uORFs with unusual translational start codons autoregulate expression of eukaryotic ornithine decarboxylase homologs

Ivaylo P. Ivanov*†, Gary Loughran*†, and John F. Atkins*‡§

*BioSciences Institute, University College Cork, Cork, Ireland; and ‡Department of Human Genetics, University of Utah, Salt Lake City, UT 84112-5330

Edited by Joan A. Steitz, Yale University, New Haven, CT, and approved May 5, 2008 (received for review February 18, 2008)

In a minority of eukaryotic mRNAs, a small functional upstream ORF (uORF), often performing a regulatory role, precedes the translation start site for the main product(s). Here, conserved uORFs in numerous ornithine decarboxylase homologs are identified from yeast to mammals. Most have noncanonical evolutionarily conserved start codons, the main one being AUU, which has not been known as an initiator for eukaryotic chromosomal genes. The AUG-less uORF present in mouse antizyme inhibitor, one of the ornithine decarboxylase homologs in mammals, mediates polyamine-induced repression of the downstream main ORF. This repression is part of an autoregulatory circuit, and one of its sensors is the AUU codon, which suggests that translation initiation codon identity is likely used for regulation in eukaryotes.

antizyme | AUU | non-AUG | polyamines | upstream ORF

Ornithine decarboxylase (ODC) catalyzes the synthesis of putrescine from ornithine. This is the first and rate-limiting reaction in the biosynthesis of polyamines in cells (1). Because of the narrow concentration range of polyamines needed for their multiple roles, ODC expression is tightly controlled. Even modest reductions in mammalian ODC activity can lead to marked resistance to tumor development (1, 2). ODC is turned over rapidly, and this, in part, is because of it being targeted to the 26S proteasome without ubiquitination by the protein antizyme, its key regulator (3, 4). Antizyme itself is negatively regulated by antizyme inhibitor, a homolog of ODC with a higher affinity for antizyme that has lost the ability to decarboxylate ornithine (5). In addition to its posttranslational regulation, ODC is under transcriptional and translational layers of control.

Appreciation of the role and prevalence of upstream open reading frames (uORFs; short coding sequences 5′ of the main coding sequence) in expression of eukaryotic genes is increasing (6–10). Although in most cases the function of the uORF does not depend on the sequence of the encoded peptide, several sequence-dependent uORFs have been studied in depth (for review, see ref. 6). As would be expected from the scanning model of eukaryotic translation initiation, most uORFs have an inhibitory effect on the expression of the main ORF. After translating a uORF, when the 40S subunit of any dissociating ribosome remains on the mRNA and resumes scanning, it may have a depleted repertoire of associated initiation factors. Further, translation of some uORFs, especially those whose sequences are crucial, leads to ribosome stalling, mostly just before termination, with consequent queuing of any trailing ribosomes. Such ribosome stalling can also inhibit expression of the main ORF indirectly via nonsense-mediated mRNA decay (NMD) effects (11).

In vitro and *in vivo* experiments have demonstrated that in mammals all codons differing by a single nucleotide from AUG can be used as initiation codons albeit at significantly reduced levels with CUG and ACG being the most, and AGG and AAG the least, efficient (12, 13). In *Saccharomyces cerevisiae*, codons differing by one nucleotide from AUG can also initiate translation but even less efficiently, and the order of initiation codon efficiency is different from that in mammals. For example, AUU is more efficient in *S. cerevisiae* than either CUG or ACG (14). Initiation at both AUG and non-AUG codons in eukaryotes is enhanced or inhibited by the identity of neighboring nucleotides, with the optimal being the sequence GCC(A/G)CCaugG (15) often referred to as the Kozak consensus.

Results

Conserved Upstream Coding Region in Homologs of ODC. Here, we report that many 5′ leaders of eukaryotic ODC homologs contain an upstream conserved coding region (uCC). A uCC is present in all 49 vertebrate antizyme inhibitor gene orthologs for which sequence is available. In these genes, it is ≈50 codons long and strikingly lacks an in-frame AUG codon [Fig. 1*Ai* and supporting information (SI) Fig. S1*A*]. Unlike the antizyme coding sequence, initiation of translation of these uCCs does not appear to start in a different frame followed by a translational frameshift (*SI Text* and Fig. S2). Instead, there is a conserved in-frame AUU near the 5′ end of the uCCs, and the possibility that it might serve as an initiation codon for the uCCs is explored below.

The 5′ leaders of all available eukaryotic ODC sequences were examined for the presence of a uCC homologous to that in vertebrate antizyme inhibitor mRNAs. At least 70 additional uCC sequences from species belonging to 9 animal phyla were identified in this search (Fig. 1*A ii–v* and Fig. S1 *B–G*). With rare exceptions, they lack appropriate in-frame initiation AUG codons and in some cases any AUG in their 5′ leaders (Figs. S3 and S4). The main exceptions are the orthologs of ODC in nonmammalian/avian vertebrates and orthologs of ODCp in tetrapods (the latter ortholog is a homolog of ODC resulting from duplication in the tetrapod lineage), which have appropriately positioned AUGs and no AUU for initiation of the uCC. This AUG, however, is present in a poor Kozak context. Mammalian ODC orthologs lack the uCC. Like the antizyme inhibitor mRNAs above, most of the invertebrate uCCs lacking an in-frame AUG have a conserved AUU codon in a good Kozak context occupying a similar position within the uCC. Where a putative AUU initiation codon can be unambiguously identified, there is a strong bias for the nucleotides in positions +5 and +6 (where the A of the AUU codon is defined as +1) to be C and G, respectively (Fig. S5). In several invertebrates, it is unclear which initiation codon is used for the expression of the uCC. In some cases, this is undoubtedly caused by an incomplete 5′ sequence, and in others insufficient close relatives are available

Author contributions: I.P.I., G.L., and J.F.A. designed research; G.L. performed research; I.P.I., G.L., and J.F.A. analyzed data; and I.P.I., G.L., and J.F.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

†I.P.I. and G.L. contributed equally to this work.

§To whom correspondence should be addressed. E-mail: j.atkins@ucc.ie.

This article contains supporting information online at www.pnas.org/cgi/content/full/0801590105/DCSupplemental.

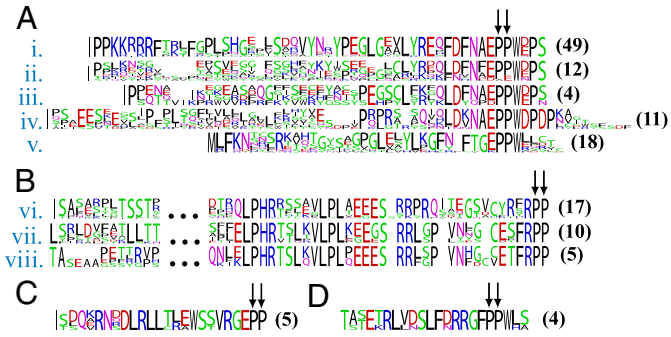

Fig. 1. WebLogo representation of the amino acid conservation of different uCCs found in 5′ leaders of eukaryotic ODC homologs: in animals (*A*), in Pezizomycotina (*B*), in Basidiomycota (*C*), and in Zygomycota (*D*). The middle, poorly conserved, portion of Pezizomycotina uCC is not shown and instead is represented by three dots. Each line represents alignment of a subset of sequences: from vertebrate antizyme inhibitors (*i*); from ODC homologs in invertebrate chordates, hemichordates, echinoderms and molluscs (*ii*); from ODC homologs in velvet worms and annelids (*iii*); from an ODC-like homolog in nematodes (*iv*); from ODC orthologs in nonmammalian, nonavian vertebrates (*v*); from Pezizomycotina uCCs likely initiated by AUU (*vi*); from Pezizomycotina uCCs likely initiated by UUG (*vii*) and from Pezizomycotina uCCs likely initiated by ACG (*viii*). The number of sequences used to compile each line is indicated in parentheses on its right. In each case, the alignment is shown starting from the putative initiation codon. Methionine is not shown as the first amino acid except for ODC orthologs in nonmammalian, nonavian vertebrates where uORF-M is likely initiated with AUG; however, all uORF-Ms are expected to start with methionine. The two adjacent prolines discussed in the text are indicated by arrows. (A complete alignment, including several subsets not show here, is available in Fig. S1.)

for comparison. It cannot be assumed that all of them would be initiated by an AUU codon. Among the different animal uCCs, similarity is highest near the C terminus of the putative peptide, notably, in at least eight animal phyla, the sequence NAEPP-WDP, or close variants. In a subset of seven different phyla, the uCCs end with the dipeptide PS (Fig. 1*A*). In plant mRNAs encoding *S*-adenosylmethionine decarboxylase, which is another key enzyme in the biosynthesis of polyamines, a short, highly conserved, regulatory, AUG-initiated uORF also ends with the dipeptide PS (16). Data supporting the assertion that the observed conservation near the C terminus of the antizyme inhibitor uCC is at the amino acid level and not at the nucleotide level is provided in *SI Text* and Fig. S6. Additional amino acid positions closer to the N terminus of the animal uCC seem conserved in a lineage-specific manner. For the remainder of this work, the uCC in animals will be referred to as uORF-M.

The 5′ leaders of nonmetazoan ODC homologs were analyzed separately for uCCs with different sequences because uORF-M could not be identified in them. Three additional orthologous uCC groups were found each in a different fungal phylum, Zygomycota, Basidiomycota, and the subphylum Pezizomycotina of Ascomycota. All 36 Pezizomycotina species with available ODC sequences have a uCC. Although none of them has an in-frame AUG, 17 have a conserved AUU codon, 10 have a conserved UUG, and 5 have a conserved ACG, all flanked with a Kozak context, near the 5′ end of the uCC (Fig. S3*B*). On the basis of these initiation codon assignments, the Pezizomycotina uORFs are between 77 and 100 codons. Again, the most highly conserved region is near the C terminus of the putative peptide with six amino acid positions, of the last ≈35, completely conserved (Fig. 1*B vi–viii* and Fig. S1 *H–J*). Prominent are the two terminal prolines, positioned slightly differently from those in uORF-M, but noteworthy given the known translational effects of proline. When present as the last or penultimate amino acid, proline has major effects on translation termination, and this feature can be exploited. [An example with tandem prolines is in the human cytomegalovirus UL4 uORF (17). An example with a single proline is in the decoding of the *Escherichia coli* tryptophanase operon (18).]

Five of the available sequences from mushrooms (Basidiomycota) have a uCC. All of these have a conserved AUU in a Kozak context near the beginning of the uCC, and none has an AUG codon available for initiation (Fig. S3*C*). Assuming initiation at that AUU, the corresponding uORFs are comparatively short, encoding 23 or 24 aa, with 17 completely conserved and again two C-terminal prolines (Fig. 1*C* and Fig. S1*K*). Four uCC sequences were identified in Zygomycota. Again, no AUG is available for initiation, and the likely start codon is a conserved ACG (Fig. S3*D*). The resulting uORFs are short, encoding 22 aa, and notably the predicted adjacent prolines in these cases are not right at the C terminus but are followed by a tryptophan that is also the case in most animal uORF-Ms (Fig. 1*D* and Fig. S1*L*).

We failed to identify uCCs in the 5′ leaders of ODC homologs in other nonanimal eukaryotes. Especially informative is the failure to find uCC in the phylum Ascomycota other than the subphylum Pezizomycotina even though multiple sequences are available for analysis. Likewise, in Basidiomycota only the sequences from mushrooms and close relatives, but not others, have the uCC identified above. These observations combined with the fact that apart from the two adjacent prolines there is little or no shared similarity between the different uCC-encoded peptides reported here suggests that each of the four groups has likely emerged and evolved independently from the others, although the possibility that they have a common origin cannot be formally ruled out.

uORF-M Regulates Expression of the Main ORF. The role of uORF-M in regulation of expression of the main coding sequence was investigated in a series of transfections of mammalian HEK-293 cells. In these experiments, a region from the cDNA (NM_018745) corresponding to the 5′ leader of mouse antizyme inhibitor mRNA, including the first two codons of the main coding sequence, was fused to *Renilla* luciferase encoding sequence in a vector, phRL-CMV (Fig. 2). Experiments with the wild-type sequence show that in polyamine-supplemented cells, expression of the main coding sequence is repressed 6.5-fold compared with polyamine-depleted cells. In addition to uORF-M, this mouse antizyme inhibitor mRNA 5′ leader has three moderately conserved conventional uORFs initiated by AUG codons (Fig. S2). Mutating any of these AUG codons to a noninitiating AAA has little or no effect, compared with wild type, on the expression of the main coding sequence either in polyamine-depleted or -supplemented cells (Fig. 2 and for full range of mutants tested see Fig. S7). When the putative initiation codon of uORF-M is mutated from AUU to a noninitiating UUU codon, expression of the main coding sequence is slightly, 1.6-fold, derepressed in polyamine-depleted cells compared with wild type (Fig. 2). More significantly, the polyamine-induced repression of expression of the main coding sequence is essentially lost (1.3-fold vs. 6.5-fold in wild type). This result is consistent with the AUU being used to initiate expression of uORF-M. Changing the AUU to a standard AUG initiation codon leads to strong, 28-fold repression of the main coding sequence in polyamine-deprived cells. This strong repression is only slightly (2.7-fold) further enhanced by spermidine supplementation, suggesting that AUU-mediated initiation of the main coding sequence could be partly responsible for the polyamine effect. A conclusive judgment is difficult based on this experiment because any test construct that contains the AUU to AUG mutant in uORF-M, even under polyamine starvation, results in *Renilla* luciferase activity close to the negative control, and this may distort the measured ratio between polyamine-depleted and -supplemented cells. To investigate the role of the conserved

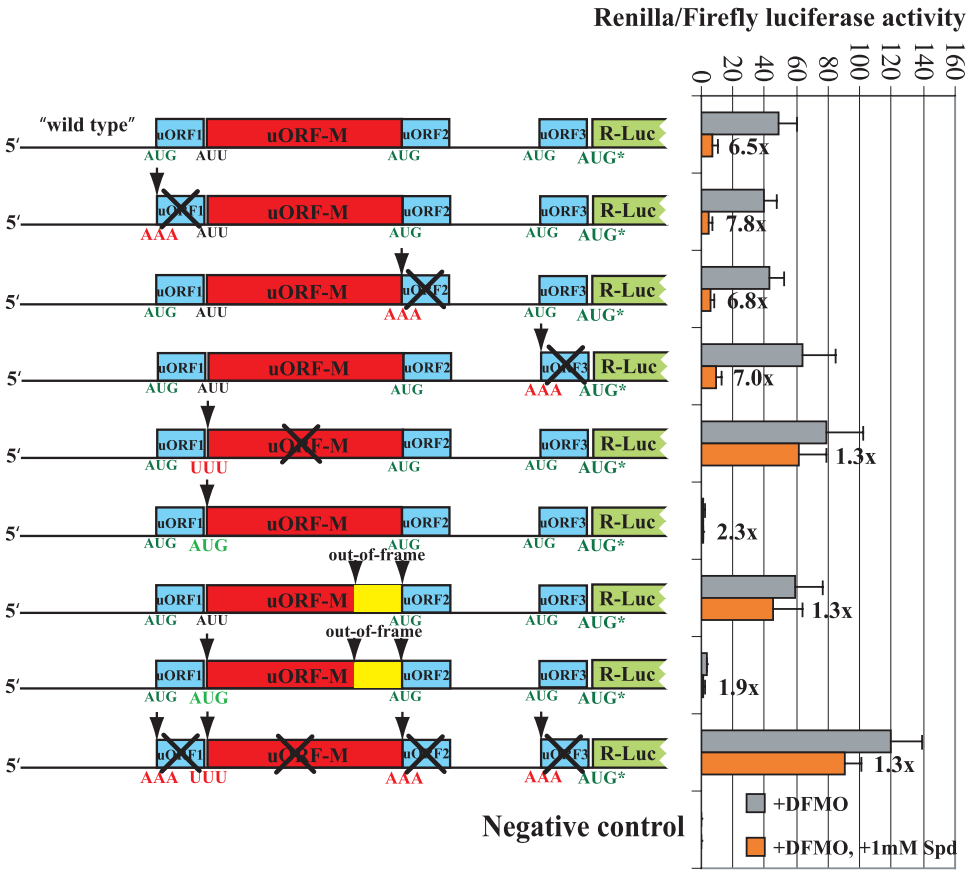

Fig. 2. Mutational analysis of the 5′ leader of mouse antizyme inhibitor mRNA in mammalian HEK-293 cells by using the dual luciferase assay. (*Left*) Schematic representation of the mutations analyzed. The conventional uORFs initiated by AUG codons are represented by light blue rectangles. uORF-M and the beginning of the *Renilla* luciferase reporter ORF are shown as red and green rectangles, respectively. All features are drawn approximately to scale. The position of each mutation is indicated by an arrow above, and mutations altering hypothetical initiation codons are shown as red-lettered triplets below the schematic 5′ leader, respectively. The mutation resulting in the last 10 codons of uORF-M being placed out-of-frame and the mutated region is represented as a yellow box. (*Right*) *Renilla* to firefly luciferase ratio either in cells depleted of polyamines by treatment with 2.5 mM DFMO, or in the same cells supplemented with 1 mM spermidine (Spd). Results from DFMO-treated cells are shown as gray and those from DFMO + Spd as orange bars, respectively. Note that the start codon of the *Renilla* luciferase ORF and the codon that follows it are the first two codons of the mouse antizyme inhibitor main coding region. Fold repression upon spermidine addition is shown above the columns.

C-terminal peptide of uORF-M, the last 10 sense codons of uORF-M were put out-of-frame by a 1-nt deletion followed by a downstream 1-nt insertion. Unlike the AUU to UUU mutant, there is no further derepression in polyamine-depleted cells, but like it, polyamine-induced repression of expression of the main coding sequence is essentially lost, and to the same extent (Fig. 2). Combining the out-of-frame mutation with the AUU to AUG mutant results in levels of repression of expression of the main coding sequence comparable with that of the AUU to AUG alone. This indicates that although the last 10 aa of uORF-M are important for mediating polyamine-induced repression, they are redundant for repression when uORF-M is initiated and translated efficiently. This result is not unexpected because translation of ORFs longer than 40 codons generally precludes ribosomal reinitiation downstream (19, 20).

Because at least some uORF-induced repression of main ORF expression can be caused by activating an mRNA NMD response (11), the steady-state mRNA levels of the wild type and three mutant constructs were investigated by Northern blot analysis. The results are shown in Fig. S8. Although AUU to AUG mutation results in lowering the steady-state levels of mRNA by half, indicative of a possible NMD response, this accounts for only a fraction of the observed drop in protein expression of the main ORF. Even more significantly, none of the observed polyamine-induced reduction of luciferase expression in the wild-type construct can be accounted for by corresponding reduction of mRNA steady-state levels. These results are consistent with the conclusion that NMD plays little or no role in mediating the uORF-M repression of the downstream ORF and strongly suggest that the observed repression is almost entirely at the translational level.

Translation initiation on the AUU codon was further investigated by fusing uORF-M, including the preceding 5′-UTR sequence of mouse antizyme inhibitor mRNA, to a firefly luciferase-encoding sequence in conjunction with an AUU to AUG and also AUU to UUU mutants in the same context. The results of these experiments (Fig. 3*A*) show that in both polyamine-depleted and supplemented cells there is no firefly activity above background (negative control) when AUU is mutated to UUU, whereas firefly activity in both the wild type and AUU to AUG constructs is clearly evident. These experiments, together with the anti-firefly Western blotting using the same cell lysates (Fig. 3*B*), are consistent with translation initiation of uORF-M starting at the AUU codon. Furthermore, these data also indicate that in polyamine-depleted cells, initiation on the AUU, at least in this context, is 18% as efficient as initiation on AUG in the same context. However, in polyamine-supplemented cells, initiation on the AUU codon is 54% as efficient as

GENETICS

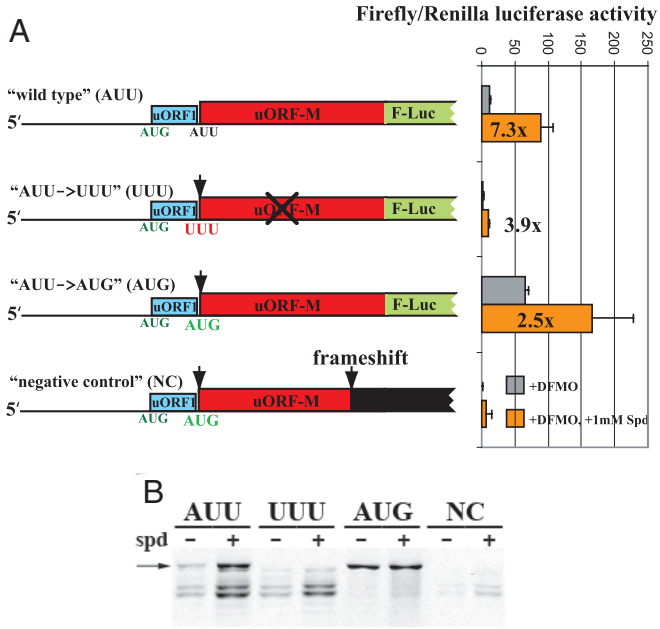

Fig. 3. Analysis of the initiation of uORF-M at AUU by fusing it N-terminally to firefly luciferase. (*A*) Analysis of firefly fusions in mammalian HEK-293 cells by using dual luciferase assay. (*Left*) Schematic representation of the mutations analyzed, analogous to that shown in Fig. 2. (*Right*) Firefly to *Renilla* luciferase ratio either in cells depleted of, or supplemented with, spermidine as described in Fig. 2. Fold stimulation upon spermidine addition is shown above the columns. (*B*) Western blot of lysates from transfected cells as described in *A* by using an antibody against firefly luciferase. The full-length fusion uORF-M-firefly-luciferase protein product is indicated by an arrow. The bands below it represent initiation on downstream codons.

initiation on AUG. This observation is again consistent with the AUU being a polyamine sensor in uORF-M-mediated regulation. The 54% initiation efficiency at the AUU codon of uORF-M at high concentrations of spermidine in these fusion experiments is significantly higher than what has been observed for generic initiation on AUU in mammals (13).

Discussion

The revelation of important uORFs, previously unsuspected because of their initiation with noncanonical start codons, especially AUU, expands our perspective of translation versatility. Until now, AUU has not been known to serve as a translation initiator for eukaryotic chromosomal gene expression, although it is used in the decoding of one animal and two plant viruses (21–23). However, three *E. coli* genes initiate with AUU. The best known is the gene encoding translation initiation factor 3 (24). This initiation factor discriminates against starting at noncanonical initiation codons. When its level decreases, the chance of initiation to replenish any deficit increases resulting in autoregulatory expression.

Initiation of eukaryotic translation on codons differing at a single position from AUG is inefficient and rare (25). The nucleotides surrounding the AUU codon in antizyme inhibitor uCC comply with the standard context for efficient initiation (15) although not to the same extent as those flanking some previously identified non-AUG-initiating codons (26). However, with the parainfluenza virus type 1 P gene GUG initiator, additional nucleotides at positions +5 and +6 also play a critical role (26). For the antizyme inhibitor uCC, the triplet after the AUU is usually CCG (Fig. S5), which differs from the preference for nucleotides +5 and +6 found in the parainfluenza P gene.

In eukaryotes, no single initiation factor performs the functions of bacterial initiation factor 3. However, eukaryotic initiation factor 1 (eIF1) is pertinent. Dissociation of eIF1 from the ternary initiation complex (eIF2, GTP, and Met-tRNA$_i^{Met}$) is a key step in initiation codon selection (27–29). Specifically, mutants in eIF1 that reduce its affinity to the ternary complex increase initiation at non-AUG codons. This phenotype is suppressed by overexpressing the mutant eIF1 protein presumably through mass action (28). eIF1 interacts with several partners associated with, or part of, the ternary complex eIF2, eIF3, and eIF5 (30). Any condition that reduces the binding of eIF1 to the ternary complex theoretically could lead to altered selectivity for the initiation codon. Mutations in eIF2 and eIF5 that lead to increased hydrolysis of GTP in the ternary complex increase initiation at non-AUG codons (31). Therefore, anything that alters the ratio of eIF2·GDP-Pi and eIF2·GTP in the ternary complex could also affect the fidelity of initiation. How polyamines might affect the activity of any of these factors leading to more efficient initiation on the AUU codon of uORF-M or whether there are other non-polyamine-related conditions that produce the same effect is unknown. Although eIF5A has the essential polyamine-derived modification, hypusine, it is not established that it is involved in the initiation step of protein synthesis (32).

uORFs with canonical start codons are important in mRNAs whose main products are involved in controlling cell growth such as receptors, growth factors, and other protooncogenes. Multiple factors, including arginine and polyamines, influence their expression, and they in turn can have positive or negative effects on downstream ORF translation (6).

With vertebrate *S*-adenosylmethionine decarboxylase mRNAs, elevated polyamines stabilize ribosomes paused, because of a special nascent peptide sequence, at the termination codon of their uORF (33). The comparable situation in plant *S*-adenosylmethionine decarboxylase 5′ leaders, which have evolved independently from vertebrates, may be even more relevant because initiation of the first of two highly conserved uORFs may be influenced by polyamine-dependent leaky scanning (16). Yet another example of translational autoregulation in genes influencing polyamine synthesis is the programmed ribosomal frameshifting required for antizyme synthesis acting as a sensor of polyamine levels (34, 35).

Studies on the 5′ leader of ODC mRNA in *Neurospora crassa* (36), showed that a region that we now recognize contains the uCC in Pezizomycotina (Fig. 1*Bvi*), causes close to 30-fold repression of expression of ODC protein. Approximately 3-fold of this could be directly attributed to translational repression of the main coding sequence, and this translational repression is partially relieved by polyamine depletion. This strongly suggests that the uCC in Pezizomycotina might have a role analogous to that of uORF-M in antizyme inhibitor.

Even if all eukaryotes share a similar translation initiation mechanism, it appears that different branches of the eukaryotic tree, not only at the kingdom level but perhaps also at the level of phylum, use uORFs differently. For example, uORFs are apparently more common in the 5′-UTRs of mammals than they are in fungi. One consequence is that to date very few, if any, uORFs have been identified that are clearly conserved between mammals and fungi and in fact between vertebrates and invertebrates (37). Computer and manual searches for uORFs almost invariably assume initiation at AUG codons. Our findings highlight the limitations of such assumptions. Although non-AUG-initiated uORFs have been reported before (38), there has not yet been a systematic attempt to investigate how widespread they might be.

The present discovery of polyamine-induced repression in homologs of ODC genes illustrates how yet another translational control device is used for the critical control of cellular polyamine levels.

Table 1. Mutations in the 5′-UTR of phRL-WT introduced by either standard one-step or two-step PCR

Mutation	Step 1	Step 2
phRL-M1*	AZIUTR/S2 + AZIM1/A	AZIM1/S + AZIUTR/A
phRL-M2*	AZIUTR/S2 + AZIM2/A	NA†
phRL-M3*	AZIUTR/S2 + AZIM3/A	AZIM3/S + AZIUTR/A
phRL-M4*	AZIUTR/S2 + AZIM4/A	AZIM4/S + AZIUTR/A
phRL-M5*	AZIUTR/S2 + AZIM5/A	AZIM5/S + AZIUTR/A
phRL-M6*	AZIUTR/S2 + AZIM6/A	AZIM6/S + AZIUTR/A
phRL-M4M5‡	AZIUTR/S2 + AZIM4/A	AZIM4/S + AZIUTR/A
phRL-M3M5‡	AZIUTR/S2 + AZIM3/A	AZIM3/S + AZIUTR/A

*phRL-WT used as template.
†NA, not applicable; one-step PCR.
‡phRL-M5 used as template.

Methods

Plasmid Construction. For primer sequences, see *SI Methods*. The 5′-UTR of mouse antizyme inhibitor was fused to *Renilla* luciferase coding sequence (phRL-WT) by standard two-step PCR (see below and Table 1). Because of the large size (4,286 nt) of the endogenous intron in antizyme inhibitor uORF-M, we replaced it with the 133-nt intron from the 5′-UTR of *Renilla* luciferase in phRL-CMV (Promega) while still maintaining the same intron/exon boundaries. First, the 5′ and 3′ sections of antizyme inhibitor 5′-UTR were amplified with primers AZIUTR/S2 and INTR/A2 (5′ end) and INTR/S2 and AZIUTR/A (3′ end) by using a mouse antizyme inhibitor cDNA (NM_018745) as template. Next, the 133-nt intron from the 5′-UTR of *Renilla* luciferase was amplified by using primers INRT/S and INTR/A with phRL-CMV as template. Equimolar amounts of each of the three first-step PCR products were mixed and used as template for the second step of the PCR with primers AZIUTR/S2 and AZIUTR/A to generate WT 5′-UTR AZI. WT 5′-UTR AZI was purified by ethanol precipitation and digested with HindIII (AAGCTT underlined in primer AZIUTR/S2; see *SI Methods*) and AvaI (CCCGAG underlined in primers AZIUTR/A and AZIM2/A; see *SI Methods*) and cloned into HindIII/AvaI-digested phRL-CMV to make phRL-WT.

All amplicons were digested with HindIII and AvaI and cloned into HindIII/AvaI-digested phRL-CMV. All clones were verified by sequencing with either phRL/S or phRL/A primers.

uORF-M-firefly fusions were generated by standard one-step PCR with primers AZIUTR/S2 + UTRD2/A BamHI using the templates indicated: phRL-WT for p2Luc-AUU-uORF-M, phRL-M3 for p2Luc-UUU-uORF-M, phRL-M4 for p2Luc-AUG-uORF-M, and phRL-M4 for p2Luc-NC-uORF-M.

All amplicons were digested with HindIII and BamHI and cloned into HindIII/BamHI-digested p2-Luc (39). All clones were verified by sequencing with primer LucCDSR. NC indicates negative control and was generated by accident when making p2Luc-AUG-uORF-M. A PCR-induced frameshift mutation places an in-frame stop codon at the 3′ end of uORF-M but before the firefly coding sequence.

Cell Culture and Transfections. HEK-293 cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% FBS, 1 mM L-glutamine, and antibiotics. Twenty-four hours before transfections, cells were passaged with medium supplemented with 2.5 mM α-difluoromethylornithine (DFMO; a kind gift from P. Woster via Dr. Michael Howard, University of Utah). Cells were transfected by using Lipofectamine 2000 reagent (Invitrogen), with the one-day protocol in which suspension cells are added directly to the DNA complexes in 96-well plates. In all, 25 ng of DNA and 0.2 μl of Lipofectamine 2000 per well in 25 μl of Opti-MEM/DFMO (Invitrogen) were incubated and plated in opaque 96-well half-area plates (Costar). Cells were trypsin-treated, washed, and added at a concentration of 4×10^4 cells per well in 50 μl of Opti-MEM/DFMO. Transfected cells were incubated overnight at 37°C in 5% CO_2, then 75 μl of DMEM/10% FBS with 1 mM final concentration aminoguanosine (Sigma), 2.5 mM DFMO ± 1 mM final concentration spermidine (Sigma) as indicated were added to each well, and the plates were incubated for an additional 48 h. For the effect of DFMO treatment and polyamine supplementation on the intracellular concentrations of polyamines in HEK-293 cell see Fig. 6 in ref. 40.

Dual Luciferase Assay. Luciferase activities were determined by using the Dual Luciferase Stop and Glo Reporter assay system (Promega). Relative light units were measured on a Veritas microplate luminometer fitted with two injectors (Turner BioSystems). Transfected cells were washed once with 1 × PBS and then lysed in 12.5 μl of passive lysis buffer (PLB; Promega), and light emission was measured after injection of 25 μl of either *Renilla* or firefly luciferase substrate. For phRL-WT and its mutants, *Renilla* luciferase activity was calculated relative to the activity of an internal control plasmid (cotransfected at 1/10 the concentration of the test plasmid) expressing firefly luciferase driven by the weak ubiquitin promoter (pUB-Luc; a kind gift from Mark Tagney, Cork Cancer Research Centre, Ireland). For each construct, all data points were averaged and the standard deviation calculated. Data shown represent the mean ± SD from three independent experiments each done in triplicate. For uORF-M firefly fusions, firefly luciferase activity was calculated relative to the activity of an internal control plasmid (phRL-CMV; cotransfected at 1/300 the concentration of the test plasmid) expressing *Renilla* luciferase. Data points and the corresponding standard deviations represent results from a single experiment done in triplicate; however, the results are representative of three additional independent experiments (data not shown).

Western Analysis. Transfected cells (HEK-293 treated with DFMO and spermidine as above) were washed once with 1 × PBS and then lysed in 20 μl of PLB; 7.5 μl was removed for Western blotting, and for the remaining 12.5 μl light emission was measured as described above as a control. Cell debris was removed from cell lysates by centrifugation at 15,000 × *g* at 4°C for 15 min, and samples were then denatured by boiling in 5 × SDS/PAGE sample buffer for 5 min. Proteins were resolved by 12% SDS/PAGE and transferred to nitrocellulose membranes (Protran), which were incubated at 4°C overnight with a 1:1,000 dilution of goat anti-firefly luciferase (Promega). The next day, membranes were incubated at room temperature for 1 h with 1:10,000 IRDye 800CW-conjugated goat secondary antibodies, and immunoreactive bands were detected on the membranes by using a LI-COR Odyssey Infrared Imaging Scanner (LI-COR Biosciences).

ACKNOWLEDGMENTS. We thank Drs. R. B. Weiss, C. W. Anderson, S. Matsufuji, and R. F. Gesteland for their reading the manuscript. This work was supported by an award from Science Foundation Ireland. J.F.A. was supported by National Institutes of Health Grant R01 GM79523.

1. Pegg AE (2006) Regulation of ornithine decarboxylase. *J Biol Chem* 281:14529–14532.
2. Nilsson JA, *et al.* (2005) Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. *Cancer Cell* 7:433–444.
3. Murakami Y, *et al.* (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* 360:597–599.
4. Zhang M, MacDonald AI, Hoyt MA, Coffino P (2004) Proteasomes begin ornithine decarboxylase digestion at the C terminus. *J Biol Chem* 279:20959–20965.
5. Murakami Y, Ichiba T, Matsufuji S, Hayashi S (1996) Cloning of antizyme inhibitor, a highly homologous protein to ornithine decarboxylase. *J Biol Chem* 271:3340–3342.
6. Morris DR, Geballe AP (2000) Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol* 23:8635–8642.
7. Sachs MS, Geballe AP (2006) Downstream control of upstream open reading frames. *Genes Dev* 20:915–921.
8. Churbanov A, Rogozin IB, Babenko VN, Ali H, Koonin EV (2005) Evolutionary conservation suggests a regulatory function of AUG triplets in 5′-UTRs of eukaryotic genes. *Nucleic Acids Res* 33:5512–5520.
9. Iacono M, Mignone F, Pesole G (2005) uAUG and uORFs in human and rodent 5′-untranslated mRNAs. *Gene* 349:97–105.
10. Galagan JE, *et al.* (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and. *A oryzae*. *Nature* 438:1105–1115.
11. Gaba A, Jacobson A, Sachs MS (2005) Ribosome occupancy of the yeast *CPA1* upstream open reading frame termination codon modulates nonsense-mediated mRNA decay. *Mol Cell* 20:449–460.
12. Peabody DS (1989) Translation initiation at non-AUG triplets in mammalian cells. *J Biol Chem* 264:5031–5035.
13. Kozak M (1989) Context effects and inefficient initiation at non-AUG codons in eukaryotic cell-free translation systems. *Mol Cell Biol* 9:5073–5080.
14. Donahue TF, Cigan AM (1988) Genetic selection for mutations that reduce or abolish ribosomal recognition of the HIS4 translational initiator region. *Mol Cell Biol* 8:2955–2963.
15. Kozak M (2005) Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* 361:13–37.
16. Hanfrey C, *et al.* (2005) A dual upstream open reading frame-based autoregulatory circuit controlling polyamine-responsive translation. *J Biol Chem* 280:39229–39237.
17. Janzen DM, Frolova L, Geballe AP (2002) Inhibition of translation termination mediated by an interaction of eukaryotic release factor 1 with a nascent peptidyl-tRNA. *Mol Cell Biol* 22:8562–8570.
18. Cruz-Vera LR, Gong M, Yanofsky C (2006) Changes produced by bound tryptophan in the ribosome peptidyl transferase center in response to TnaC, a nascent leader peptide. *Proc Natl Acad Sci USA* 103:3598–3603.

19. Luukkonen BGM, Tan W, Schwartz S (1995) Efficiency of reinitiation of translation on human immunodeficiency virus type 1 mRNAs is determined by the length of the upstream open reading frame and by intercistronic distance. *J Virol* 69:4086–4094.
20. Pöyry TA, Kaminski A, Connell EJ, Fraser CS, Jackson RJ (2007) The mechanism of an exceptional case of reinitiation after translation of a long ORF reveals why such events do not generally occur in mammalian mRNA translation. *Genes Dev* 21:3149–3162.
21. Li J, Tong S, Wands JR (1999) Identification and expression of glycine decarboxylase (p120) as a duck hepatitis B virus pre-S envelope-binding protein. *J Biol Chem* 274:27658–27665.
22. Schmitz J, Prüfer D, Rohde W, Tacke E (1996) Noncanonical translation mechanisms in plants: Efficient *in vitro* and *in planta* initiation at AUU codons of the tobacco mosaic virus enhancer sequence. *Nucleic Acids Res* 24:257–263.
23. Fütterer J, *et al.* (1996) Position-dependent ATT initiation during plant pararetrovirus rice tungro bacilliform virus translation. *J Virol* 70:2999–3010.
24. Sacerdot C, *et al.* (1996) The role of the AUU initiation codon in the negative feedback regulation of the gene for translation initiation factor IF3 *in Escherichia coli. Mol Microbiol* 21:331–346.
25. Becerra SP, Rose JA, Hardy M, Baroudy BM, Anderson CW (1985) Direct mapping of adeno-associated virus capsid proteins B and C: A possible ACG initiation codon. *Proc Natl Acad Sci USA* 82:7919–7923.
26. Boeck R, Kolakofsky D (1994) Positions +5 and +6 can be major determinants of the efficiency of non-AUG initiation codons for protein synthesis. *EMBO J* 13:3608–3617.
27. Lomakin IB, Shirokikh NE, Yusupov MM, Hellen CUT, Pestova TV (2006) The fidelity of translation initiation: Reciprocal activities of eIF1, IF3, and YciH. *EMBO J* 25:196–210.
28. Cheung Y-N, *et al.* (2007) Dissociation of eIF1 from the 40S ribosomal subunit is a key step in start codon selection *in vivo. Genes Dev* 21:1217–1230.
29. Passmore LA, *et al.* (2007) The eukaryotic translation initiation factors eIF1 and eIF1A induce an open conformation of the 40S ribosome. *Mol Cell* 26:41–50.
30. Valasek L, Nielsen KH, Zhang F, Fekete CA, Hinnebusch AG (2004) Interactions of eukaryotic translation initiation factor 3 (eIF3) subunit NIP1/c with eIF1 and eIF5 promote preinitiation complex assembly and regulate start codon selection. *Mol Cell Biol* 21:9437–9455.
31. Donahue T (2000) Genetic approaches to translation initiation in *Saccharomyces cerevisiae. Translational Control of Gene Expression*, eds Sonenberg N, Hershey JWB, Mathews MB (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY) pp 487–502.
32. Huang Y, Higginson DS, Hester L, Park MH, Snyder SH (2007) Neuronal growth and survival mediated by eIF5A, a polyamine-modified translation initiation factor. *Proc Natl Acad Sci USA* 104:4194–4199.
33. Law GL, Raney A, Heusner C, Morris DR (2001) Polyamine regulation of ribosome pausing at the upstream open reading frame of *S*-adenosylmethionine decarboxylase. *J Biol Chem* 276:38036–38043.
34. Matsufuji S, *et al.* (1995) Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. *Cell* 80:51–60.
35. Ivanov IP, Atkins JF (2007) Ribosomal frameshifting in decoding antizyme mRNAs from yeast and protists to humans: Close to 300 cases reveal remarkable diversity despite underlying conservation. *Nucleic Acids Res* 35:1842–1858.
36. Hoyt MA, Broun M, Davis RH (2000) Polyamine regulation of ornithine decarboxylase synthesis in *Neurospora crassa. Mol Cell Biol* 20:2760–2773.
37. Hayden CA, Jorgensen RA (2007) Identification of novel conserved peptide uORF homology groups in *Arabidopsis* and rice reveals ancient eukaryotic origin of select groups and preferential association with transcription factor-encoding genes. *BMC Biol* 5:32.
38. Warnakulasuriyarachchi D, Ungureanu NH, Holcik M (2003) The translation of an antiapoptotic protein HIAP2 is regulated by an upstream open reading frame. *Cell Death Differ* 10:899–904.
39. Grentzmann G, Ingram JA, Kelly PJ, Gesteland RF, Atkins JF (1998) A dual-luciferase reporter system for studying recoding signals. *RNA* 4:479–486.
40. Howard MT *et al.* (2001) Cell culture analysis of the regulatory frameshift event required for the expression of mammalian antizymes. *Genes Cells* 6:931–942.