|
| Wild type (MAGDIS) | 4.0 |
| AUG → GUG | 100 |
| Substitutions for D (codon 4) | |
| Glu | 75 |
| Thr | 70 |
| Ser | 57 |
| Arg | 87 |
| Met | 64 |
| Substitutions for I (codon 5) | |
| Val | 16 |
| Leu | 77 |
| Ala | 98 |
| Substitutions for S (codon 6) | |
| Thr | 3.2 |
| Lys | 23 |
| Ile | 26 |
| Ala | 83 |
| Val | 68 |
| Pro | 53 |
| Asp | 91 |

* The rate of hGH synthesis was determined as under “Experimental Procedures” and normalized for comparison to the construct with the initiator AUG mutated to GUG.
DISCUSSION

Based on studies of translational suppression by the uORFs from the AdoMetDC and gpUL4(gp48) genes, a general model has been suggested to account for inhibition by sequence-dependent uORFs (1). In this model, a scanning ribosome encounters the initiator AUG of the uORF and initiates translation. Upon reaching the termination codon of the uORF, amino acids toward the carboxyl terminus of the nascent peptide are proposed to interact with a target, which is thought to be part of the translational apparatus. This interaction is thought to reversibly inhibit a step of either translational termination or release of the completed peptide, which in turn arrests the translating ribosome over the termination codon. The arrested ribosome creates a blockade to scanning by additional ribosomes entering at the cap, thus inhibiting translation of the downstream cistron. This model was based initially on observations that 1) uORFs must be translated to suppress translation, 2) the amino acid sequence at the carboxyl-terminal end of the encoded peptides is critical for suppression of downstream translation, 3) uORFs appear to act only in cis on the ribosome that translated them, and 4) mRNAs whose translation is suppressed by uORFs are associated with a single ribosome. Consistent with this model, recent studies have identified a ribosome paused over the termination codon of the sequence-dependent uORF of the gpUL4(gp48) gene (12), retaining the product of the uORF as a peptidyl-tRNA (21). The majority of the translationally suppressed mRNA identified in the current study was also found in the monosome fraction in extracts from yeast.

Specificity of regulation by the sequence-dependent uORFs presumably resides in the interactions of the nascent peptides or peptidyl-tRNAs with their targets. Suggested targets are 1) a component of the peptide channel of the 60 S ribosomal subunit, 2) the peptidyltransferase of the 60 S subunit, or 3) one of the eukaryotic release factors. In the case of the uORF of the AdoMetDC gene, it is apparent from the current study that the sequence requirements for interaction of the encoded peptide are quite stringent and are conserved across species. The wild type peptide has the sequence MAGDIS, with the fourth position D and the fifth position I being critical residues. Of the 20 possibilities tested at each position in yeast, only aspartic acid gives full suppressive activity in the fourth position, and only the homologue of isoleucine, valine, will substitute at position 5. The suppressive aspartyl-isoleucyl (or -valyl) sequence apparently must be located precisely with respect to the carboxyl terminus of the peptide. Termination codons at the sixth position of the AdoMetDC uORF abolish suppression, as does extending the peptide by just one amino acid at the carboxyl terminus. Consistent with the sixth amino acid in the peptide simply acting as a spacer, this position seems to be quite forgiving in the amino acids allowed, albeit more so in yeast than in mammalian cells.

Our previous results (9), obtained from scrambling the codons of the uORF or altering the codons at degenerate positions, argued that the sequence of the peptide and not the possible occurrence of rare codons, produced the translational suppression. This conclusion is substantiated here. The results of random mutagenesis followed by expression in yeast show recovery of multiple, degenerate codons at many positions (Fig. 4). In a number of instances, the frequency of utilization in yeast of the recovered codons differed widely with no significant effect on the suppressive activities of the resultant uORFs (data not shown). For example, four leucine codons at position 4, which varied in frequency of use from 13% (CUU) to 28% (UUG), gave identical relief of suppression. At position 5, two quite differently used arginine codons, CGG (4%) and AGG (22%), again yielded uORFs with identical activities. Thus, there is no reason to think that the suppressive activity of the AdoMetDC uORF is related to the frequency of codon usage.

The uORF from the cytomegalovirus gene gpUL4(gp48), like that from AdoMetDC, is sequence-dependent. Interestingly, however, the carboxyl-terminal amino acid sequence of this uORF, KYIPP, bears no resemblance to the critical residues of the uORF from AdoMetDC. In fact, in the currently available compendium of uORFs (5), there is none that encodes a peptide resembling MAGDIS, although there are several with carboxyl termini rich in proline, as in the gpUL4(gp48) uORF. The uniqueness of the AdoMetDC uORF is underscored by the recent finding that it is a polyamine-responsive element and that interaction with its target may be controlled by intracellular polyamine levels (15). The lack of sequence relatedness, together with polyamine regulation of the AdoMetDC uORF, argues strongly that different peptides encoded by uORFs interact with different cellular targets or with distinct sites on the same target. Another, seemingly unrelated sequence-dependent uORF is that, associated with genes in lower eukaryotes that encode the small subunit of the arginine-specific carbamoyl phosphate synthetase (CP1 in S. cerevisiae and ARG2 in Neurospora crassa). Nonsense or missense mutations within this uORF abolish suppressive activity (22, 23), and the uORF functions as a cis-acting repressor of translation through a ribosome-stalling mechanism (24). In addition to the peptide product of the CP1 uORF, arginine and the product of the regulatory gene, CPAR, are also required for translational repression. Because of the regulation by arginine and a lack of sequence homology with the AdoMetDC uORF, one suspects that both the target of the peptide and the mechanism of regulation may be different for these uORFs as well.

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