**In Vitro** Translation of the Upstream Open Reading Frame in the Mammalian mRNA Encoding S-Adenosylmethionine Decarboxylase*

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The upstream open reading frame (uORF) in the mRNA encoding S-adenosylmethionine decarboxylase is a polyamine-responsive element that suppresses translation of the associated downstream cistron in vitro. In this paper, we provide the first direct evidence of peptide synthesis from the S-adenosylmethionine decarboxylase uORF using an in vitro translation system. We examine both the influence of cation concentration on peptide synthesis and the effect of altering the uORF sequence on peptide synthesis. Synthesis of wild type and altered peptides was similar at all concentrations of magnesium tested. In contrast, synthesis of the wild type peptide was more sensitive than that of altered peptides to elevated concentrations of the naturally occurring polyamines, spermidine and spermine, as well as several polyamine analogs. The sensitivity of *in vitro* synthesis to spermidine was influenced by both the amino acid sequence and the length of the peptide product of the uORF. Findings from the present study correlate with the effects of the uORF and polyamines on translation of a downstream cistron *in vivo* and support the hypothesis that polyamines and the structure of the nascent peptide create a rate-limiting step in uORF translation, perhaps through a ribosome stalling mechanism.

The naturally occurring polyamines putrescine, spermidine, and spermine are small positively charged molecules that occur ubiquitously in living cells and are required for cell growth (1, 2). They have been implicated in many cellular processes, including DNA replication, transcription, and translation (1, 2). Control of intracellular polyamine levels is important, because depletion of polyamines leads to decreased cell growth and alterations in cellular differentiation (3–5), whereas elevation of polyamines can be associated with cell toxicity and with oncogenic transformation (2, 6). Thus, polyamine levels are tightly regulated by a variety of mechanisms, including feedback regulation of the expression, activity, and stability of key enzymes involved in polyamine biosynthesis (2, 6–8). S-Adenosylmethionine decarboxylase (AdoMetDC)* is a key regulated enzyme in the pathway of polyamine biosynthesis.

*AdoMetDC catalyzes the decarboxylation of S-adenosylmethionine, thus generating the n-propylamine donor for the conversion of putrescine to spermidine and of spermidine to spermine. Expression of *AdoMetDC* is regulated by exogenous signals and also by the endogenous level of polyamines, which influence its expression at multiple levels including transcription, translation, and enzyme stability (9–12). Translational regulation of *AdoMetDC* may serve as a fast response mechanism (13), turning off polyamine synthesis quickly when intracellular levels reach a critical level.

The mammalian *AdoMetDC* transcript contains an upstream open reading frame (uORF) specifying a peptide of six amino acids with the sequence MAGDIS (14). Studies in mammalian cells suggest that translation of this uORF is necessary for polyamine regulation of *AdoMetDC* (15). A point mutation in the AUG start codon abolishes regulation, and insertion of the uORF into the 5′ leader of a heterologous reporter gene confers polyamine regulation similar to that seen with the endogenous *AdoMetDC* transcript (15). Inhibition of downstream translation by the *AdoMetDC* uORF requires its amino acid coding sequence but not its precise nucleotide sequence, because missense codon substitutions but not synonymous substitutions abolish the suppressive influence of the uORF (15). Saturation mutagenesis of the last three codons of the uORF in yeast demonstrate that only aspartic acid is tolerated in the fourth position, and only the homolog of isoleucine, valine, can substitute in the fifth position (16). The precise positioning of these two amino acids relative to the stop codon may also be important, because carboxyl-terminal extensions and truncations of the peptide product of the uORF abolish its suppressive influence (16, 17).

These studies imply a rather precise interaction between the MAGDIS peptide and its regulatory target. According to the ribosome stalling model of polyamine regulation of *AdoMetDC* translation (17, 18), polyamines act to modulate an interaction of the fourth and fifth amino acids of the peptide product of the *AdoMetDC* uORF with some component in the translational apparatus, such as the peptidyl transferase center, the peptide channel of the ribosome, or a termination factor. The role of the sixth residue may be to position these residues in the correct orientation for this interaction to occur. This model predicts that a step in translation, such as chain termination or peptide release, would be inhibited through this interaction, causing the ribosome to stall over the termination codon. Ribosome stalling would block further scanning of ribosomes and suppress translation of both the uORF and the downstream cistron. This model implies that low polyamine levels would lead to relief of the blockade and allow translation of both the uORF and the downstream cistron. The ribosome stalling model of putrescine, spermidine, and spermine is supported by this new in vitro translation assay.

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The abbreviations used are: AdoMetDC, S-adenosylmethionine decarboxylase; uORF, upstream open reading frame; HPLC, high performance liquid chromatography; AP3, N-(3-aminopropyl)-1,3-propanediamine; AP6, N-(3-aminopropyl)-1,6-hexanediamine; BESPM, N7-N7′-bis(ethyl) spermine.
AdoMetDC translation is similar to that proposed for another sequence-dependent regulatory uORF found in the Neurospora crassa arg-2 transcript, with the exception that in this transcript the intracellular level of arginine regulates the stalling (19, 20).

The ribosome stalling model makes certain predictions that can be tested in an in vitro translation system. First and foremost, the AdoMetDC uORF should be translated, and its translation should be influenced by the concentration of polyamines. The effect of polyamines on translation of the uORF should be influenced by specific changes in the amino acid sequence of the encoded peptide or in its length but not by changes in the ribonucleotide sequence that retain the amino acid coding content. Furthermore, for regulation of AdoMetDC to be a fine-tuned biological mechanism, we predict that uORF translation should be influenced by polyamines in a unique fashion not shared by other cellular cations such as magnesium. In the present study, these predictions were tested using an in vitro wheat germ translation system and a newly developed analytical procedure to measure synthesis of the six-residue peptide product of the uORF.

EXPERIMENTAL PROCEDURES

Plasmid Construction for RNA Synthesis—Constructs were designed to produce capped transcripts driven by the T7 promoter that contain the AdoMetDC 5' leader with wild type or altered uORFs (14) but not any of the open reading frame encoding AdoMetDC. The full 5' leader is intact in these constructs, with the exception that the 14 nucleotides from the 5' cap to the uORF in the mammalian transcript is replaced with 80 nucleotides from the pBluescript SK+ polylinker region. Furthermore, HindIII and Bgl II restriction enzyme sites were engineered for insertion of synthetic oligonucleotides encoding the uORFs. All constructs were verified by sequencing.

Plasmid constructs were linearized with XhoI, purified, and used as templates for in vitro transcription reactions using a T7 transcription kit (Ambion, Austin, TX). Capped transcripts were made using trace amounts of [32P]UTP (3000 Ci/mol; NEN Life Science Products) to quantitate RNA. RNA concentration was determined in triplicate by thioribarbituric acid precipitation and liquid scintillation counting, using the specific activity of the [32P]UTP. The integrity of the RNA and also the relative concentration was verified by resolving duplicate samples on an 8% denaturing acrylamide gel, followed by quantitation using the STORM PhosphorImager system and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). RNA was stored in aliquots at -80 °C with RNasin (Promega, Madison, WI).

Preparation of Wheat Germ Extract for in Vitro Translation—Wheat germ extract was prepared essentially as described previously (21–23).

In Vitro Translation of Wild Type and Altered uORFs—In vitro translation was performed in a total volume of 50 μl using a modified procedure (21–23). The final concentration of the components in the translation reactions were: 1.2 mM ATP, 80 μM GTP, 0.4 mM each amino acid (excluding methionine), 9.6 mM creatine phosphate, 2.0 mM potassium hydroxide, 64 μg/ml creatine phosphokinase, 20 mM HEPES, 2 mM dithiothreitol, 1.65 mM magnesium (unless otherwise stated), 53 mM potassium acetate, 2 μCi/μl [35S]methionine (specific activity, 1175 Ci/mmol; NEN Life Science Products), RNA (8 ng/μl), RNasin (0.4 unit/μl), and 2 mM bestatin aminopeptidase inhibitor (Sigma). To each reaction, 20 μl of wheat germ lysate was added. The concentration of endogenous polyamines in three independent lysate preparations was determined (24), and the lysates were found to contain 40–70 μM spermidine, 15–25 μM spermine, and no detectable putrescine, to the final reaction volume. Additional spermidine (Sigma) or spermine (Sigma) was added to the reactions as indicated under "Results." Reactions were incubated 20 min (unless otherwise stated) at room temperature and were terminated by placing tubes on ice. Unlabeled carrier peptides (30 nmol) with the sequence MAGDIS (Alpha Diagnostic, San Antonio, TX) or MAGIES, MAGDVS, MAGDLS, or MAGDI (United Biochemical Research, WA) were added to samples as appropriate. Peptides were extracted from the translation mixtures by vortexing the samples with methanol (60 μl) and n-butanol (120 μl), incubating the samples at -80 °C (1 h), centrifuging the samples at 20,000 × g (10 min), and evaporating the resulting supernatant solutions to dryness (25).

HPLC/TLC Analyses of in Vitro Translation Products—Extracted translation samples were resuspended in 30 μl of water (HPLC grade), followed by 130 μl water plus 0.1% trifluoroacetic acid (HPLC grade; Pierce). Samples were loaded onto a C18 reverse phase column (inner diameter, 4.6 mm × 75 mm; Altex, Berkeley, CA), linked to a BioCAD Sprint Perfusion Chromatography System (PerSeptive Biosystems, Framingham, MA). Prior to loading, the column was pre-equilibrated with water plus 0.1% trifluoroacetic acid. Peptides were eluted from the column using a gradient of acetonitrile (HPLC grade) plus 0.09% trifluoroacetic acid and a flow rate of 0.75 ml/min. The elution profile was monitored by absorbance at 210 nm, and the peptides were eluted from the column at 21.5 ± 1.5 min, depending on the sequence of the peptide. This was determined by the HPLC abundance profiles of standard, radioactive peptides (data not shown). Fractions containing the peptide (4 fractions, 250 μl each) were collected, pooled, and evaporated. Samples were resuspended in 20 μl of 50% acetonitrile and spotted on silica gel 60 plates (EM Separations, Gibbstown, NJ). Plates were developed 5 h in n-butanol/glacial acetic acid/water (4:1:1) and dried. Carrier peptides were visualized by spraying the plates with 0.5% ninhydrin in acetone. Plates were wrapped in plastic wrap, and in vitro translation products were quantified using the STORM PhosphorImager system and ImageQuant software.

HPLC/HPLC Analyses of in Vitro Translation Products—HPLC/HPLC analyses were performed as described above, except evaporated samples from the first HPLC run were derivatized (26) with acetic anhydride (Sigma) and anhydrous methyl alcohol (Sigma) and then reanalyzed by HPLC. Fractions of the acetylated peptide peak were collected and analyzed by liquid scintillation counting to quantitate translation products. The identity of the acetylated peptide peak was confirmed by mass spectroscopy.

In Vitro Translation of Brome Mosaic Virus RNA—Control translation reactions containing brome mosaic virus RNA (Promega, Madison, WI) were performed as described above, except the final reaction volume was 25 μl, and reactions contained final concentrations of 0, 1, or 2 mM bestatin, 2.5 mM magnesium acetate, 140 mM potassium acetate, and 70 μM spermine. Reactions were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Protein synthesis was quantified by densitometry of the autoradiographs (Bio-Rad model GS-700 Imaging Densitometer).

Determination of Peptide Stability—Synthetic peptides (30 nmol) were added to wheat germ translation mixtures containing 2 mM bestatin and 700 μM spermidine. Samples were incubated at room temperature or on ice (10 min), extracted, and analyzed by HPLC. The stability of the peptide was determined by comparing the HPLC peak heights of peptides incubated at room temperature versus those incubated on ice.

Determination of RNA Stability—Transcripts were added to wheat germ translation mixtures containing 700 μM spermidine and incubated at room temperature or on ice for 15 min. Transcripts were recovered by phenol/chloroform extraction and ethanol precipitation and were resolved on an 8% denaturing acrylamide gel. Transcript stability was determined in duplicate by comparing the intensity of samples incubated at room temperature versus samples incubated on ice, using the STORM PhosphorImager system.

RESULTS

In Vitro Translation of the AdoMetDC uORF—Capped transcripts of the AdoMetDC 5' leader, containing either the wild type uORF or an altered version (Fig. 1), were synthesized and incubated in wheat germ translation mixtures with [35S]methionine. The stabilities of the transcripts in the wheat germ lysate were determined, and uniform recovery of all RNAs was obtained after incubation in translation mixtures containing 700 μM spermidine (data not shown). The peptide products encoded by the uORFs have half-lives of <1 min in wheat germ extracts (data not shown), and, thus, to stabilize the synthesized peptides, 2 mM of a aminopeptidase inhibitor (bestatin) was added to translation reactions. Using this concentration of bestatin, the recovery of wild type and altered peptides was found to be 75 ± 5% after incubation (10 min) in a wheat germ translation mixture containing 700 μM spermidine (data not shown). A similar situation was reported for the stability of other small peptides in rabbit reticulocyte lysates (25). To ensure that the presence of bestatin did not interfere with the translational machinery, control reactions using brome mosaic virus RNA were performed. The addition of a final concentra-
tion of 2 mM bestatin had no effect on brome mosaic virus RNA translation (data not shown).

Peptide synthesis from the AdoMetDC uORF was analyzed according to the scheme in Fig. 2. Briefly, after incubation of the translation mixtures, unlabeled synthetic peptide was added to act as a carrier and to monitor recovery during extraction and chromatographic separations. Unlabeled and 35S-labeled peptides were then extracted from the translation mixtures using a combination of methanol and n-butanol and purified by sequential HPLC and TLC. As shown by ninhydrin staining of the TLC plate in Fig. 2, uniform recovery of the carrier peptides was achieved through the chromatographic separations. A PhosphorImager scan of the same TLC plate showed co-migration of the 35S-labeled products with the ninhydrin-stained carrier peptides. 35S-Labeled products were detected from reaction mixtures containing either the wild type uORF (MAGDIS) or the altered uORF (MAGEIS) after incubation for 20 min at room temperature (lanes 3 and 5). In contrast, no signal was observed in reaction mixtures lacking added RNA (lane 1) or incubated on ice (lanes 2 and 4).

To confirm peptide production from the uORF, translation reactions were analyzed by an alternate procedure (Fig. 3). After initial extraction and HPLC as in Fig. 2, the recovered peptide peak from HPLC was acetylated. Acetylation lengthens the elution time of the peptides from the HPLC column by 2 min, as determined by mass spectroscopy of HPLC fractions (data not shown). As shown in Fig. 3, the acetylated radioactive products of a translation reaction containing the wild type uORF co-eluted from the HPLC column with the absorbance peak of the acetylated MAGDIS carrier peptide. A similar observation was made for translation mixtures containing the MAGEIS uORF, but no radioactive products were detected in control reactions lacking added RNA or incubated on ice (data not shown). This result, together with the co-migration of radioactive products and the corresponding carrier peptides on TLC, establishes that uORF-encoded peptides are synthesized in the wheat germ translation reactions.

**FIG. 1. Transcripts used in wheat germ translation reactions.** Transcripts contain 80 nucleotides of the pBluescript SK+ vector (thin black line), engineered HindIII or BglII restriction enzyme sites, wild type or altered uORFs (gray box), and the full-length AdoMetDC intercistronic region (thick black line). Other details are described under “Experimental Procedures.” Transcripts were named after the expected peptide product of the uORF, except the WOBBLE uORF, which encodes the wild type MAGDIS peptide, but using synonymous codon substitutions.

**FIG. 2. HPLC/TLC analyses of 35S-labeled peptides.** In vitro translation reactions were carried out as described under “Experimental Procedures.” After translation, unlabeled carrier peptides were added, and the peptides were extracted with methanol and n-butanol. The peptides were then purified by HPLC, and fractions were collected of the peptide peak. The retention time of the peptide on the HPLC column was determined by comparison with a standard (30 nmol of unlabeled carrier peptide; data not shown). A representative HPLC absorbance profile is shown (A), and the arrow marks the position of the MAGDIS peptide peak, which elutes from the column at 21.2 min. The bestatin peak is positioned to the right of the arrow, with an HPLC retention time 5.75 min longer than the MAGDIS peptide. Peptides recovered from HPLC were further purified using TLC. The TLC plates were stained with ninhydrin to mark the position of the peptides on the plate and also to examine peptide recovery through the purification steps. 35S-Labeled peptides were quantified by PhosphorImager scan analyses. Shown is a representative ninhydrin-stained TLC plate (B) and a PhosphorImager scan of the same plate (C). Translation reactions contained either no added RNA (lane 1, both MAGDIS and MAGEIS carrier peptides were added), the MAGDIS uORF (lanes 2 and 3), or the MAGEIS uORF (lanes 4 and 5). Samples were either incubated 20 min at room temperature (lanes 1, 3, and 5) or on ice (lanes 2 and 4).

The peptides were not far from physiological levels (30, 34). Panels A and C of Fig. 4 show the final concentrations of magnesium in the translation reactions, whereas panels B and D show the final concentrations of added spermidine. The wheat germ lysate contributes an additional 40–70 μM spermidine, 15–25 μM spermine, and no detectable putrescine to the final reaction volume of the reactions, as described under “Experimental Procedures”). At all concentrations of magnesium, synthesis of wild type and altered peptides was comparable, and, if anything, the wild type peptide was synthesized at a slightly greater rate (Fig. 4A). Likewise, at concentrations of spermidine less than 300 μM, synthesis of the two peptides was comparable, but at concentrations of 500 μM and higher, synthesis of the wild type peptide was inhibited more strongly than that of the altered (Fig. 4B). The differences in peptide synthesis in response to magnesium and spermidine concentrations are observed clearly in the ratios of altered to wild type peptide synthesis plotted in Fig. 4 (C and D). This spermidine dose-response experiment was repeated three times, with different preparations of both wheat germ extract and RNA, with reproducible results.
To investigate further the differences in peptide synthesis in response to cation concentration, time courses using low and high concentrations of magnesium and spermidine were carried out (Fig. 5). As shown in Fig. 5, translation time courses of both wild type and altered uORFs exhibited the expected lag period (5 min), followed by linear translation (28, 30). In translation mixtures lacking added spermidine and including low (1.65 mM) or high (4.5 mM) magnesium concentrations, the rate of translation of the wild type uORF was somewhat greater than that of the altered (Fig. 5, A and B). Specifically, the translation rate of the altered uORF is 0.64 of the wild type uORF at low magnesium (Fig. 5A), and 0.88 at high magnesium (Fig. 5B), as calculated from the 5–20 min portion of the curve. In contrast, in translation mixtures containing 700 μM spermidine, the effect was reversed and the translation rate of the altered uORF was 2.4-fold higher than the wild type (Fig. 5C). This last experiment (Fig. 5C) was repeated three times, with different preparations of wheat germ extract and RNA, and a 2–3-fold difference in translation rates was consistently observed. This difference was even more pronounced in translation mixtures containing 900 μM spermidine (4.5-fold; data not shown). The stabilities of the wild type and altered peptides and also the corresponding transcripts were shown to be identical after incubation in a wheat germ lysate containing an increased concentration of spermidine (700 μM; data not shown). Taken together, these results clearly demonstrate that translation of the wild type uORF is more sensitive to increased concentrations of spermidine than an altered uORF, and furthermore, this differential effect was not observed with magnesium.

**Sequence Dependence of the Sensitivity of uORF Translation to Spermidine**—It was of interest to investigate whether the sensitivity of wild type uORF translation to spermidine resulted from the ribonucleotide sequence of the uORF, the sequence of the resulting peptide, and/or the length of the peptide product. To this end, translation of the wild type uORF and various altered uORFs (Fig. 1) were compared in wheat germ translation reactions with and without added spermidine (750 μM). As shown in Table I, translation of the wild type uORF, the MAGDVS uORF, and a uORF encoding the wild type peptide but with synonymous codon substitutions (WOBBLE), were highly sensitive to the addition of 750 μM spermidine. The influence of spermidine on translation of the wild type and WOBBLE uORFs was comparable, whereas translation of the MAGDVS uORF may have been slightly more sensitive. In contrast, translation of the MAGDLS and MAGDI uORFs were much less sensitive to spermidine than were either the MA-
GEIS or wild type uORFs. In fact, translation of MAGDLS and MAGDI uORFs may have been slightly stimulated by 750 μM spermidine (Table I). In a similar experiment, alteration of the termination codon from wild type (UAG) to UAA yielded a transcript equally as sensitive to spermidine as wild type, but an alteration to the UGA termination codon resulted in 1.6-fold less sensitivity, as determined by comparing the ratios (no added spermidine/750 μM spermidine; data not shown).

**Influence of Polyamine Structure on Translation of Wild Type and Altered uORFs**—The sensitivity of wild type uORF translation to compounds with structural similarity to the natural polyamines was examined by comparing translation of the wild type uORF to an altered uORF (MAGDLS) in translation reactions with varying concentrations either of the natural polyamine spermidine or spermine or of several polyamine analogs (Fig. 6). As shown in Fig. 7, the spermine analogs N-(3-aminopropyl)-1,3-propanediamine (AP3) and N-(3-aminopropyl)-1,6-hexanediamine (AP6) behaved similarly to spermidine in their inhibition of translation of the wild type uORF relative to the altered uORF (although AP6 appears to have a somewhat different influence on translation of the MAGDLS uORF). This difference in translation between wild type and altered uORFs in response to varied concentrations of the spermidine analogs is observed in the ratios plotted in Fig. 7 (E and F). A similar experiment was done with spermine and the spermine analog, N,N′,N″-tris(ethyl)spermine (BESPM), as shown in Fig. 8. Qualitatively, the influence of these two polyamines on translation of the wild type and altered uORFs was very similar to that of spermidine and the spermine analogs, except that spermine and BESPM were effective at approximately 4-fold lower concentrations. This difference in the effective concentration ranges between spermine and spermidine has been reported for many other cellular functions (29, 33).

**DISCUSSION**

In this paper, we provide the first direct evidence of peptide synthesis from the AdoMetDC uORF. This conclusion is based on the fact that the 35S-labeled translation product co-purifies with the MAGDIS carrier peptide during various purification steps, including methanol and n-butanol extraction, HPLC and TLC chromatographic separations and HPLC separation after peptide acetylation. Furthermore, the detection of the 35S-labeled product was dependent on the addition of RNA to the reaction mixture and on incubation of the reaction at the required temperature.

The effect of cation concentration on translation of the wild type uORF was examined by comparing its translation with the translation of two different altered uORFs, encoding the peptide MAGD from the PhosphorImager scan. These values were normalized to the translation sample with the highest intensity (MAGDLS, 750 μM spermidine), which was set at 100.

**TABLE I**

| uORF       | No added spermidine | 750 μM spermidine | No Added/750 μM spermidine |
|------------|---------------------|-------------------|---------------------------|
| MAGDIS     | 42.8 ± 0.1          | 9.6 ± 0.3         | 4.5 ± 0.1                 |
| MAGDYS     | 61 ± 3              | 11 ± 1            | 5.8 ± 0.4                 |
| WOBBLE    | 30 ± 6              | 7.3 ± 0.7         | 4.2 ± 0.5                 |
| MAGEIS     | 39 ± 2              | 19 ± 3            | 2.2 ± 0.2                 |
| MAGDI      | 77 ± 4              | 83 ± 2            | 0.94 ± 0.02               |

**FIG. 7.** Influence of spermidine and spermidine analogs on uORF translation. The wild type MAGDIS uORF (open circles) or the altered MAGDLS uORF (filled squares) were used in wheat germ translation reactions containing the added concentrations of 0, 250, 700, or 900 μM spermidine (A), AP3 (B), or AP6 (C). The reactions were analyzed using the HPLC/TLC method (Fig. 2 and “Experimental Procedures”). The top panels of the figure show the ratio of altered uORF translation to wild type uORF translation at each cation concentration (A–C). The y axis values were normalized to the translation sample with the highest intensity (MAGDLS, 250 μM spermidine), which was set at 100. The bottom panels of the figure show the ratio of altered uORF translation to wild type uORF translation at each cation concentration (D–F). The ratios at 900 μM spermidine and 900 μM AP3 are not shown, because the relative intensities corresponding to MAGDIS translation are too close to background to allow accurate quantitation.

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The wild type MAGDIS uORF (open circles) or the altered MAGDLS uORF (filled squares) were used in wheat germ translation reactions containing the added concentrations of 0, 70, 140, or 210 \( \mu \text{M} \) spermine (A and C) or BESPM (B and D), and the reactions were analyzed using the HPLC/TLC method (Fig. 2 and “Experimental Procedures”). The top panels of the figure show the response of wild type and altered uORF translation to cation concentration (A and B). The y axis values were normalized to the translation sample with the highest intensity (MAGDLS, 70 \( \mu \text{M} \) spermine), which was set at 100. The bottom panels of the figure show the ratio of altered uORF translation to wild type uORF translation at each cation concentration (C and D). The ratio at 210 \( \mu \text{M} \) spermine was not shown, because the relative intensity corresponding to MAGDLS translation was too close to background to allow accurate quantification.

**TABLE II**

**Comparison of uORF translation with translation of a downstream cistron in vivo**

The effect of the sequence of the uORF on its translation is summarized from “Results.” The influence of uORF sequence on polyamine regulation of downstream translation was determined by inserting wild type and altered uORFs into the 5′ leader of a heterologous reporter gene and monitoring reporter activity in yeast. The yeast strain used in this experiment was deficient in the pathway of polyamine biosynthesis, and reporter activity was assayed in yeast grown either in the presence or absence of polyamines added to the culture media.

| uORF        | uORF (No added/750 \( \mu \text{M} \) spermine) | Downstream cistron (polyamine-deficient yeast cells starved/unstarved) |
|-------------|-------------------------------------------------|---------------------------------------------------------------------|
| MAGDIS      | 4.5 ± 0.1                                       | 4.5                                                                 |
| MAGDYS      | 5.8 ± 0.4                                       | 6.2                                                                 |
| WOBBLE      | 4.2 ± 0.5                                       | ND*                                                                |
| MAGDEIS     | 2.2 ± 0.2                                       | 2.4                                                                 |
| MAGDLS      | 0.80 ± 0.03                                     | 1.2                                                                 |
| MAGDI       | 0.94 ± 0.02                                     | 1.2                                                                 |

*The WOBBLE uORF was not tested in yeast but was shown to confer polyamine regulation on a downstream reporter gene in Chinese hamster ovary cells, ND, not determined.

MAGDVS, MAGDEIS, and MAGDLS and also a uORF encoding the shortened peptide product, MAGDI, had different sensitivities to spermidine than the wild type uORF. For instance, translation of the MAGDVS uORF was slightly more sensitive to spermidine than the wild type uORF, whereas translation of MAGDEIS, MAGDLS, and MAGDI uORFs were less sensitive. In fact, translation of MAGDLS and MAGDI uORFs appeared to be stimulated slightly by 750 \( \mu \text{M} \) spermidine. In contrast, translation of a transcript encoding the wild type peptide but using an altered ribonucleotide sequence (WOBBLE) had a similar sensitivity to 750 \( \mu \text{M} \) spermidine as the wild type uORF. Interestingly, the pattern of the influence of spermidine on uORF translation *in vitro* parallels the pattern of polyamine regulation of an associated downstream cistron *in vivo*. For instance, translation of the three uORFs that confer polyamine regulation on a downstream reporter gene (the wild type, MAGDVS, and WOBBLE uORFs), are the most sensitive to spermidine. Likewise, translation of altered uORFs that relieve polyamine regulation of a downstream reporter gene (the MAGDEIS, MAGDLS, and MAGDI uORFs) are not as sensitive to spermidine. A closer look at the data shows that the MAGDVS uORF has a somewhat greater influence than the wild type uORF on polyamine regulation of the downstream cistron *in vivo*, and translation of this uORF is also somewhat more sensitive to spermidine. Furthermore, the MAGDEIS uORF has an intermediate regulatory effect on the downstream cistron, and translation of this uORF is also somewhat more inhibited by spermidine than altered uORFs that fully relieve polyamine regulation (MAGDLS and MAGDI). Overall, the magnitude of the effect of elevated polyamines on translation of the downstream cistron *in vivo* and translation of the uORF *in vitro* is comparable (Table II). Taken together, these observations are consistent with a mechanism whereby polyamines influence the translational efficiency of the uORF, which in turn influences the translational efficiency of the downstream cistron.

According to the ribosome stalling hypothesis for translational regulation of AdoMetDC, the peptide product of the AdoMetDC uORF must be synthesized, and its synthesis should be influenced by the same parameters that influence translation of the downstream cistron, namely the structure of the peptide product of the uORF and the concentration of polyamines. The present study confirms this prediction for *in vitro* synthesis of the uORF-encoded peptide. These results are consistent with the peptide product of the uORF being involved in the inhibitory mechanism, perhaps by interacting with polyamines to create a rate-limiting step in uORF translation. This reduction in the efficiency of translation of the uORF could in turn create a regulated blockade to the further scanning of ribosomes to the downstream cistron.

Examples of uORFs that are translated and whose peptide products influence the translational efficiency of the downstream cistron are found in both prokaryotes and eukaryotes. In prokaryotes, the leader peptide of the cat-86 transcript interacts in a sequence-dependent fashion with the peptidyl transferase center of the 50 S ribosomal subunit and interferes with translational elongation (35, 36). This causes the ribosome to stall on the transcript and destabilizes a downstream stem-loop structure that normally masks the ribosome-binding site for the cat-86 gene (35, 36). In eukaryotes, the peptide product of the uORF in the human cytomegalovirus gpUL4 transcript inhibits translation of the downstream cistron by a mechanism that requires the amino acid sequence of the encoded peptide (37, 38). Inhibition is associated with the detection of paused ribosomes over the termination codon of the uORF, as well as the detection of peptidyl-tRNA\textsuperscript{Pc}; tRNA\textsuperscript{Pc} incorporates the last amino acid of the 22-amino acid uORF (37–39). These results support a model where the nascent peptide interacts with the translational apparatus in such a way as to inhibit peptidyl-tRNA hydrolysis, thus interfering with translational termination (37–39). Another example of a sequence-dependent uORF in eukaryotes is in the *N. crassa* arg-2 transcript (19). In this transcript, translational inhibition is regulated by the intracellular level of arginine and is associated with the detection of a paused ribosome over the termination codon of the uORF (19, 20). Translational control of arg-2 and AdoMetDC transcripts provide prototypes for regulated interactions between uORF-encoded peptides and their cellular targets in eukaryotes. However, the different regulatory effects...
for Arg-2 and AdoMetDC and also the lack of sequence homology between the two uORFs suggest that both the targets of the uORF-encoded peptides and the mechanisms of regulation of these transcripts may be different.

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