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Inhibition of Nascent-Peptide Release at Translation Termination

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The transcript leader of the human cytomegalovirus (CMV) gpUL4 (gp48) gene contains a 22-codon upstream open reading frame (uORF2) that represses translation of the downstream cistron. Previous work demonstrated that ribosomes stall at the termination codon of uORF2 and, remarkably, that the coding information of uORF2 is required for both the translational repression and ribosomal stalling. We now provide evidence that the peptide product of uORF2 is synthesized and is retained in the ribosome in the form of a peptidyl-tRNA. Translation of the gp48 transcript leader in cell extracts produces the 2.4-kDa uORF2 peptide and a second product migrating with an apparent molecular mass of 20 kDa that represents the uORF2 peptide covalently linked to $tRNA^{Pro}$, the tRNA predicted to decode the carboxy-terminal codon of uORF2. The uORF2 peptidyl-tRNA is only detected after translation of RNAs containing uORF2 sequences that also inhibit downstream translation and cause ribosomal stalling. These data support a model in which the nascent uORF2 peptide blocks translation termination prior to hydrolysis of the peptidyl-tRNA bond. This blockade results in ribosomal stalling on the transcript leader which in turn impedes the access of ribosomes to the downstream cistron. This system illustrates that translation termination may be a critical step controlling expression of some eukaryotic genes.

Although only ~10% of eukaryotic mRNAs possess AUG codons upstream from the initiation codon of the major cistron, two-thirds of oncogenes and many genes encoding growth factors and cellular receptors contain at least one upstream AUG codon and associated upstream open reading frame (uORF) in their transcript leaders (19). According to the scanning model of eukaryotic translation (18), ribosomes generally initiate only at the most 5′ proximal AUG codon on the mRNA, which predicts that upstream AUG codons should inhibit translation of the downstream cistron. However, in some cases, ribosomes bypass the first AUG codon (6, 18). In other examples, after initiating at an upstream AUG codon and translating the uORF, ribosomes reinitiate translation at a downstream AUG codon (14, 18). Thus, the presence of an upstream AUG codon is insufficient for repression of downstream translation by an uORF, and translational events in addition to initiation at an upstream AUG codon must be considered in evaluating the regulatory effects of an uORF.

A few uORFs, including the 22-codon second uORF (uORF2) in the human cytomegalovirus (CMV) gpUL4 (gp48) transcript leader, share an unusual property: their inhibitory effects on downstream translation depend on their amino acid coding information (4, 5, 13, 22, 24, 30; reviewed in reference 8). This sequence dependence suggests that the uORF peptide products are synthesized and mediate the regulatory effects of the uORF. Though none of these putative regulatory peptides have been identified, peptide products encoded by a few other short uORFs have been detected (10, 17).

Previous studies demonstrated that the same uORF2 coding sequences required for inhibition of downstream translation are also associated with ribosomal stalling at the uORF2 termination codon (2). These results suggest a model in which repression of downstream translation is a consequence of inhibition of translation termination mediated by the uORF2 peptide. We now confirm that the uORF2 peptide is indeed synthesized as predicted by the model. Moreover, the detection of the peptide as ribosome-associated peptidyl-$tRNA^{Pro}$ bolsters the hypothesis that the nascent uORF2 peptide blocks translation termination prior to peptide release.

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MATERIALS AND METHODS

Plasmids, synthetic peptides, and antiserum. The plasmids pEQ307, pEQ367, pEQ438, pEQ439, pEQ505, pEQ509, and pEQ543, containing wild-type or mutant gp48 transcript leader sequences, were described previously (2). A full-length 22-mer uORF2 peptide and a multiple antigenic peptide on an octovalent core matrix (28) with each arm containing the N-terminal 15 amino acids (MQPLVLSAKKLSSLL) of uORF2 were synthesized. Polyclonal rabbit antiserum raised to the high-performance liquid chromatography-purified multiple antigenic peptide was affinity purified by using the 22-mer synthetic uORF2 peptide and an ImmunoPure Ag/Ab Immobilization kit (Pierce).

In vitro transcription and translation. Plasmids digested with Asp-718I, which cuts all constructs at the end of the gp48 transcript leader, were used as templates for in vitro transcription with an AmpliScribe T3 transcription kit (Epicentre Technologies, Madison, Wis.). Thus, all the transcripts contain only the gp48 leader in its wild-type or mutant form. Cell-free translation in nuclease-treated rabbit reticulocyte lysate (RRL, Promega) was performed as previously described (8) except that the RNA concentration was changed to 40 μg/ml and 300 μCi of [^{35}S]methionine (Amersham) per ml was added for labeling. To pellet ribosomes, the translation mixture was centrifuged at 100,000 × *g* for 75 min at 4°C in a Beckman TL100 centrifuge using a TLV100.3 rotor. Where indicated, EDTA was added to a concentration of 10 mM to dissociate the ribosomes from transcripts.

Radioimmunoprecipitation. After cell-free translation, typically in a 25-μl reaction mixture, an equal volume of 5 M urea–2% sodium dodecyl sulfate (SDS) was added and vortexed for 30 s. After dilution with 500 μl of low-salt wash buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 0.1% Nonidet P-40), 5 μl of crude or affinity-purified antiserum was added. Following overnight incubation at 4°C, 50 μl of protein G agarose (Boehringer Mannheim) was added, and the reaction mixture was incubated for 30 min at 4°C with rocking. The agarose beads were washed once with low-salt buffer, once with high-salt buffer (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 0.1% Nonidet P-40), and then twice more with low-salt buffer. For RNase digestion, the washed agarose beads were incubated for 15 min at 37°C with 10 μg of RNase A per ml in 100 μl of Tris-EDTA or with

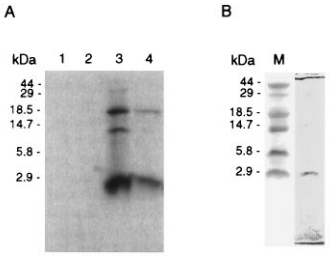

FIG. 1. Immunoprecipitation of the uORF2 peptide. (A) The gp48 transcript leader in pEQ438 was transcribed in vitro and translated in RRL in presence of [^{35}S]methionine for 5 min at 30°C. After immunoprecipitation, samples were electrophoresed on a 15% SDS-polyacrylamide gel as described in Materials and Methods. The autoradiograph shows results of adding no RNA (lane 1) or pEQ438 RNA (lanes 2 to 4) to the translation reaction mixture and immunoprecipitating with preimmune serum (lane 2), crude immune serum (lane 3) or affinity-purified immune serum (lanes 1 and 4). (B) The synthetic 22-mer uORF2 peptide (3 μg) was electrophoresed and stained with Coomassie blue. The sizes of protein markers (M) are indicated.

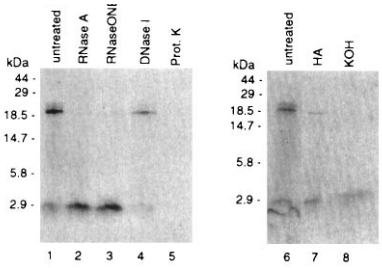

FIG. 2. Covalent linkage of RNA to the uORF2 peptide. In two separate experiments (left and right panels), pEQ438 RNA was translated in RRL and following immunoprecipitation with affinity-purified antiserum, the immune complexes were left untreated (lanes 1 and 6) or were incubated with RNase A (lane 2), RNaseONE (lane 3), RNase-free DNase I (lane 4), proteinase K (lane 5), hydroxylamine (lane 7), or KOH (lane 8) as described in Materials and Methods. Protein size markers are indicated.

10 U of RNaseONE (Promega), which cleaves at all four bases, in 100 μl of Tris-HCl, pH 8.0. For proteinase K treatment, the agarose beads were incubated with 20 μg of proteinase K per ml for 60 min at 37°C. For the treatment with alkali, the agarose beads were incubated with either 200 μl of 1 M hydroxylamine for 8 h at room temperature or with 100 μl of 300 mM KOH at room temperature for 16 h and then neutralized with 1 M Tris-HCl, pH 8.0. After the pelleted protein G agarose beads were boiled twice in SDS gel loading buffer for 3 min, samples were separated electrophoretically on 15% SDS-tricine-polyacrylamide gels as described previously (25), except that the spacer gel was eliminated and glycerol was omitted from the separating gel.

RT-PCR. After cell-free translation and immunoprecipitation, the protein G agarose beads were washed sequentially twice with low-salt buffer, twice with high-salt buffer, and twice with low-salt buffer. Following incubation with hydroxylamine as described above, the beads were pelleted and RNA in the supernatant was precipitated with ethanol. The pellet was resuspended in 10 μl of diethylpyrocarbonate-treated H_2O, 1 μl of which was denatured for 2 min at 94°C in 10 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.25 mM dNTPs, and 1 μM antisense primer (oligonucleotide 98: 5′-GGGCTCGTCCGGGATTTG-3′). After the cooling to 42°C, 0.5 μl of Superscript II reverse transcriptase (RT) (100 U; Gibco BRL) was added and incubated for 60 min at 42°C. After the enzyme was inactivated at 94°C, 1 μl of the RT reaction mixture was amplified with oligonucleotides 98 and 97 (5′-GG CTCGTTGGTCTAGGGG-3′) for 25 cycles of 30 s at 94°C, 15 s at 60°C, and 30 s at 72°C. The PCR products were analyzed by 8% polyacrylamide gel electrophoresis (PAGE). The gel-purified DNA fragments were sequenced by the Taq DyeDeoxy terminator cycle sequencing method (Applied Biosystems) using primer 97.

RESULTS

Synthesis of the uORF2-encoded peptide. Previous studies demonstrated that uORF2 in the gp48 transcript leader represses downstream translation and that ribosomes stall at the uORF2 termination codon (2, 26). The dependence of both translational inhibition and ribosomal stalling on the uORF2 coding information suggests that the peptide encoded by uORF2 mediates these effects. To determine whether the uORF2 peptide is in fact synthesized, we employed the in vitro transcription and translation system used in our previous study (2). In vitro transcripts containing the gp48 transcript leader were translated in RRL in the presence of [^{35}S]methionine. Synthesis of the uORF2 peptide was assessed by immunoprecipitation with antiserum specific for the 15 N-terminal amino acids of uORF2.

First, we translated pEQ438 RNA (containing the wild-type uORF2 except for mutations that create an optimal AUG codon context), the leader of which inhibits downstream translation and causes ribosomal stalling at the uORF2 termination codon (2). Precipitation with crude or affinity-purified immune serum revealed a 2- to 3-kDa band (Fig. 1A, lanes 3 and 4) migrating at the same size as the synthetic 22-amino-acid-long uORF2 peptide (Fig. 1B). No peptide was detected if RNA was omitted from the translation reaction mixture or if preimmune serum was used for the precipitation (Fig. 1A, lanes 1 and 2). These data reveal that the predicted 2.4-kDa peptide encoded by uORF2 is synthesized, at least in this cell-free translation system.

In addition to the 2.4-kDa peptide, a 20-kDa band was precipitated with both crude and affinity-purified immune serum and at least two other minor bands were precipitated with the crude immune serum. We focused on determining the nature of the 20-kDa band, since it was consistently detected and was the only band, besides the free peptide band, precipitated by the affinity-purified immune serum.

Linkage of the uORF2 peptide to tRNA^{Pro}. Although the 20-kDa band in Fig. 1 immunoprecipitated with immune serum, it cannot be any simple protein product resulting from translation of the gp48 transcript leader, since uORF2, encoding a 2.4-kDa peptide, is the longest ORF. Even if the entire transcript used in this assay were translated from end to end, the protein product would be no more than 9 kDa. Therefore, we postulated that the 20-kDa band represents uORF2 linked to another molecule.

To determine the chemical content of the material linked to the uORF2 peptide, we treated the immunoprecipitates with RNases, DNase, proteinase K, and bases (Fig. 2). The 20-kDa band disappeared and shifted to the peptide band after digestion with RNase A and RNaseONE, but it was resistant to DNase I. Incubation in 0.3 M KOH, which in control experiments degraded RNA (data not shown), eliminated the 20-kDa material, supporting the conclusion that the 20-kDa material contains RNA. The minor bands precipitated with crude immune serum (Fig. 1, lane 3) were also eliminated by RNase digestion (data not shown). Degradation of the RNA component of the 20-kDa material during the radioimmunoprecipitation may explain the presence of these minor bands as well as variation in the relative abundance of the 20-kDa band compared to the 2.4-kDa band in different experiments (e.g., compare Fig. 1, lane 4, to Fig. 2, lane 1). When the translation products were digested with RNase prior to immunoprecipitation, only the 2.4-kDa peptide band was detected (data not shown), confirming that the immunogenic determinant in the 20-kDa molecule is the uORF2 peptide. Proteinase K digestion eliminated both the 20- and 2.4-kDa bands. These data indicate that the 20-kDa molecule is a hybrid molecule containing the uORF2 peptide linked to RNA.

If the RNA component of the 20-kDa material is tRNA, as expected if translation of uORF2 is blocked prior to release of

the nascent chain, then hydroxylamine should hydrolyze the ester bond linking the tRNA to the peptide (3). Incubation of the translation products with hydroxylamine caused a significant reduction in the abundance of the 20-kDa material (Fig. 2). The absence of a corresponding increase in the free peptide band may result from instability of the uORF2 peptide in hydroxylamine (data not shown). Nonetheless, the relatively marked reduction in intensity of the 20-kDa band compared to the 2.4-kDa free peptide band suggests that the 20-kDa band is in fact uORF2 peptidyl-tRNA.

If uORF2 translation is blocked at termination, immediately prior to hydrolysis of the peptidyl-tRNA bond, then uORF2 would be expected to be linked to proline tRNA, since the final uORF2 codon is CCU. To test this hypothesis, we analyzed the RNA component of the immunoprecipitated products by RT-PCR and DNA sequence analyses. After immunoprecipitation, the antigen-antibody complexes bound to protein G agarose beads were treated with hydroxylamine to release the RNA which was then ethanol precipitated and analyzed by RT-PCR using primers specific for proline-tRNA. As shown in Fig. 3, a ~75-bp product was amplified from RNA immunoprecipitated with the pEQ438 translation products (lane 2). A product of the same size resulted from amplification of total RNA purified from RRL (lane 6). No abundant PCR product was detected when RNA was omitted from the translation reaction mixture, when preimmune serum was used, or when RT was omitted. As well, no specific PCR product was evident if the translation extract was programmed with RNA containing a missense mutation changing the C-terminal proline codon to alanine (P22A), a mutation that eliminates translational inhibition and ribosomal stalling by uORF2 (2, 5).

The RT-PCR products were gel purified, and their sequences were determined with the sense proline tRNA primer. The sequence corresponding to the anticodon of the RRL $tRNA^{Pro}$ found after RT-PCR of whole RRL RNA revealed a mixture of GGG and CGG (Fig. 3B). In contrast, the anticodon sequence associated with uORF2 peptidyl-$tRNA^{Pro}$ was uniquely GGG. The GGG sequence likely resulted from RT-PCR of the IGG anticodon of the tRNA which is expected to decode the C-terminal proline codon, CCU, of uORF2 (see Discussion).

Synthesis of uORF2 peptide and peptidyl-tRNA from gp48 transcript leader mutants. Previous studies identified uORF2 missense mutations that eliminated the translational inhibition and ribosomal stalling effects of the wild-type uORF2 (2, 5). In contrast, mutants with synonymous changes within uORF2 or with deletions of transcript leader sequences flanking uORF2 retained these properties. To investigate the effect of these mutations on synthesis of uORF2 peptide and the peptidyl-$tRNA^{Pro}$, we translated mutant RNAs and immunoprecipitated the products (Fig. 4). Mutation of the AUG2 codon (pEQ367) resulted in no uORF2 products, as expected. The gp48 transcript leader with the wild-type AUG2 context (pEQ307) produced both the uORF2 and the peptidyl-tRNA bands. The reduced intensity of these products compared to those resulting from pEQ438 RNA translation likely reflects inefficient initiation at the wild-type AUG codon (1), shown previously to result in less inhibition of downstream translation and less evident ribosome pausing (2) than the optimal context AUG2 codon. Translation of the missense mutants P22A (pEQ439) and I20L-P22T (pEQ509), which release translational inhibition in vivo and in cell extracts and eliminate ribosomal stalling (2, 5), yielded abundant uORF2 peptide bands but little or no peptidyl-tRNA band. The relatively large amount of free peptide produced by the missense mutants compared to peptide produced by wild-type uORF2 constructs

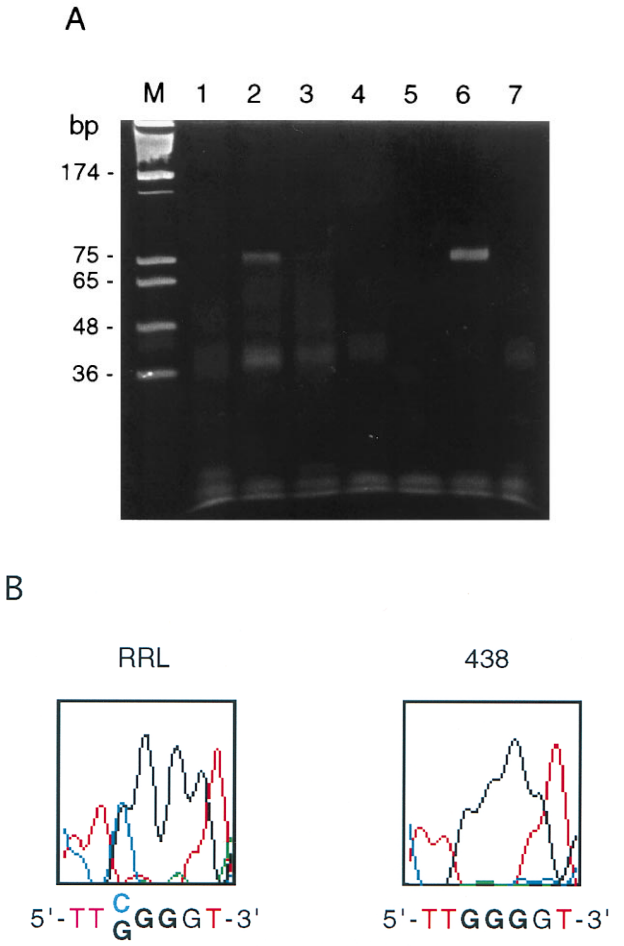

FIG. 3. Identification of uORF2 peptidyl-$tRNA^{Pro}$. (A) After cell-free translation of no RNA (lane 1), pEQ438 RNA (lanes 2, 3, and 4), or pEQ439 RNA (lane 5) and immunoprecipitation with preimmune serum (lane 3) or affinity-purified immune serum (lanes 1, 2, 4, and 5), the immune complexes on the beads were incubated with hydroxylamine for 8 h at room temperature. Following pelleting of the beads, nucleic acids in the supernatant were ethanol precipitated and analyzed by RT-PCR using proline-specific oligonucleotides as described in Materials and Methods. RT was omitted from the RT-PCR shown in lane 4. RT-PCR was also performed using 10 ng of total RRL RNA (lane 6) or no RNA (lane 7) as templates. DNA size markers (lane M) are indicated. (B) RT-PCR products amplified from total RRL RNA (RRL) and from the RNA released by hydroxylamine treatment of the immunoprecipitated products of pEQ438 RNA translation (438) were gel isolated and sequenced by using the proline tRNA sense primer. The anticodon loop sequences are shown.

suggests that, in addition to repressing translation of the downstream cistron, the wild-type uORF2 inhibits its own synthesis.

The uORF2 peptidyl-tRNA was synthesized by mutants which retain the wild-type coding information of uORF2. For example, 543 RNA, containing synonymous mutations affecting the wobble bases of codons 18 through 21 of the uORF2, produced the peptidyl-tRNA band. Since the RNAs shown thus far contain another upstream AUG codon and associated 3-codon uORF (uORF3), we also analyzed the construct pEQ505 that has a 36-nucleotide deletion immediately downstream from uORF2 which eliminates uORF3. Translation of this RNA produced the same uORF2 peptidyl-tRNA band. Together, these data demonstrate that gp48 leader sequences required for synthesis of the uORF2-peptidyl-$tRNA^{Pro}$ are identical to those necessary for uORF2-mediated translational inhibition and ribosomal stalling (2, 5).

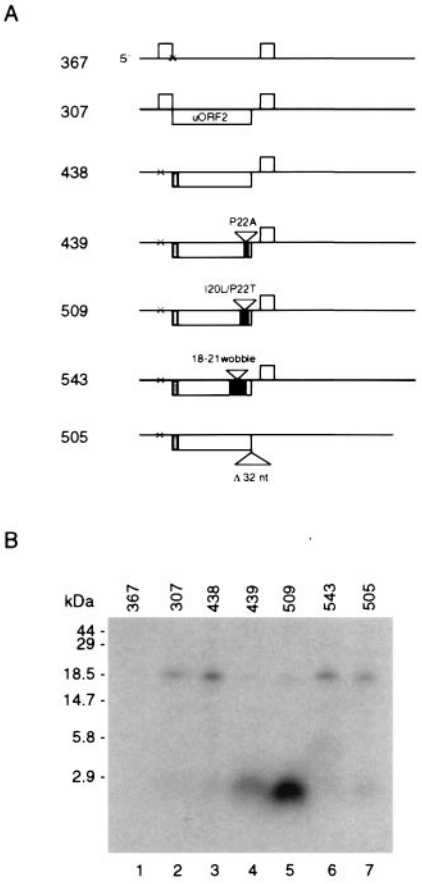

FIG. 4. Effects of uORF2 mutations on uORF2 peptide and peptidyl-tRNA synthesis. (A) Wild-type and mutant gp48 transcript leader RNAs contain the wild-type AUG2 codon (307), mutation of the AUG2 codon to AAG (367), or mutation of the AUG1 codon to AAG (×) and of the nucleotides flanking AUG2 to the optimal context ACCaugG (gray boxes in 438 through 505). Additional changes in leader sequences were missense mutation P22A (439); missense mutation I20L-P22T (509), a synonymous mutation affecting the third bases in codons 18 to 21 (543); and a deletion of the 32 nucleotides immediately downstream from the uORF2 termination codon (505). (B) These indicated RNAs were translated in RRL and, following immunoprecipitation with affinity-purified immune serum, were analyzed by SDS-PAGE. Protein size markers are shown.

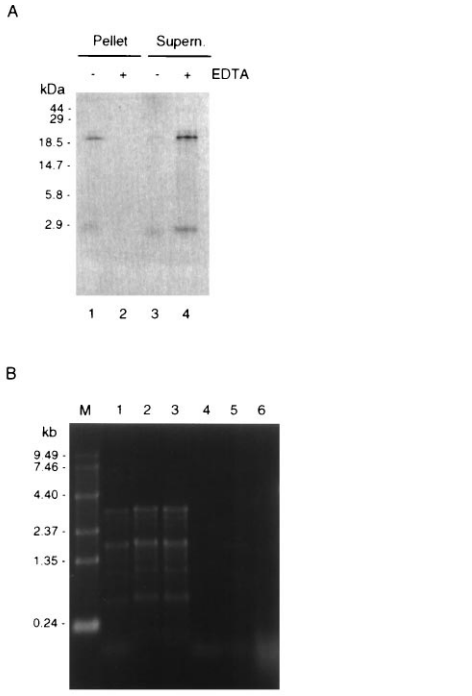

FIG. 5. Ribosomal association of the uORF2 peptidyl-tRNA^{Pro}. (A) After translation of 438 RNA in RRL, extracts were incubated with EDTA (lanes 2 and 4) or left untreated (lanes 1 and 3) and then ultracentrifuged as described in Materials and Methods. The pelleted ribosome (lanes 1 and 2) and supernatant (lanes 3 and 4) fractions were immunoprecipitated with affinity-purified immune serum. Protein size markers are indicated. (B) RNA was extracted from unfractionated RRL (lane 1) or from the same ribosomal pellet (lanes 2 and 3) or supernatant (lanes 4 and 5) fractions as were used for the experiment in panel A. EDTA was added prior to pelleting of the sample shown in lanes 3 and 5. These RNAs and 1 μg of rabbit tRNA (lane 6) were analyzed by ethidium bromide staining after electrophoretic separation on a formaldehyde agarose gel. RNA size markers are indicated (lane M).

Association of uORF2 peptidyl-tRNA^{Pro} with ribosome. If the hypothesis that the uORF2 peptide inhibits translation is correct, then the uORF2 peptidyl-tRNA^{Pro} should reside within the ribosome. To evaluate this prediction, we pelleted ribosomes after translation and analyzed the pellet and supernatant fractions for the presence of uORF2 peptide and the uORF2 peptidyl-tRNA^{Pro} by immunoprecipitation. In parallel reaction mixtures, we added EDTA to dissociate ribosomes from the mRNA and peptidyl-tRNA prior to pelleting the ribosomes. As shown in Fig. 5A, the peptidyl-tRNA^{Pro} was detected mainly in the ribosome pellet fraction. Upon the addition of EDTA, it was released into the supernatant. The free uORF2 peptide was present in both the pellet and supernatant fractions in the absence of EDTA and was found entirely in the supernatant after EDTA incubation. The efficiency of the ribosome pelleting was monitored by gel electrophoresis of RNA purified from the pellet and supernatant fractions (Fig. 5B). While rRNAs were detected exclusively in the pellet fraction, tRNA was present predominantly in the supernatant fractions. These data suggest the uORF2 peptidyl-tRNA^{Pro} is localized predominantly within the ribosome.

DISCUSSION

Inhibition of downstream translation by uORFs in a few eukaryotic genes depends on the uORF coding content (7). In the case of the CMV gp48 uORF2, the coding information is also necessary to effect ribosomal stalling at the uORF2 termination codon. This sequence dependence implicates the uORF2 peptide product as a mediator of translational repression and ribosomal stalling.

In this article, we demonstrate that the 2.4-kDa peptide encoded by the gp48 uORF2 is synthesized in cell-free translation assays (Fig. 1). Although no peptide products of other sequence-dependent regulatory uORFs have been reported (7), peptides encoded by uORFs in a few other viral genes have been detected. A heptapeptide encoded by the first uORF in the Rous sarcoma virus transcript leader is synthesized in cell-free translation extracts (10). Similarly, translation of a 23-codon uORF in a simian virus 40 mRNA produces a 2.7-kDa protein in cell extracts and in infected cells (17). The Rous sarcoma virus and simian virus 40 uORFs affect downstream translation or genome packaging, though, in contrast to what is known about gp48 uORF2, these effects are not known to depend on the uORF coding content.

In addition to the free 2.4-kDa uORF2 peptide, the products of uORF2 translation include the uORF2 peptide linked to

tRNA (Fig. 2 and 3). Liberation of the uORF2 peptide from the hybrid molecule by hydroxylamine suggests that the RNA component is tRNA linked to the uORF2 peptide by the usual ester bond present in aminoacyl-tRNAs (3).

Analyses of RNA recovered from the supernatant after immunoprecipitation with uORF2 antiserum and hydroxylamine treatment enabled precise identification of the tRNA associated with the uORF2 peptide. Since previous studies showed that ribosomes stall at the end of uORF2 and the last codon of uORF2 encodes proline, we hypothesized that the tRNA linked to uORF2 might be $tRNA^{Pro}$. In eukaryotes except for yeasts, $tRNA^{Pro}$ genes differ only in the first nucleotide of the anticodon (15, 27, 29), thus enabling detection of any $tRNA^{Pro}$ by RT-PCR using one set of primers that anneal to the perfectly conserved tRNA body sequences. RT-PCR of the RNA associated with uORF2 peptide revealed a band consistent with $tRNA^{Pro}$. The absence of a similarly intense tRNA amplification product in the controls (Fig. 3A) is one indication that detection of $tRNA^{Pro}$ is not due to proline tRNA contamination of the immunoprecipitated material. Moreover, sequence analysis revealed that the $tRNA^{Pro}$ was unique, unlike the mixture detected in total RRL RNA (Fig. 3B). If the PCR product resulted from nonspecific tRNA contamination, we would have detected the same mixture of $tRNA^{Pro}$ in the RNA associated with the immunoprecipitated uORF2 peptide products as we did using whole RRL RNA.

The results of the $tRNA^{Pro}$ sequence analysis are strong evidence that the 20-kDa immunoprecipitated band represents tRNA decoding the terminal codon of uORF2 (CCU) linked to the nascent uORF2 peptide chain. The only proline tRNAs reported in vertebrates have the anticodon sequences IGG (I [inosine] is modified from A [adenine]) and ncm^5UGG (where ncm^5U [5-carbamoylmethyluridine] is modified from U [uridine]) (12, 16). There is an additional $tRNA^{Pro}$ gene with the anticodon sequence CGG, although the corresponding tRNA has not previously been detected. Most likely, the tRNA having the anticodon IGG decodes both CCC and CCU proline codons, while ncm^5UGG decodes CCA and CGG decodes CCG (16). Our analysis of the DNA sequence of the products of RT-PCR of RNA isolated from RRL revealed proline tRNAs with the anticodons GGG and CGG (Fig. 3B). The GGG sequence likely resulted from reverse transcription of tRNA having the anticodon IGG (11). Our detection of the CGG anticodon sequence may represent the first evidence for this predicted tRNA. We do not know why we did not detect $tRNA^{Pro}(ncm^5UGG)$, though low levels in RRL or problems with reverse transcription of the modified base in this tRNA are possible explanations. Regardless, since the only tRNA immunoprecipitated by the uORF2 antiserum is the one expected to decode the last codon of uORF2, these data suggest that all 22 codons of uORF2 are translated, but when the ribosome reaches the termination codon, the nascent peptidyl-$tRNA^{Pro}$ is not hydrolyzed.

If the uORF2 peptidyl $tRNA^{Pro}$ is responsible for a block to translation termination and ribosomal stalling at the end of uORF2, then the uORF2 peptidyl-tRNA should be imbedded in the ribosome. Indeed, the peptidyl-tRNA is found predominantly in the ribosome pellet (Fig. 5), and upon dissociation of ribosomes by incubation in EDTA, the uORF2 peptidyl-tRNA is released into the supernatant fraction. In addition to the peptidyl-tRNA, a portion of the free uORF2 peptide is present in the ribosome pellet in the absence of EDTA. This finding could be the result of tRNA degradation during the immunoprecipitation assay, a problem that might also account for variation in the ratio of free uORF2 peptide to uORF2 peptidyl-tRNA in different experiments. Alternatively, the termination block may be only temporary, allowing a low level of hydrolysis of the peptidyl-tRNA ester bond. If the uORF2 peptide functions through interactions with ribosomal components or translation factors, then the free uORF2 peptide might tend to remain associated with the ribosome even after hydrolysis.

The demonstration that the uORF2 peptide is associated with the ribosome as a peptidyl-$tRNA^{Pro}$(IGG) indicates that after synthesis of all 22 amino acids of uORF2, translation stops before release of the nascent peptide chain. The peptide itself is responsible for the block to termination, since missense but not synonymous mutations allow efficient release of the peptide (Fig. 4). Moreover, the same uORF2 sequences mediate autoinhibition of uORF2 translation (Fig. 4), inhibition of downstream translation (2, 5), and ribosomal stalling (2). These data support a model of inhibition by uORF2 in which the nascent peptide chain inhibits its own termination. This failure to terminate causes the ribosomes to stall at the termination codon, which in turn blocks other ribosomes from scanning to the downstream AUG codon (1, 2).

In several other examples, nascent peptide chains affect translation in *cis*. Translation of the signal peptide of secreted proteins results in arrest of translation elongation after the nascent peptide exists from the ribosome and binds to the signal recognition particle (23). Certain prokaryotic antibiotic resistance genes are regulated by translation attenuation, a mechanism that depends on the peptide product of an uORF (21). In this case, the interaction of the nascent peptide with the ribosomal peptidyl transferase interferes with translation elongation or termination (9). Studies of the mitochondrial protein rhodanese suggest that the N-terminal peptide acts to block translation termination but not elongation (20).

The molecular mechanism by which the nascent uORF2 peptide blocks peptide release is unknown. It does not block translation elongation since carboxy-terminal extensions, even by only 1 codon, eliminate its inhibitory effects (5). The specificity for termination suggests that uORF2 peptide acts by interfering with the binding or activity of a release factor, such as eRF1 or eRF3, when a termination codon is in the ribosomal A site. Alternatively, the uORF2 peptide might interfere with the ribosomal peptidyl transferase, since this activity differs during elongation and termination. Peptidyl transferase catalyzes transfer of the nascent peptide chain in the ribosomal P site to the next aminoacyl-tRNA during elongation but to a H_2O molecule during termination. Further studies of the molecular interactions of the uORF2 peptide are needed to elucidate its precise mechanism of action.

There are no clear examples in which translation termination regulates eukaryotic gene expression. Limited data suggest that termination in eukaryotes is a relatively slow process (31). However, during translation of a typical monocistronic mRNA, the duration of termination is likely to contribute only a small proportion to the total time required for synthesis of the protein, since initiation, often a slow step, and multiple elongation reactions are also involved. However, in the case of an uORF, termination may assume greater importance, becoming a primary determinant of the ability of ribosomes to initiate at the AUG codon of the downstream cistron. In the case of gp48 uORF2, the poor context of the uORF2 AUG codon predicts that most ribosomes loading on to the transcript near the 5′ end bypass the AUG codon and translate the downstream ORF by a leaky scanning mechanism (1). However, the block at termination of uORF2 translation means that even only a few ribosomes translating uORF2 can profoundly repress downstream translation. Whether a similar mechanism accounts for regulation by other uORFs, particu-

larly those that act in a sequence-dependent manner, will require additional investigations.

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