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Coding Sequence-Dependent Ribosomal Arrest at Termination of Translation

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A remarkably high percentage of proto-oncogene, growth factor, cellular receptor, and viral transcript leaders contain short upstream open reading frames (uORFs), yet the significance and regulatory effects of these uORFs have not been well characterized. In the case of the human cytomegalovirus gpUL4 (gp48) transcript, the second of three uORFs (uORF2) inhibits translation of the downstream cistron by a process that depends on the uORF2 amino acid coding information. To investigate the mechanism underlying this unusual regulatory element, we adapted the toeprinting (or reverse transcriptase extension inhibition) assay for use in detecting positions of ribosomal stalling on gp48 transcripts. Using a cell-free translation system, we demonstrate that ribosomes arrest at the termination codon of uORF2 by a uORF2 coding sequence-dependent mechanism. Further, the sequence requirements for ribosomal stalling are the same as for inhibition of downstream translation. We also provide evidence for ribosomal stalling in vivo, on the natural viral mRNA. These data support the hypothesis that the inhibition of downstream translation results from uORF2 peptide-dependent ribosomal arrest at termination and suggest that translation termination may be a regulatory step in expression of some eukaryotic genes.

Short upstream open reading frames (uORFs) are present in a minority of eukaryotic mRNA leaders but are unusually prevalent in certain subsets of genes, including those involved in the control of cellular growth (14). The regulatory effects of uORFs depend in part on parameters influencing translation initiation at the AUG codon of the uORF (uAUG). Thus, if the context of nucleotides surrounding the uAUG codon is optimal for translation initiation (13), ribosomes are more likely to translate the uORF instead of the downstream ORF, while the presence of an internal ribosomal entry site downstream from the uAUG codon enables ribosomes to bypass the uORF and translate the downstream ORF efficiently (17).

In several cases, however, the impact of a uORF is not accounted for by the efficiency of initiation at the uAUG codon. For example, some uORFs have little or no effect on downstream translation even though the uAUG codons are efficiently recognized, presumably because ribosomes reinitiate at the downstream AUG codon (1, 2, 9, 11, 12). Conversely, uORFs such as the second one (uORF2) in the human cytomegalovirus (CMV) gp48 gene transcript profoundly inhibit downstream translation even though initiation at the uAUG codon is very inefficient (2). These observations suggest that at least in some cases, translational events other than initiation determine the overall effect of the uORF.

A remarkable feature of gp48 uORF2 is that its inhibitory effect depends on its amino acid coding information (5), a property reported for uORFs in very few other eukaryotic genes (reviewed in reference 8). Despite this coding sequence dependence, uORF2 inhibits downstream translation only in *cis* (5). As well, the inhibitory effect requires a termination codon at the authentic position immediately following the uORF coding sequences; carboxy-terminal extension of the uORF by only a single codon relieves the inhibition (5). The current studies were designed to test the hypothesis, suggested by these data, that inhibition by uORF2 results from a block at termination of uORF2 translation mediated by the nascent uORF2 peptide (2).

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MATERIALS AND METHODS

Plasmids, synthetic RNAs, and sequencing. Plasmid pEQ4, containing the β-galactosidase (β-Gal) ORF in the in vitro transcription vector pBS+ (Stratagene) has been described previously (7). The wild-type gp48 transcript leader construct pEQ307 and the AUG2$^-$ mutant construct pEQ367 were constructed by inserting the *Hin*dIII-*Eco*RV fragments from pEQ239 and pEQ325 (20), respectively, into the *Hin*dIII-*Eco*RV sites in pEQ4. Plasmids pEQ438 and pEQ439 were constructed by insertion of the *Hin*dIII-*Bam*HI fragments from pEQ422 and pEQ429 (2), containing the gp48 leader and β-Gal ORF, into pBS+. Plasmids pEQ505, pEQ542, and pEQ543 were generated by PCR amplification of pEQ366, pEQ406, and pEQ419 (5), respectively, using primers 64 (CCTTACCATGGAGCCGCTGG) and 17 (20). After cutting with *Nco*I and *Afl*II, each amplimer was inserted into the *Nco*I-*Afl*II sites of pEQ438. Thus, the uORF in these plasmids initiates at an optimal context AUG codon (underlined in the primer 64 sequence). The same cloning strategy was used to construct pEQ507 and pEQ509, using plasmids pEQ409 and pEQ413, respectively, as templates for PCR. Plasmids pEQ409 and pEQ413, each containing two uORF2 missense mutations, were constructed by using a degenerate primer-based PCR strategy as previously described (5). The uORF2 sequences in pEQ409 and pEQ507 contain mutations of the 10th uORF2 codon, AAA (encoding lysine) to GAA (encoding glutamine), designated K10Q, and of CCG to ACG at codon 21 (P21T). The mutations in pEQ413 and pEQ509 are ATC to CTC at codon 19 (I19L) and CCT to ACT at codon 22 (P22T).

For in vitro transcription, the plasmids were linearized at the *Bam*HI site located downstream from the β-Gal termination codon, and transcripts were synthesized by using an AmpliScribe T3 transcription kit (Epicentre Technologies) with 1.25 μCi of [α-^{32}P]UTP (Amersham). Although the data shown in this report resulted from experiments using uncapped transcripts, very similar translational inhibition and toeprint data were obtained with capped transcripts (not shown).

Plasmid sequences were verified by the *Taq* DyeDeoxy terminator cycle sequencing method (Applied Biosystems) with the assistance of the Fred Hutchinson Cancer Research Center Biotechnology Laboratory. The dideoxy sequencing of pEQ438 shown in Fig. 2c was carried out by using a Sequenase version 2.0 sequencing kit (U.S. Biochemical) and primer 17 (20).

Cell-free translation. After cell-free translation in nuclease-treated rabbit reticulocyte lysates (RRL; Promega, Inc.), β-Gal protein synthesis and activity

were determined as described previously (7). A standard translation reaction in this study was performed with 0.2 ng of RNA in a 6.3-μl reaction mixture.

Toeprint assay in RRL. The toeprint assay was carried out by annealing 10 nM ^{32}P-end-labeled primer 17 (20) to 2 μl of the translation reaction mix for 5 min at 40°C in 10 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.01 M dithiothreitol, and 0.25 mM deoxynucleoside triphosphates. After addition of reverse transcriptase (50 U; Superscript II; Gibco/BRL) and incubation for 60 min at 40°C, samples were extracted with phenol-chloroform and were analyzed on a 6% denaturing polyacrylamide gel.

Magnesium precipitation of ribosomes was performed as described previously (18). Briefly, after addition of 3 volumes of 0.2 M $MgCl_2$, 2% Triton N-101, and 2 mg of heparin per ml and incubation on ice for 1 h, the samples were layered on a 4-volume cushion of 1 M sucrose–20 mM Tris-HCl (pH 7.4)–10 mM NaCl–0.2 M $MgCl_2$. Following centrifugation at 16,000 × *g* for 15 min at 4°C, the pellet was resuspended in 10 μl of H_2O.

Toeprinting and primer extension of RNA from infected cells. Human fibroblasts and CMV (Towne) were grown in Dulbecco's modified Eagle's medium supplemented with 10% Nu-serum (Collaborative Research, Inc., Bedford, Mass.). Cytoplasmic extracts were prepared from 150-mm-diameter plates of uninfected cells or cells infected at a multiplicity of infection of 10 PFU per cell as for polyribosome analysis (20) except that no cycloheximide was added. RNA for primer extension analysis was purified from the cytoplasmic extracts by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation.

The toeprint assay was carried out by using the cytoplasmic extracts as described for translation in RRL except that a 20-μl reaction mixture containing 11 μl of cytoplasmic extract was incubated for 5 min at 55°C prior to adding 0.1 μM ^{32}P-labeled primer 17. After addition of the primer, the reaction mixture was incubated for 2 min at 55°C and then for 5 min at 40°C. Reverse transcriptase (100 U) was added, and the reaction mixture was incubated for 1 h at 40°C prior to phenol-chloroform extraction and analysis on a denaturing polyacrylamide gel.

RESULTS

Inhibition of downstream translation in cell-free translation assays. To simplify the analysis of the uORF2 inhibitory mechanism, we used a cell-free translation assay in RRL. Using plasmids containing the wild-type or mutant gp48 leader sequences upstream of the β-Gal ORF (Fig. 1a), we synthesized transcripts (Fig. 1b) and assessed their efficiencies as templates for β-Gal translation as determined by using both [^{35}S]methionine labeling (Fig. 1c) and β-Gal enzymatic activity assays (Fig. 1d).

The wild-type leader in pEQ307 RNA (Fig. 1a) expressed only slightly less β-Gal than did the control pEQ4 RNA, which lacks gp48 leader sequences. However, mutating the nucleotides surrounding AUG2 to the optimal context for translation initiation reduced β-Gal translation 5- to 10-fold. As in transfection assays (2, 5, 20), mutation of the uORF2 AUG codon (AUG2, pEQ367) or of the last sense codon in uORF2 (pEQ439) eliminated the inhibitory effect of the gp48 leader in the RRL assay. RNA containing uORF2 as the only uORF (pEQ505) expressed a low level of β-Gal in the RRL system (Fig. 1c and d), demonstrating that neither of the other two uORFs nor the wild-type sequences immediately 3′ from the uORF2 termination codon are required for the inhibitory effect of the gp48 leader. Thus, even though the magnitude of the inhibitory effect of the gp48 leader was less in the RRL assays than in transfection assays, inhibition by the gp48 leader depended on the uORF2 coding sequences in cell-free translation as in vivo.

Toeprint analysis after cell-free translation. We next used the RRL assay to examine the mechanism of inhibition by uORF2 and in particular to determine whether ribosomes arrest after translating uORF2, as has been hypothesized (2). We adapted the toeprint or extension inhibition assay, first developed to study prokaryotic translation (10), to detect predominant positions of ribosomes on the gp48 transcript leader. In this assay, ribosomes associated with an mRNA block the extension of an antisense radiolabeled primer by reverse transcriptase at a position corresponding to the 3′ boundary of the ribosome. The sizes of the reverse-transcribed products, as

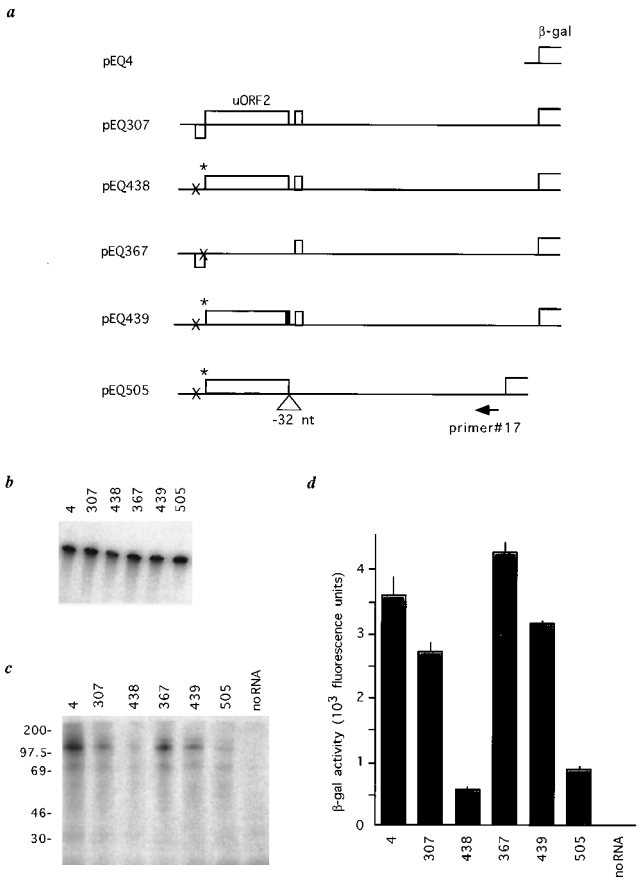

FIG. 1. Inhibition of downstream translation by the uORF2 leader in RRL. (a) Transcript leaders expressed from the indicated β-Gal plasmids contain no gp48 sequences (pEQ4), the wild-type gp48 transcript leader (pEQ307), or the gp48 leader with mutations of the first or second AUG codon to noninitiator sequences (×). In pEQ438, pEQ439, and pEQ505, the bases surrounding AUG2 codon are the optimal context for translation initiation, ACCAUGG (*). The carboxy-terminal missense mutation (proline to alanine at codon 22; black rectangle) present in pEQ439 and a 32-nucleotide (nt) deletion downstream from uORF2 in pEQ505 are indicated. The position of the antisense primer 17 (20) used in subsequent toeprint assays is indicated (arrow). (b) The quantity and integrity of in vitro transcripts (0.5 ng), synthesized by using the indicated plasmids templates and labeled with [^{32}P]UTP as described in Materials and Methods, were assessed by separation on a 6% denaturing polyacrylamide gel followed by autoradiography. (c) The transcripts shown in panel b were translated in RRL in presence of [^{35}S]methionine, and the products were analyzed on a sodium dodecyl sulfate–5% polyacrylamide gel. Molecular weight standards (in kilodaltons) are shown on the left. (d) The β-Gal activities in duplicate 2-μl aliquots from RRL translation assays were determined as described in Materials and Methods, and the means (plus standard deviations) are shown.

detected by electrophoresis and autoradiography, identify sites of ribosomal stalling on the mRNA. Since a single ribosome protects a 30- to 35-nucleotide fragment of mRNA from RNase digestion and the decoding site is approximately in the middle (22), the toeprint for a ribosome stalled at termination of uORF2 is predicted to map approximately 15 to 17 nucleotides downstream from the uORF2 termination codon.

Because RNA secondary structure or binding proteins, rather than ribosomes, could create a barrier to reverse transcriptase in the toeprint assay, we first analyzed the pattern generated by toeprint analysis of wild-type and mutant gp48 leader transcripts in RRL prior to translation (Fig. 2a). After mixing of the gp48 transcript in RRL on ice without addition of amino acids, the predominant reverse transcription product for each transcript mapped to the expected 5′ end of the RNA.

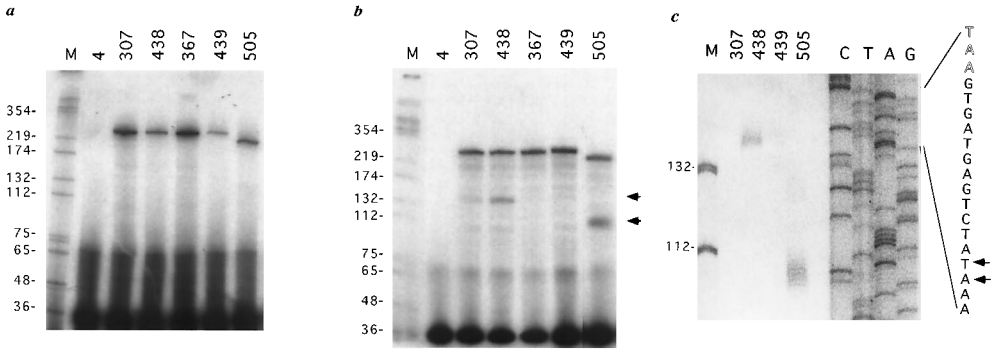

FIG. 2. Ribosome stalling at the gp48 uORF2 termination site. (a) Control toeprint assay of RNA samples prior to translation. The indicated pEQ RNA samples were mixed on ice with RRL in preparation for cell-free translation. Prior to addition of amino acids, a 2-μl aliquot from the mixture was reverse transcribed by using ^{32}P-end-labeled primer 17, and the products were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. In all panels, the lengths (in nucleotides) of markers (M) are indicated on the left. (b) Toeprint analysis of RNAs translated in RRL. After the transcripts represented in panel a were translated in the RRL assay for 30 min at 30°C, 2 μl of each mixture was analyzed by using the toeprint assay as described in Materials and Methods. Arrows indicate toeprint bands appearing after translation of pEQ307, pEQ438, and pEQ505 RNAs. (c) Precise mapping of the uORF2-related toeprint site. By separation of a subset of the samples used for panel b on a sequencing gel and comparison with the sequencing reactions using pEQ438 as a template (right), the toeprint bands in translations programmed with pEQ438 and pEQ505 RNAs map to a position 16 to 17 nucleotides downstream from the uORF2 termination codon.

The primer extension product obtained by using pEQ505 RNA was shorter than the other gp48 leader constructs because of the 32-nucleotide deletion in pEQ505 (Fig. 1a). As expected, no signal was detected with pEQ4 RNA, since it does not contain the gp48 leader sequences complementary to the primer used in this assay. In other experiments, using a primer corresponding to downstream β-Gal sequences, the expected 5′ end of pEQ4 was detected (data not shown).

We next analyzed the products generated by toeprint assay of the same transcripts after translation. In addition to bands corresponding the 5′ end of the transcripts, we detected a new band after translation of pEQ438 and pEQ505 RNAs, both of which contain the optimal context AUG2 codon and an otherwise wild-type uORF2. As well, the same band was faint but detectable after translation of pEQ307 RNA, containing the wild-type gp48 leader. This toeprint band depended on initiation at AUG2 and on the coding information of uORF2 since it was not detected when either pEQ367 (AUG2$^-$) or pEQ439 (proline 22 mutation) RNA was used.

Analyses of the samples as shown in Fig. 2b on a sequencing gel mapped the toeprint band to a position approximately 116 nucleotides from the 5′ end of the mRNA or 16 to 17 nucleotides downstream from the T of the uORF2 termination codon, TAA (Fig. 2c). The difference in the size of the toeprint band obtained by pEQ505 RNA compared with pEQ438 RNA is consistent with the 32-nucleotide deletion in pEQ505. The same toeprint site was detected after translation of pEQ307, pEQ438, and pEQ505 RNAs, but not pEQ367 or pEQ439 RNA, in a toeprint assay using a different primer (data not shown). Thus, the toeprint band maps to the position expected if a ribosome is stalled at the uORF2 termination codon.

Generation of the toeprint band and inhibition of downstream translation depend on the same uORF2 coding sequences. In previous transfection experiments (5), several missense mutations of uORF2 which alleviated the inhibitory effects on downstream translation were identified. In contrast, synonymous mutations within uORF2 consistently retained the inhibitory effect. To determine whether these same sequences were required for inhibition in the cell-free translation assay, we translated synthetic RNAs containing uORF2 missense mutations or synonymous mutations in RRL (Fig. 3). A high level of β-Gal was expressed from each of the three RNAs (pEQ507, pEQ509, and pEQ542) containing uORF2 missense mutations which eliminate the inhibitory effects of uORF2 in transfection assays (reference 5 and data not shown). In contrast, the mutant with four synonymous mutations in uORF2 (pEQ543) expressed low levels of β-Gal. These data reveal that the sequence requirements for inhibition by uORF2 are the same in the cell-free assay as in vivo.

We next assessed the effects of these same mutations on the toeprint assay. The toeprint band was eliminated by the missense mutations but not by the synonymous mutations (Fig. 3). Thus, the uORF2 sequence requirements for inhibition of downstream translation are identical to those required for generation of the toeprint band.

Role of translating ribosomes in the stalling mechanism. To determine whether the toeprint signal was produced from transcripts that were associated with ribosomes, we precipitated ribosomes with magnesium after translation in RRL and analyzed the pelleted mRNA-ribosome complexes in the toeprint assay (Fig. 4a). The toeprint band was evident when ribosome-

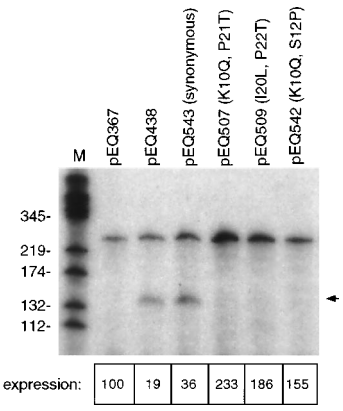

FIG. 3. Dependence of ribosomal stalling and translational inhibition on uORF2 sequences. Plasmid pEQ543 contains four synonymous mutations in codons 18 to 21 of uORF2, while the pEQ507, pEQ509, and pEQ542 each contain two missense mutations in uORF2. After translation of the RNAs synthesized from these plasmids, β-Gal activity was measured and a toeprint analysis was performed as described in Materials and Methods. The β-Gal values are indicated as a percentage of expression from pEQ367 RNA. Lengths of markers (M) are indicated in nucleotides.

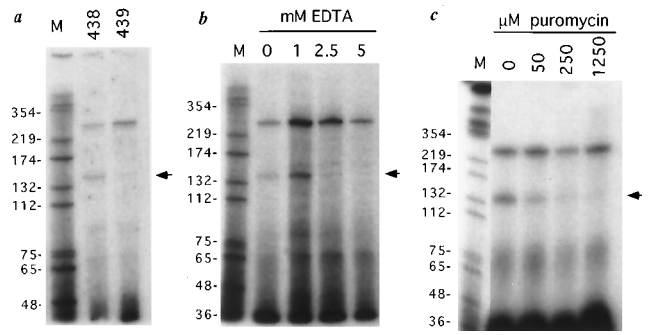

FIG. 4. Characterization of the ribosome stalling signal in the toeprint assay. (a) Toeprint analysis of magnesium-precipitated ribosome-associated RNA. After translation of pEQ438 and pEQ439 RNA in RRL, ribosomes in a 10-μl translation mixture were precipitated with magnesium and pelleted through a sucrose cushion as described in Materials and Methods. After resuspension, 2 μl of the ribosomal pellet was analyzed in the toeprint assay. Arrow indicates the toeprint band. In all panels, lengths of markers (M) are indicated in nucleotides. (b) Effects of EDTA incubation on the toeprint assay. After translation the pEQ438 RNA in RRL, EDTA was added to the indicated final concentration for 5 min at 30°C. After addition of $MgCl_2$ to 20 mM to samples with 1, 2.5, and 5 mM EDTA, the toeprint assay was performed. (c) Puromycin eliminates the toeprint. After translation of pEQ438 RNA in RRL, puromycin was added to the indicated concentrations. Following incubation for 5 min at 30°C, the toeprint assay was performed.

associated RNA corresponding to pEQ438 but not pEQ439 was used.

The role of ribosomes in generating the toeprint band was also assessed by toeprint analysis after dissociation of ribosomes from pEQ438 mRNA by using EDTA followed by addition of Mg^{2+} to enable the reverse transcriptase reaction (Fig. 4b). Incubation in 2.5 or 5 mM EDTA caused the toeprint band to vanish. The band corresponding to the 5′ end of the RNA was still evident, demonstrating that reverse transcriptase functioned under these conditions. Together, the data in Fig. 4a and b support the conclusion that ribosomes loaded on the gp48 leader at the uORF2 termination codon are responsible for the toeprint band.

To determine whether translating ribosomes are needed to generate the toeprint band, we assessed the impact of puromycin, a drug which releases nascent peptides from translating ribosomes, on the toeprint assay. Incubation of the translation reaction mixture containing pEQ438 RNA with puromycin reduced the intensity of the toeprint (Fig. 4c). The band corresponding to the 5′ end remained detectable, confirming that puromycin did not affect reverse transcriptase activity. This reduction in the toeprint signal by puromycin suggests that translation is required for ribosomal stalling at the uORF2 termination codon.

Ribosomal arrest in vivo. Since the uORF2 coding sequences required for translational inhibition are the same in cell-free translation and in vivo assays, we tested the prediction that ribosomes arrest at termination of uORF2 translation in vivo as in the cell-free translation system. We first analyzed that 5′-end structure of the authentic gp48 mRNA in infected cell extracts at early (16 h) and late (71 h) times after CMV infection. By primer extension analysis of purified RNA (Fig. 5a), we detected the expected abundant early mRNA and the minor late gp48 transcripts (3).

We next performed a toeprint assay using mock-infected or CMV-infected cell cytoplasmic extracts. Because these extracts possessed an activity that degraded or dephosphorylated the labeled oligonucleotide primer used in the toeprint assay (data not shown), we heated the extracts as described in Materials and Methods prior to adding the primer. Using this assay, we detected a toeprint band at exactly the same position as the one observed in cell-free assays (Fig. 5b). The 5′-end band of 438 RNA was slightly larger than that of the viral mRNA as a result of sequences derived from the polylinker in the in vitro transcription plasmid.

Finally, we prepared mock-infected and CMV-infected cell extracts after addition of puromycin (Fig. 5c). Incubation with puromycin in vivo eliminated the toeprint band just as occurred in the cell-free assay (Fig. 4c). These data suggest that the mechanism responsible for ribosomal arrest at the termination codon of uORF2 in the cell free system also operates on the authentic viral mRNA in vivo.

DISCUSSION

Several features of gp48 uORF2-mediated inhibition are not fully accounted for by the hypothesis that efficiency of initiation at an uAUG codon is the primary factor responsible for

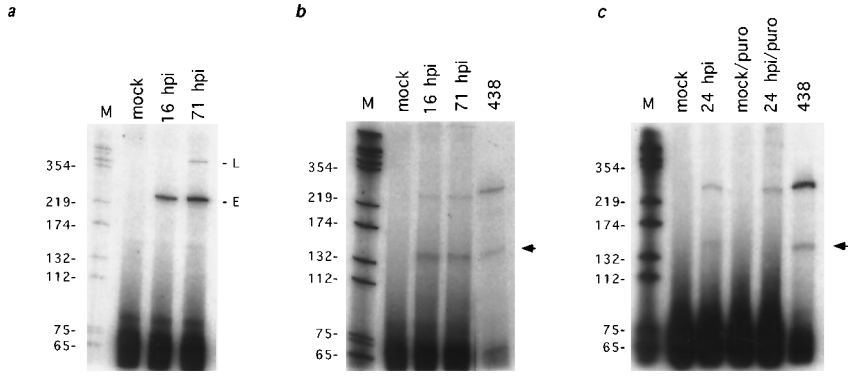

FIG. 5. Toeprint analysis of gp48 transcripts in CMV-infected cell lysates. (a) Primer extension reaction. Human foreskin fibroblasts (10^7) were mock infected or infected with human CMV until 16 or 71 h postinfection (hpi); 0.3 μg of RNA purified from cytoplasmic extracts was reverse transcribed at 50°C after annealing to primer 17. The positions of the predominant early (E) and the minor late (L) transcription start sites are indicated. In all panels, lengths of markers (M) are indicated in nucleotides. (b) Toeprint of gp48 transcript in CMV-infected cell lysates. The cytoplasmic extracts used for panel a were subjected to toeprint analysis adapted for use with cell lysates as described in Materials and Methods. The arrow indicates the toeprint band. Lane 438 shows the toeprint analysis of pEQ438 RNA after translation in RRL and demonstrates the same size toeprint as seen in infected cells. A slightly larger band corresponding to the transcript 5′ end in lane 438 result from polylinker sequences in the in vitro transcription plasmid. (c) Effect of puromycin in vivo. Toeprint analysis was performed on mock-infected or 24-h-infected cytoplasmic extracts as for panel b or after incubation for 30 min in medium containing 100 μM puromycin.

inhibition of downstream translation. For example, uORF2 is a potent inhibitory sequence even though initiation at its AUG codon is very inefficient (2). A second unusual property of uORF2 is that its coding content and termination codon are required for inhibition, implicating the putative uORF2 peptide as the mediator of the inhibitory effect, yet uORF2 functions only in *cis* (5). These observations led to the suggestion that termination, rather than initiation, of uORF2 translation is primarily responsible for inhibition of downstream translation. The studies reported above support a model in which ribosomes arrest at termination of uORF2 translation by a mechanism that depends on the nascent uORF2 peptide and that these stalled ribosomes obstruct other ribosomes from gaining access to the downstream ORF (2).

To investigate the mechanism of gp48 uORF2 inhibition, we first assessed the effects of uORF2 on downstream translation in a cell-free translation assay. The key criterion that this cell-free assay must fulfill to be considered reliable for these studies is that inhibition by the gp48 transcript leader display the same *cis*-acting sequence requirements for inhibition as were determined previously using in vivo assays. Indeed, inhibition by the gp48 leader in the RRL assays is dependent on the AUG2 codon and on the coding information of uORF2, identical to results of in vivo assays (2, 5, 20). The strong inhibitory effect of the optimal context uORF2 construct (Fig. 1, pEQ438) compared with the wild-type construct (pEQ307) in the cell-free translation system is consistent with the conclusion, derived from in vivo studies, that a leaky scanning mechanism enables ribosomes to bypass the wild-type AUG2 codon.

One difference between the cell-free and in vivo systems is that the magnitude of the inhibitory effect of the gp48 leader is less in the RRL assay than in in vivo assays. For example, the optimal context AUG2 constructs inhibit downstream translation approximately 100-fold in intact cells (2) but only 5- to 10-fold in the RRL assay (Fig. 1 and 3). Similarly, the wild-type leader inhibits translation 10-fold in vivo (2, 5, 20) but is, at most, minimally inhibitory in the cell-free assay (Fig. 1). This diminished inhibitory effect in the RRL assay may reflect a reduced translational capacity of the RRL compared with intact cells. A prediction of the ribosomal stalling model (2) is that the impact of a stalled ribosome will be most evident after multiple additional scanning 40S ribosomal subunits have encountered the blockade created by the stalled ribosome. If the translational capacity of RRL is short-lived, then the ability to detect the effect of the blockade will be limited. Regardless of the cause of this difference in the sensitivity, the uORF2 coding sequence-dependent inhibition in the RRL assay suggests that this cell-free system is useful for evaluating the mechanism of uORF2 inhibition.

Experiments using the toeprint assay (10), adapted for use in our system, suggest that ribosomes stall at termination after translating uORF2. The toeprint band (i) maps to the position expected to result from a ribosome stalled at termination codon of uORF2 (Fig. 2), (ii) depends on the association of the RNA template with ribosomes (Fig. 4), and (iii) requires translation of uORF2 (Fig. 2 and 4). The absence of the toeprint band in assays using missense mutants reveals that the ribosomal stalling is prolonged at termination after translation of the wild-type uORF2 compared with the mutants.

Although ribosomal pausing has been detected at termination codons in other eukaryotic mRNAs (6, 15, 22), we are not aware of any examples in which termination of translation has been demonstrated to be the primary regulatory event controlling gene expression in a eukaryotic system. In our studies, detection of the toeprint band correlates well with inhibition of downstream translation; it is present after translation of RNAs with inhibitory gp48 transcript leaders but not after translation of RNAs with uORF2 mutations that alleviate the inhibition (Fig. 1 to 3). These data support the hypothesis that the ribosomal stalling is responsible for inhibition of downstream translation.

Alleviation of the translational inhibition both in vivo (5) and in cell-free translation assays (Fig. 1 and 3) implicates the peptide product of uORF2 in the inhibitory mechanism. Similarly, elimination of the toeprint band by missense mutations in the cell-free assays (Fig. 2 and 3) suggests that the uORF2 peptide mediates ribosomal stalling. An alternative possibility is that the mutations act by altering the RNA sequence or structure in the vicinity of termination codon. However, synonymous mutations of four nucleotides near the carboxy terminus of uORF2 do not eliminate the uORF2-mediated translational inhibition or ribosomal stalling (Fig. 3). Moreover, elimination of the uORF2 effects by missense mutations of sequences more than 30 nucleotides upstream from the termination codon (pEQ542; Fig. 3) and thus external to the stalled ribosome (22) is unlikely to result from changes in the RNA structure near the termination codon. Finally, inhibition of translation by puromycin eliminates the toeprint band. Thus, although we have not yet been able to detect the peptide product of uORF2, these data strengthen the hypothesis that the nascent peptide of uORF2 is synthesized and mediates the ribosomal stalling.

Nascent peptides cause ribosomal stalling in other systems (16, 22). For example, ribosomal pausing occurs after translation of the signal peptide in proteins destined for entry into the secretory pathway (22). The small size (22 codons) of uORF2, the critical role of its carboxy-terminal codons (5), and the requirement for the termination codon immediately after the coding sequences suggest that, in contrast to signal peptides which induce ribosomal pausing after emerging from the ribosome, gp48 uORF2 likely acts within the ribosome. Though we do not know the structural basis for the inhibitory effect of the nascent uORF2 peptide, we hypothesize that it interacts either with an intrinsic component of the ribosome, such as a ribosomal protein, or with a translation factor, such as eukaryotic peptide release factor, in a manner that delays or prevents a step in the termination reaction. Further studies of the uORF2 should aid in dissecting the mechanism of delayed as well as normal termination in eukaryotic translation.

Finally, in previous in vivo assays (2, 5, 20) as well as the RRL assays reported here, we analyzed translation of chimeric RNAs containing the gp48 transcript leader fused upstream of reporter gene cistrons. The successful adaptation of the toeprint assay to cytoplasmic extracts enabled an assessment of the translational state of the authentic viral mRNA. The detection of the same toeprint signal by using viral mRNA expressed in vivo and by using chimeric RNAs in the cell-free translation system provides the first evidence that control of translation of the viral gp48 gene operates through the same ribosomal stalling mechanism characterized in studies using reporter gene constructs. The significance of this translational mechanism for the virus or the infected cell remains unknown. Since, in its current form, the toeprint assay is not quantitative, the presence of the toeprint band at early and late times after infection does not reveal whether the ribosomal stalling is constitutive or regulated during the CMV infectious cycle. We anticipate that refinements in the toeprint assay will enable more informative investigations of gp48 translational control during CMV infection and will facilitate a broad range of studies of ribosomal pausing during translation and of protein-mRNA interaction in other in vivo systems.

CMV gp48 is one of only five eukaryotic genes reported thus far in which the inhibitory effect of a uORF appears to depend on its amino acid coding sequence (2, 4, 11, 21; reviewed in reference 8). Additional examples of sequence-dependent regulatory uORFs will likely emerge from studies of translational control of the large number of uORF-containing eukaryotic mRNAs involved in control of cellular growth and differentiation (14). On the basis of studies of gp48 uORF2-mediated inhibition, the potential for regulation of other genes at the level of translational termination deserves investigation.

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REFERENCES

1. **Biegalke, B. J., and A. P. Geballe.** 1990. Translational inhibition by cytomegalovirus transcript leaders. Virology **177:**657–667.
2. **Cao, J., and A. P. Geballe.** 1995. Translational inhibition by a human cytomegalovirus upstream open reading frame despite inefficient utilization of its AUG codon. J. Virol. **69:**1030–1036.
3. **Chang, C.-P., C. L. Malone, and M. F. Stinski.** 1989. A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. J. Virol. **63:**281–290.
4. **Damiani, R. D., Jr., and S. R. Wessler.** An upstream open reading frame represses expression of *Lc*, a member of the *R*/*B* family of maize transcriptional activators. Proc. Natl. Acad. Sci. USA **90:**8244–8248.
5. **Degnin, C. R., M. R. Schleiss, J. Cao, and A. P. Geballe.** 1993. Translational inhibition mediated by a short upstream open reading frame in the human cytomegalovirus gpUL4 (gp48) transcript. J. Virol. **67:**5514–5521.
6. **Doohan, J. P., and C. E. Samuel.** 1992. Biosynthesis of reovirus-specified polypeptides: ribosome pausing during the translation of reovirus S1 mRNA. Virology **186:**409–425.
7. **Geballe, A. P., and M. K. Gray.** 1992. Variable inhibition of cell-free translation by HIV-1 leaders sequences. Nucleic Acids Res. **20:**4291–4297.
8. **Geballe, A. P., and D. R. Morris.** 1994. Initiation codons within 5′ leaders of mRNAs as regulators of translation. Trends Biochem. Sci. **19:**159–164.
9. **Grant, C. M., P. F. Miller, and A. G. Hinnebusch.** 1994. Requirements for intercistronic distance and level of eukaryotic initiation factor 2 activity in reinitiation on *GCN4* mRNA vary with the downstream cistron. Mol. Cell. Biol. **14:**2616–2628.
10. **Hartz, D., D. S. McPheeters, R. Traut, and L. Gold.** 1988. Extension inhibition analysis of translation initiation complexes. Methods Enzymol. **164:** 419–425.
11. **Hill, J. R., and D. R. Morris.** 1993. Cell-specific translational regulation of S-adenosylmethionine decarboxylase mRNA: dependence on translation and coding capacity of the *cis*-acting upstream open reading frame. J. Biol. Chem. **268:**726–731.
12. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eukaryotic ribosomes. Mol. Cell. Biol. **7:**3438–3445.
13. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
14. **Kozak, M.** 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. J. Cell Biol. **115:**887–903.
15. **Lodish, H. F., and M. Jacobsen.** 1972. Regulation of hemoglobin synthesis. Equal rates of translation and termination of α- and β-globin chains. J. Biol. Chem. **247:**3622–3629.
16. **Lovett, P. S.** 1990. Translational attenuation as the regulator of inducible *cat* genes. J. Bacteriol. **172:**1–6.
17. **Meerovitch, K., and N. Sonenberg.** 1993. Internal initiation of picornavirus RNA translation. Semin. Virol. **4:**217–227.
18. **Palmiter, R. D.** 1974. Magnesium precipitation of ribonucleoprotein complexes. Expedient techniques for the isolation of undergraded polysomes and messenger ribonucleic acid. Biochemistry **13:**3606–3615.
19. **Parola, A., and B. K. Kobilka.** 1994. The peptide product of a 5′ leader cistron in the β_2 adrenergic receptor mRNA inhibits receptor synthesis. J. Biol. Chem. **269:**4497–4505.
20. **Schleiss, M., C. R. Degnin, and A. P. Geballe.** 1991. Translational inhibition mediated an upstream reading frame within the cytomegalovirus gp48 gene transcript leader. J. Virol. **65:**6782–6789.
21. **Werner, M., A. Feller, F. Messenguy, and A. Pierard.** 1987. The leader peptide of yeast gene cpal is essential for the translational repression of its expression. Cell **49:**805–813.
22. **Wolin, S. L., and P. Walter.** 1988. Ribosome pausing and stacking during translation of a eukaryotic mRNA. EMBO J. **7:**3559–3569.