Regulated Translation Termination at the Upstream Open Reading Frame in S-Adenosylmethionine Decarboxylase mRNA*

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The upstream open reading frame (uORF) in the mRNA encoding S-adenosylmethionine decarboxylase is a cis-acting element that confers feedback control by cellular polyamines on translation of this message. Recent studies demonstrated that elevated polyamines inhibit synthesis of the peptide encoded by the uORF by stabilizing a ribosome paused in the vicinity of the termination codon. These studies suggested that polyamines act at the termination step of uORF translation. In this paper, we demonstrate that elevated polyamines stabilize an intermediate in the termination process, the complete nascent peptide linked to the tRNA that decodes the final codon. The peptidyl-tRNA molecule is found associated with the ribosome fraction, and decay of this molecule correlated with release of the paused ribosome from the message. Furthermore, the stability of this complex is influenced by the same parameters that influence regulation by the uORF in vivo, namely the concentration of polyamines and the sequence of the uORF-encoded peptide. These results suggest that the regulated step in uORF translation is after formation of the peptidyl-tRNA molecule but before hydrolysis of the peptidyl-tRNA bond. This regulation may involve an interaction between the peptide, polyamines, and a target in the translational apparatus.

S-Adenosylmethionine decarboxylase (AdoMetDC)† is a key regulated enzyme in the pathway of polyamine biosynthesis. AdoMetDC catalyzes the decarboxylation of S-adenosylmethionine, generating the n-propylamine donor for the conversion of putrescine to spermidine and spermine to spermine. These polyamines are required for cell growth and have been implicated in many cellular processes including DNA replication, transcription, and translation (1, 2).

Spermidine and spermine feedback inhibit AdoMetDC translation by acting through the peptide produced from a small upstream open reading frame (uORF) located in the leader of the AdoMetDC transcript (3–5). The peptide product of the uORF is six amino acids long and has the sequence MAGDIS. This peptide seems to act in cis on the mRNA that produced it, and features of the carboxyl-terminal sequence are of particular importance (4, 6). Saturation mutagenesis of the last three codons of the uORF demonstrated that only aspartic acid is tolerated in the fourth position and only the isoleucine homologue, valine, can substitute in the fifth position (4). The specific structural requirements of the fourth and fifth amino acids are demonstrated by the fact that glutamic acid does not fully substitute for aspartic acid, and substitution of isoleucine with another homologue, leucine, completely abolishes regulation (4).

These studies imply a precise interaction between the MAGDIS peptide and its regulatory target. In one model of AdoMetDC regulation, polyamines could act to modulate an interaction affecting the fourth and fifth amino acids of the peptide product of the AdoMetDC uORF with a component of the translational apparatus, such as the peptidyltransferase center of the ribosome or a termination factor. This interaction could inhibit a step in translation such as chain termination or peptide release, causing the ribosome to stall over the termination codon. The stalled ribosome could then block scanning of additional ribosomes and suppress translation of the associated downstream cistron. In agreement with this model, a ribosome was found paused in the vicinity of the uORF termination codon (7). Elevated polyamine levels increased the stability of the paused ribosome, whereas alterations in the critical fourth and fifth residues of the peptide sequence eliminated this effect of polyamines (7). Although these studies clearly demonstrated a polyamine-enhanced stalling of the ribosome in the vicinity of the termination codon of the wild-type AdoMetDC uORF, they did not establish the identity of the rate-limiting step that produced the ribosome stall.

In this paper we describe the presence of an intermediate in the uORF termination process, the complete nascent peptide linked to the tRNA that decodes the final codon. This complex is associated with the ribosome and is stabilized by elevated polyamine levels. Alterations in the critical fourth and fifth residues of the nascent peptide eliminate this stabilization. These results are consistent with a regulated translation termination mechanism for the AdoMetDC uORF, with the regulated step being at or just prior to hydrolysis of the peptidyl-tRNA bond.

EXPERIMENTAL PROCEDURES

Translation Materials—Details of plasmid construction and in vitro synthesis of capped mRNAs were described previously (5). Wheat germ extract was prepared as described previously (8–10).

Detection and Characterization of 35S-Peptidyl-tRNAs—Wheat germ translation reactions were performed, as described previously (5), with the exceptions that no bestatin was added to translation mixtures, and the samples were incubated 20 min at 26 °C instead of at room temperature. The concentration of endogenous polyamines in the wheat germ translation reactions is ~40–70 μM spermidine, 15–25 μM spermine, and no detectable putrescine (5). Additional spermidine (Sigma) was added to the reactions as indicated under "Results." A procedure described previously (11) for detection of aminoacyl tRNAs was adapted for this purpose. Reactions (25 μl) were terminated by addition of 235 μl of pH 5.0 buffer (0.1 M sodium acetate, 0.5 M sodium chloride, 1 mM...
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EDTA, 1% SDS) and 260 µl of phenol equilibrated with the same solution but without EDTA. The aqueous layer (200 µl) was precipitated with 3 volumes of ethanol and 0.067 volume of 3 mM sodium acetate, pH 5.2. Precipitates were washed with 75% ethanol containing 10 mM sodium acetate, pH 5.2, and resuspended in 6 µl of acid resuspension buffer, pH 5.0 (10 mM sodium acetate, 1 mM EDTA). Acid loading buffer, pH 5.0 (9 µl; 0.1 M sodium acetate, 7 mM urea, 0.05% bromophenol blue, 0.05% xylene cyanol), was added, and samples were electrophoresed on an acid urea gel, pH 5.0 (0.1 mM sodium acetate, 6.5% acrylamide, 7 mM urea), at 500 V for 7 h at 4 °C. 32P-Labeled bands were quantitated using the STORM PhosphorImager system and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The intensity values of the 32P-peptidyl-tRNAs were adjusted for recovery by normalizing to the summed intensities of the 32P-Met-tRNA Met bands. In control experiments, the intensities of the 32P-S-trNAMet bands were not influenced by spermidine concentration or by the sequence of the uORF-encoded peptide (data not shown). For RNase A digestion, extracted and precipitated wheat germ translation reactions were resuspended in 100 µl of Tris-EDTA, pH 7.0. RNase A (10 µg; Sigma) was added, and samples were incubated for 15 min at 37 °C. For proteinase K treatment, reactions were resuspended in 100 µl of 1% SDS, and after addition of proteinase K (20 µg; Sigma), samples were incubated for 60 min at 37 °C. For alkali treatments, samples were resuspended either in 100 µl of 0.3 M KOH and incubated for 60 min at room temperature in pH 9.0, or in 0.5 M Tris-HCl, pH 9.0, and incubated for 30 min at 37 °C. Untreated controls were resuspended in 100 µl of 10 mM sodium acetate, pH 5.2, and incubated for 60 min on ice.

Detection of 32P-Labeled Peptide Component of Complex—Peptidyl-tRNA complexes were isolated as described above. Ethanol precipitates were resuspended in 100 µl of 0.5 M Tris-HCl, pH 9.0, and incubated for 30 min at 37 °C to hydrolyze the peptidyl-tRNA bond (12). Unlabeled synthetic carrier peptides (30 nmol), with the sequences MAGDIS (Alpha Diagnostic, San Antonio, TX), MAGEIS, MAGDVS, MAGDLS, or MAGDI (United Biochemical Research, Inc., Seattle, WA), were added to samples as indicated. Peptides were purified by addition of 900 µl of water containing 0.1% trifluoroacetic acid, followed by C18 column chromatography using Sep-Pak Light C18 Cartridges (Waters Associates) as per the manufacturer’s instructions. The purified peptides were resolved on thin layer chromatography plates as described previously (5).

Centrifugation of Translation Reactions—After incubation, three 50-µl reactions were combined to give a total volume of 150 µl. Ice-cold buffer (350 µl; 10 mM magnesium acetate, 20 mM potassium acetate, 20 mM HEPEs, pH 7.5, and 1 mM dithiothreitol) was added, followed by centrifugation at 100,000 × g for 90 min at 4 °C using a Beckman TL 100 centrifuge and a TLA 45 rotor (Beckman Instruments, Fullerton, CA). Ribosome-containing pellets were resuspended in 500 µl of pH 5.0 buffer (0.1 M sodium acetate, 0.5 mM sodium chloride, 1 mM EDTA, 1% SDS), and the pellet and supernatant fractions were analyzed for 32P-peptidyl-tRNA and 32P-Met-tRNA 32S (see above) or for rRNA and tRNA by ethidium bromide staining of a formaldehyde-agarose gel.

RESULTS

In Vitro Formation of Peptidyl-tRNA Complexes—Capped transcripts of the AdoMetDC 5′ leader containing the wild-type uORF (Fig. 1) were synthesized and incubated in wheat germ translation mixtures with [35S]methionine and 750 µM spermidine. This elevated concentration of spermidine led to reduced accumulation of the final peptide product from the wild-type uORF compared with altered derivatives (5). Peptidyl-tRNA complexes were recovered from the translation mixtures as described under “Experimental Procedures” using a procedure that was initially developed to separate charged and uncharged forms of tRNA species (11, 15). At all steps during the process, care was taken to avoid hydrolysis of the peptidyl-tRNA which included maintaining a pH 7.0 and a temperature < 37 °C. By using this procedure, multiple intensely labeled bands were observed in translation samples (Fig. 2, lanes 3–6; marked with bracket). Because these bands do not co-migrate with [35S]methionine (lanes 1 and 2), and because they are present in samples lacking added mRNA (lanes 3 and 4), they are thought to contain charged initiator [35S]-Met-tRNA Met and elongator [35S]-Met-tRNA Met species (see below for further evidence). In translation samples containing the wild-type AdoMetDC uORF, a slower migrating (less intensely labeled) band was detected (lanes 5 and 6, arrow). To characterize the [35S]methionine-containing bands, in vitro translation reactions containing the wild-type uORF and 750 µM spermidine were extracted, precipitated, resuspended in suitable buffers, and subjected to various treatments (Fig. 3A). Treatment with RNase A completely eliminated all of the 32S-containing bands (compare lanes 2 and 3). Treatment of samples with proteinase K eliminated the slower migrating band but not the multiple intensely labeled bands (lane 4). All of the bands were eliminated by treatment with 0.3 M KOH (lane 5) or 0.5 M Tris-HCl, pH 9.0 (data not shown), whereas none of the bands was eliminated by treatment with DNase I (not shown). Overall, these results are consistent with the multiple intensely labeled bands containing [35S]-Met-tRNA Met species and the slowest migrating species containing a [35S]—
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FIG. 3. Properties of the slowly migrating complex. In vitro translation reactions containing 750 μM spermidine and the wild-type AdoMetDC uORF were performed as described in the legend for Fig. 2. A, after extraction and precipitation, samples were resuspended in suitable buffers and were either left untreated (lanes 1 and 2) or were incubated with RNase A (lane 3), proteinase K (lane 4), or 0.3 M KOH (lane 5). After incubation for an appropriate length of time, samples were extracted and precipitated under acid conditions and electrophoresed on an acid urea gel. Lane 1 contains a reaction that was resuspended in water, and lane 2 contains a reaction that was resuspended in the buffer used for RNase A treatment, but no enzyme was added. B, translation reactions either contained no inhibitor (lane 1) or the following inhibitors added prior to incubation: cycloheximide (1 μM, lane 2), hygromycin B (8 mM, lane 3), puromycin (2.6 mM, lane 4), or edeine (10 μM, lane 5). After incubation, samples were extracted with phenol and precipitated with ethanol under acid conditions. Resuspended samples were electrophoresed on an acid urea gel.

To determine whether formation of the putative 35S-peptidyl-RNA complex requires ongoing translation, samples containing the wild-type uORF and 750 μM spermidine were incubated with various translation inhibitors (Fig. 3B). Incubated with RNase A (lane 3), proteinase K (lane 4), or 0.3 M KOH (lane 5). After incubation for an appropriate length of time, samples were extracted and precipitated under acid conditions and electrophoresed on an acid urea gel. Lane 1 contains a reaction that was resuspended in water, and lane 2 contains a reaction that was resuspended in the buffer used for RNase A treatment, but no enzyme was added. Translation reactions either contained no inhibitor (lane 1) or the following inhibitors added prior to incubation: cycloheximide (1 μM, lane 2), hygromycin B (8 mM, lane 3), puromycin (2.6 mM, lane 4), or edeine (10 μM, lane 5). After incubation, samples were extracted with phenol and precipitated with ethanol under acid conditions. Resuspended samples were electrophoresed on an acid urea gel.

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FIG. 4. Identity of the peptide component of the slower migrating complex. In vitro translation samples containing the wild-type AdoMetDC uORF and 750 μM spermidine were carried out and then were phenol-extracted and ethanol-precipitated under acid conditions. Samples were resuspended in 0.5 M Tris-HCl, pH 9.0, and were incubated for 30 min at 37 °C to hydrolyze the peptidyl-tRNA bond (12). Control experiments demonstrated that hydrolysis is complete under these conditions (data not shown). After incubation, unlabeled synthetic carrier peptides were added (MAGDIS (lanes 2 and 3), MAGDI (lane 5), or MAGEIS (lane 7), followed by C18 reverse phase column chromatography and thin layer chromatography. The positions of the carrier peptides on the thin layer chromatography plate were determined by ninhydrin-staining (left panel), and the migration of 35S-labeled material on the same plate was determined by autoradiography (right panel). Lanes 1, 4, and 6 contain carrier peptides only, without prior C18 chromatography, as a measure of recovery from the purification steps. Lane 2 contains a reaction that was resuspended in water instead of 0.5 M Tris-HCl, pH 9.0. This lane indicates the amount of peptide contamination without base hydrolysis arising from released peptide products of the AdoMetDC uORF.
Fractionation of MAGDIS-tRNA\textsuperscript{Ser} with the Ribosome—In vitro translation reactions were carried out and then centrifuged at 100,000 × g. Pellet and supernatant fractions were extracted and precipitated as described previously and electrophoresed on an acid urea gel. In Fig. 6A, \textsuperscript{35}S-MAGDIS-tRNA\textsuperscript{Ser} was detected almost exclusively in the pellet (lane 3), whereas the multiple \textsuperscript{35}S-Met-tRNA\textsuperscript{Met} species were highly enriched in the supernatant (lane 2). In contrast, no \textsuperscript{35}S-MAGDIS-tRNA\textsuperscript{Ser} was present in either fraction in reactions that were incubated with cycloheximide (lanes 5 and 6). The efficiency of ribosome pelleting was monitored by formaldehyde-agarose gel electrophoresis of RNA purified from the same translation mixtures (Fig. 6B). Although rRNAs were enriched in the pellet fractions (lanes 3 and 6), tRNA was localized in the supernatant fractions (lanes 2 and 5). These results are consistent with the MAGDIS-tRNA\textsuperscript{Ser} generated in these reactions being associated with the ribosome.

Correlation of Rate of MAGDIS-tRNA\textsuperscript{Ser} Decay with Rate of Ribosome Release from the uORF Termination Codon—Translation reactions, containing the wild-type uORF and either no added spermidine or 750 \textmu M spermidine, were incubated for 20 min. Cycloheximide was then added to inhibit further translation, and incubation was continued for varying lengths of time. A portion of each sample was analyzed for \textsuperscript{35}S-MAGDIS-tRNA\textsuperscript{Ser}, and a second portion was subjected to a primer extension inhibition assay to analyze for paused ribosomes as detailed below. In Fig. 7A, \textsuperscript{35}S-MAGDIS-tRNA\textsuperscript{Ser} (marked by arrow) is seen to disappear after cycloheximide addition. With the assumption that the disappearance of this signal is first order, a semi-log plot of \textsuperscript{35}S-MAGDIS-tRNA\textsuperscript{Ser} intensity versus time of incubation after cycloheximide addition is shown in Fig. 7A (3rd panel). The rate of decay is clearly slowed in the presence of spermidine. Specifically, the half-life of \textsuperscript{35}S-MAGDIS-tRNA\textsuperscript{Ser} is calculated to be ~15 min with spermidine and ~5 min without.

To examine ribosome release from the uORF termination site, a primer extension inhibition assay was used (7). In this assay, after incubation for the indicated time in the presence of cycloheximide, a radiolabeled primer is annealed to the mRNA downstream of the uORF sequence. Reverse transcriptase is added, which extends the primer until it encounters a block, such as a paused ribosome. Reactions are electrophoresed on a denaturing acrylamide gel alongside a sequencing ladder. The precise locations (within one or two nucleotides) of the blocks are determined by comparing the migration of the corresponding extension products with the ladder. Blocks to reverse transcriptase are found at both the uORF initiation and termination sites, and these blocks have been shown to be due to paused ribosomes (7). The ribosome paused over the termination codon is released from the message after cycloheximide addition (Fig. 7B), consistent with previous data (7). The rate of ribosome release is slowed with elevated spermidine, as shown by a semi-log plot of intensity versus time of incubation after cycloheximide addition (3rd panel, Fig. 7B). Specifically, the half-life of the stalled ribosome is calculated to be ~17 min with spermidine and ~6 min without. Interestingly, the rates of ribosome release from the termination site and the rates of \textsuperscript{35}S-MAGDIS-tRNA\textsuperscript{Ser} decay, as measured in the same reactions, are similar both in the presence and absence of spermidine. A similar observation was made using another translation inhibitor, edeine (data not shown).

Influence of Peptide Sequence on Accumulation of Peptidyl-tRNA in Vitro—To determine the effect of peptide sequence on accumulation of peptidyl-tRNA, translation reactions containing 750 \textmu M spermidine were performed either with no added mRNA or with uORFs encoding wild-type or altered peptide products (Fig. 1). It has been shown previously that the stabilities of these mRNAs are identical under these conditions (5). In Fig. 8A, comparable \textsuperscript{35}S-peptidyl-tRNA intensities are obtained from reactions with the wild-type uORF (lane 2), a uORF that encodes the wild-type peptide but with synonymous codon substitutions (lane 3), and a uORF encoding the MAGDYS peptide (lane 4). A UORF sequence encoding the MAGDYS peptide demonstrated reduced accumulation of peptidyl-tRNA (~37% of the wild-type level, lane 6), as did a UORF encoding the MAGEIS peptide (~60% of the wild-type level, lane 5). Similar results were obtained using three different batches of wheat germ extract and 2–4 different lots of RNA (depending on the particular RNA species). To verify the identity of the \textsuperscript{35}S-peptidyl-tRNAs in Fig. 8A, the \textsuperscript{35}S-labeled peptide compo-
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To determine the influence of the sequence of the uORF-encoded peptide on the stability of peptidyl-tRNA, reactions containing 750 μM spermidine were performed with either no added mRNA (lane 1), the wild-type uORF (lane 2), a uORF encoding the wild-type peptide but with synonymous codon substitutions (MAGDIS*-UCC uORF, lane 3), or uORFs encoding the altered peptide products MAGDYS (lane 5), or MAGDLS (lane 6). A, peptidyl-tRNAs were isolated from the translation mix and electrophoresed on an acid urea gel. B, peptides were hydrolyzed from isolated peptidyl-tRNAs by incubation with 0.5 M Tris-HCl, pH 9.0. The appropriate synthetic carrier peptide was added, followed by C18 reverse phase column chromatography and thin layer chromatography. Synthetic peptide migration was determined by ninhydrin staining (left panel, B; lane 1 contains a mixture of MAGEIS, MAGDIS, and MAGDVS synthetic peptides). 35S-Labeled peptides were visualized by autoradiography (right panel, B). The amount of 35S-peptidyl-tRNAs (A) and 32S-labeled peptides (B) was determined by PhosphorImager analysis.

Intensity values were normalized to the wild-type value, which was set at 100 (values indicated at the top of each figure).

FIG. 7. Correlation of the rate of peptidyl-tRNA decay with the rate of ribosome release from the uORF termination codon. Translation reactions containing the wild-type AdoMetDC uORF and either no added spermidine (SPD) or 750 μM spermidine. After a 20-min incubation, cycloheximide was added to inhibit further translation, and samples were incubated for the indicated lengths of time. To examine peptidyl-tRNA decay (A), a portion of each sample was extracted and precipitated under acid conditions and electrophoresed on an acid urea gel. The amount of peptidyl-tRNA at each time point was quantitated by PhosphorImager analysis. PhosphorImager intensities were normalized within each data set to the value at time 0, which was set at 100. A plot of the log of peptidyl-tRNA intensity against time of incubation after cycloheximide addition is shown (3rd panel, A; open circles, no added spermidine; filled squares, 750 μM spermidine). To examine ribosome release from the uORF termination site (B), the remaining portion of each sample was subjected to the primer extension inhibition assay (7). A block to reverse transcriptase consistent with a ribosome paused at the uORF termination codon is indicated by termination, whereas initiation indicates the position of a ribosome paused at AUG. The block at termination was quantitated by PhosphorImager analyses. Intensities were normalized within each data set and plotted as described above (3rd panel, B; open circles, no added spermidine; filled squares, 750 μM spermidine). The ice lane contains samples incubated for 20 min on ice, instead of 26°C. The C lane contains a reaction where cycloheximide was added before the first 20-min incubation period. This reaction illustrates the efficiency of cycloheximide inhibition.

FIG. 8. Effect of uORF-encoded peptide sequence on accumulation of peptidyl-tRNA. Translation reactions containing 750 μM spermidine were performed with either no added mRNA (lane 1), the wild-type uORF (lane 2), a uORF encoding the wild-type peptide but with synonymous codon substitutions (MAGDIS*-UCC uORF, lane 3), or uORFs encoding the altered peptide products MAGDYS (lane 4), MAGEIS (lane 5), or MAGDLS (lane 6). A, peptidyl-tRNAs were isolated from the translation mix and electrophoresed on an acid urea gel. B, peptides were hydrolyzed from isolated peptidyl-tRNAs by incubation with 0.5 M Tris-HCl, pH 9.0. The appropriate synthetic carrier peptide was added, followed by C18 reverse phase column chromatography and thin layer chromatography. Synthetic peptide migration was determined by ninhydrin staining (left panel, B; lane 1 contains a mixture of MAGEIS, MAGDIS, and MAGDVS synthetic peptides). 35S-Labeled peptides were visualized by autoradiography (right panel, B). The amount of 35S-peptidyl-tRNAs (A) and 32S-labeled peptides (B) was determined by PhosphorImager analysis.

Intensity values were normalized to the wild-type value, which was set at 100 (values indicated at the top of each figure).
was calculated to be 11 min, whereas the half-life of MAGDLS-tRNA\textsuperscript{Ser} was 3 min. This slower rate of decay of $^{35}$S-MAGDIS-tRNA\textsuperscript{Ser} would seem to explain its increased accumulation in reactions in comparison with $^{35}$S-MAGDLS-tRNA\textsuperscript{Ser} (Fig. 8).

**DISCUSSION**

In a previous study (5), synthesis of the MAGDIS peptide in wheat germ translation reactions was found to be uniquely sensitive to elevated levels of spermidine and spermine in comparison with altered derivatives of this peptide. Under the same conditions where specific inhibition of wild-type peptide synthesis was observed, recent studies (7) demonstrated a stabilized ribosome paused in the vicinity of the termination codon of the AdoMetDC uORF. These results suggest, but do not prove, that synthesis of the peptide product of the wild-type uORF is inhibited at the termination step by spermine and spermidine. 

The current study investigates an intermediate in the AdoMetDC uORF termination process, i.e. the complete nascent peptide (MAGDIS) linked to the tRNA species that decodes the last codon (tRNA\textsuperscript{Ser}). The identification of this intermediate is based on several observations. First, we detected an $^{35}$S-labeled compound in translation reactions that was degraded by both proteinase K and RNase A, consistent with the complex containing a peptide and an RNA component. The $^{35}$S-labeled peptide component of this complex co-migrated with the MAGDIS peptide on thin layer chromatography plates. Furthermore, a synonymous codon substitution of the last serine codon of the uORF altered the electrophoretic mobility of the complex, suggesting the RNA component is a wheat tRNA\textsuperscript{Ser} species. Finally, this complex pelleted with the ribosome fraction and was not detectable in reactions incubated with various translation inhibitors, suggesting that it is formed by protein synthesis in association with a ribosome.

Accumulation of MAGDIS-tRNA\textsuperscript{Ser} was influenced by the same parameters that have been demonstrated to regulate AdoMetDC translation in vivo (3, 4), to influence the rate of peptide synthesis from the AdoMetDC uORF (5), and to stabilize a ribosome paused close to the uORF termination codon (7). These parameters include polyamine concentration and the sequence of the uORF-encoded peptide, specifically in the critical fourth and fifth residues. In this paper, we found that translation reactions containing elevated concentrations of spermidine led to increased stability of MAGDIS-tRNA\textsuperscript{Ser}. Accumulation of the peptidyl-tRNA complex also followed the previously defined trend with regard to alterations in the encoded peptide sequence in the critical fourth and fifth residues. For example, alteration of the fifth codon from isoleucine to leucine completely abolished uORF regulation in vivo (4, 23), and this particular alteration also led to instability and reduced accumulation of MAGDLS-tRNA\textsuperscript{Ser} in comparison with MAGDIS-tRNA\textsuperscript{Ser}. Similar parallels were demonstrated for other alterations in the encoded peptide sequence, including substitution of valine in the fifth codon (enhanced regulation) and substitution of glutamic acid in the fourth codon (reduced regulation).

The peptidyl-tRNA intermediate is stabilized approximately 3-fold by elevated polyamines in the wheat germ extracts studied here. This is similar in magnitude to the regulatory effects observed previously with the AdoMetDC uORF. In drug-treated mammalian cells (3) and starved yeast auxotrophs (23), the influence of added polyamines on expression of genes downstream of this uORF is 3–5-fold. In wheat germ extracts (7), the influence of added polyamines on translation of genes downstream of the uORF varies from 2- to 5-fold, depending on the particular wheat germ preparation and construct tested. In making these comparisons, it is important to recognize that one is comparing intact polyamine-depleted cells with gel-filtered extracts, and therefore, variables such as the concentrations of residual polyamines, magnesium ion, and monovalent cations are not controlled. Consequently, it is impossible to do more than state that the magnitude of the effect on termination of the AdoMetDC uORF is similar to previous results measuring expression of associated downstream genes. However, the related and perhaps more important question is whether there is any reason to think that there are other sites of polyamine action besides termination, or can the observed regulatory properties be accounted for through the effects on MAGDIS termination? No specific effects of polyamines on initiation of translation of the uORF were observed in our previous studies (7). The possibility that polyamines exert specific effects on peptide elongation at the uORF has not been tested directly; however, the fact that the sequence-specific properties of the uORF are abolished by extending the length of the coding region at the 3′ end (6) would seem to argue against elongation as a target. Therefore, there is no compelling reason at this point to invoke other sites of regulation besides termination at the AdoMetDC uORF.

Translation termination in eukaryotes is a multistep process, which includes presentation of the stop codon in the ribosomal A site, binding of eukaryotic release factor 1 (eRF1), hydrolysis of the peptidyl-tRNA bond, followed by dissociation of the ribosome, de-acylated tRNA, and peptide from the mRNA (reviewed in Ref. 24). That we detect a peptidyl-tRNA complex and that the stability of this complex is influenced by the same parameters that influence regulation by the uORF in vivo suggest that the regulated step in termination must be after formation of the peptidyl-tRNA molecule but before hydrolysis of the peptidyl-tRNA bond. Consistent with this hypothesis, peptidyl-tRNA decay is correlated temporally with ribosome release from the uORF termination site. If the regulated step were after hydrolysis, perhaps through an effect on ribosome release, then peptidyl-tRNA decay would occur faster than ribosome release. One possible site of regulation could be at the interaction of eRF1 with the ribosome. eRF1 binding could be compromised if the structure of the ribosomal A site were to be

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\textsuperscript{2} A. Raney, G. L. Law, and D. R. Morris, unpublished results.
altered by an interaction between the nascent peptide, polyamines, and a target in the ribosome or associated factors. Alternatively, eRF1 could bind, but its activity might be compromised. Because eRF1 must act in conjunction with the peptidyltransferase center of the 60S ribosomal subunit to catalyze the transfer of the nascent peptide chain in the ribosomal P site to a water molecule, the activity of eRF1 could be impaired by interaction of the nascent peptide either with eRF1 directly or alternatively with peptidyltransferase.

Another example of a regulatory peptidyl-tRNA molecule that has been identified in eukaryotes is the product of the uORF2 sequence of the human cytomegalovirus gene gpUL4 (25, 26). Similar to the situation described for AdoMetDC, accumulation of uORF2-peptidyl-tRNAPro within the ribosome depends on the peptide sequence encoded by the uORF and is associated with a rate-limiting step at uORF2 termination (25). This is manifested by the stability of a ribosome paused over the uORF2 termination codon (25, 27). Despite the similarities of the regulatory mechanisms for AdoMetDC and gpUL4, there are a few important differences. Most apparent, the structures of the two uORF-encoded peptides bear no resemblance to one another, suggesting different targets of interaction. In addition, uORF2 termination does not appear to be regulated by a small molecule, as is the case for polyamine regulation of the AdoMetDC uORF (5, 7, 25–27). Finally, studies suggest that the ribosome may be released from the uORF2 termination codon prior to hydrolysis of the peptidyl-tRNAPro bond (26), whereas this seems not to be the case for AdoMetDC. Further studies of both AdoMetDC and gpUL4 transcripts should elucidate the cellular targets of the uORF-encoded peptides, and also the mechanism by which polyamines modulate this regulation in the case of AdoMetDC. Because these two sequence-specific uORFs differ greatly in both size and sequence from each other and also from the sequence-specific uORF associated with the fungal arg-2 (CPA1) gene (summarized in Ref. 28), it seems reasonable to postulate that there are multiple targets available in the translational machinery for this class of regulatory mechanisms.

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