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Initiation of Protein Synthesis from the A Site of the Ribosome

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Summary

Positioning of the translation initiation complex on mRNAs requires interaction between the anticodon of initiator Met-tRNA, associated with eIF2-GTP and 40S ribosomal subunit, and the cognate start codon of the mRNA. We show that an internal ribosome entry site located in the genome of cricket paralysis virus can form 80S ribosomes without initiator Met-tRNA, eIF2, or GTP hydrolysis, with a CCU triplet in the ribosomal P site and a GCU triplet in the A site. P-site mutagenesis revealed that the P site was not decoded, and protein sequence analysis showed that translation initiates at the triplet in the A site. Translational initiation from the A site of the ribosome suggests that the repertoire of translated open reading frames in eukaryotic mRNAs may be greater than anticipated.

Introduction

Initiation of translation of most mRNAs in eukaryotic cells is accomplished by a scanning process in which ribosomal 40S subunits, carrying eIF3 and the eIF2-GTP/Met-tRNA$_i$ complex, are recruited at or near the 5′ end of the capped mRNAs (Merrick and Hershey, 1996). Subsequently, 40S complexes are postulated to processively scan the mRNA in a 5′ to 3′ direction until an appropriate start codon (usually an AUG, or less commonly a GUG or CUG triplet) is correctly positioned in the P site of the 40S ribosomal subunit. Then, eIF5 promotes GTP hydrolysis which is required for the dissociation of eIF2 from the 40S subunit. Finally, in the presence of eIF5B, 60S ribosomal subunits join to form an 80S ribosome (Merrick and Hershey, 1996; Gingras et al., 1999; Pestova et al., 2000).

An alternate mechanism is the direct binding of 40S subunits to so-called internal ribosome entry sites (IRES) in eukaryotic mRNAs (reviewed in Jackson, 1996). Initially discovered to be located in the 5′ noncoding regions of picornavirus RNAs, IRES elements have been detected in cellular mRNAs as well (reviewed in Sachs et al., 1997; Johannes et al., 1999). Picornavirus-like IRES elements recruit functional 48S ribosomal complexes by an as yet unknown mechanism which requires all canonical initiation factors necessary for the scanning mechanism, with the exception of eIF1, eIF1A, and the cap binding protein eIF4E (Pestova et al., 1996). In contrast, the IRES elements in the hepatitis C and classical swine fever RNA viral genomes require only eIF2-GTP/Met-tRNA$_i$ and eIF3 for the assembly of functional 48S ribosomes (Pestova et al., 1998b).

The recent sequencing of several insect viral RNA genomes revealed these viral mRNAs to be dicistronic; an upstream open reading frame (ORF) encoding viral nonstructural genes is separated by an intergenic region (IGR) from a downstream ORF encoding the structural proteins (Johnson and Christian, 1998; Moon et al., 1998; Sasaki et al., 1998; Nakashima et al., 1999; Domier et al., 2000; Wilson et al., 2000). These IGR elements have been shown to function as IRESs (Sasaki and Nakashima, 1999; Domier et al., 2000; Wilson et al., 2000). Curiously, the IGR did not contain an AUG, CUG, or GUG triplet that could be used as the start site for the synthesis of the structural proteins, implying an unusual mechanism of translational initiation. Recently, Sasaki et al. (Sasaki and Nakashima, 2000) have shown that *Plautia stali intestine* virus (PSIV) IGR-IRES directs translation initiation at a CAA codon in the absence of Met-tRNA$_i$. Here, we show that the cricket paralysis virus (CrPV) IGR-IRES can assemble 80S ribosomes from purified 40S and 60S ribosomal subunits in the absence of eIF2, Met-tRNA$_i$, or GTP hydrolysis and without a coding triplet in the ribosomal P site. Addition of tRNA to the ribosomal A site results in translocation of the 80S ribosome, suggesting that translation can commence at the A site of the ribosome by a hitherto unprecedented mechanism.

Results

Assembly of 40S Complexes on the IGR-IRES

Protein sequence analysis has revealed that the N-terminal alanine in the structural protein precursor of CrPV is encoded by GCU$_{6217–6219}$ (Wilson et al., 2000). Mutational analyses have indicated that both this GCU codon and the preceding CCU triplet (Figure 1A), which is part of an inverted repeat sequence element (Figure 1B), are important for IGR-IRES activity (Wilson et al., 2000). To determine the roles of the GCU and CCU triplets in translation initiation, the locations of these triplets within bound ribosomal 40S subunits were determined by toeprinting assays.

As expected (Pestova et al., 1996), 40S subunit binding to the well-studied encephalomyocarditis virus (EMCV) IRES required a variety of eukaryotic initiation factors (eIFs) and Met-tRNA$_i$, and resulted in two strong toeprints (Figure 2A, lane 2), indicating that AUG$_{834–836}$ or AUG$_{826–828}$ of the EMCV IRES occupied the ribosomal P site. The compound edeine, which is known to interfere with AUG codon recognition by 40S-eIF2-GTP/Met-tRNA$_i$ complexes (Kozak and Shatkin, 1978; Odom et al., 1978) inhibited the binding of 40S subunits to the EMCV IRES (Figure 2A, lane 3). In contrast, 40S subunits could bind the IGR-IRES RNA in the absence of all factors (Figure 2A, lane 6), a scenario reminiscent of 40S recruitment by the hepatitis C-viral (HCV) IRES (Pestova

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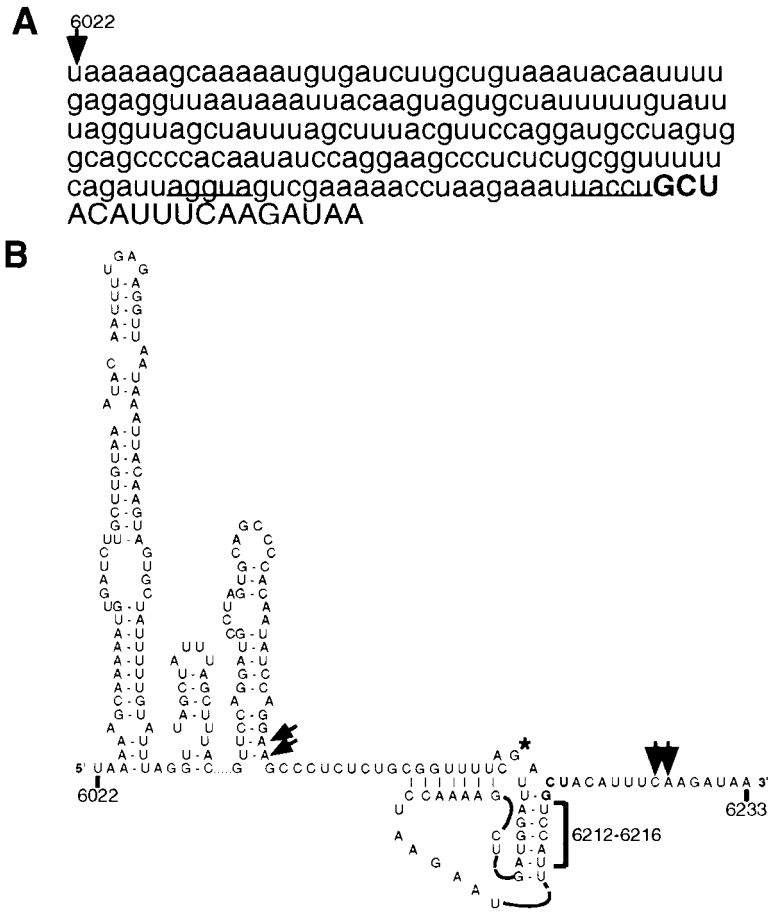

Figure 1. Nucleotide Sequence and Predicted Folding of the IGR and the First 17 Nucleotides of the Coding Region of the Viral ORF2

(A) IGR nucleotide sequence (nucleotides 6022–6216; GenBank accession number AF218039). Stop codon $UAA_{6022-6024}$ of ORF1 is indicated in italics. Coding sequences are indicated in uppercase letters, inverted repeat sequences are underlined, and the predicted A-site GCU triplet of the viral ORF2 is marked in bold.

(B) Secondary structure for the IGR was predicted by alignment with putative helices in *Drosophila* C virus, *Plautia stali intestine* virus, *Rhopalosiphum padi* virus, and *Himetobi P* virus intergenic regions (Johnson and Christian, 1998; Moon et al., 1998; Sasaki et al., 1998; Nakashima et al., 1999). Inverted repeat sequences are indicated with brackets, and the A-site GCU triplet is marked in bold. The arrows indicate the positions of the primary toeprints observed upon 40S ribosomal subunit binding, and an asterisk that of an additional toeprint seen in the presence of 40S and 60S subunits (see Figure 2).

et al., 1998b). 40S subunit binding produced predominant toeprints at nucleotides C_{6226} and A_{6227} and at A_{6161} and A_{6162} (Figure 2A, lanes 6 and 7). These specific toeprints were caused by bound 40S subunits, because they were not detected when assays were performed on naked RNA (Figure 2A, lane 5) or in the presence of factors omitting 40S subunits (Figure 2B, lane 6). The presence of the $CA_{6226–6227}$, but not the $AA_{6161–6162}$ toeprint correlated with the ability of the IRES to mediate translation initiation, because both the functional IGRmut17 and the inactive IGRmut16 (see Figure 4) variants produced a toeprint at $AA_{6161–6162}$, while a toeprint at $CA_{6226–6227}$ was seen only with IGRmut17 (data not shown). Curiously, binding of 40S subunits to the IGR-IRES was not inhibited by edeine, providing a first hint that positioning of 40S subunits on the EMCV- and IGR-IRES elements is accomplished by different mechanisms.

The nucleotides in an mRNA that occupy the P and the A site of the ribosome can be inferred from the toeprint pattern. For example, 48S complexes, composed of HCV-IRES and 40S subunits, carrying initiator $tRNA^{met}$/eIF2-GTP complexes, arrest primer extension 15 to 17 nucleotides downstream of the AUG start codon located in the ribosomal P site (Pestova et al., 1998b). By convention, the A of the AUG is designated as the "+1" position. However, binary 40S/HCV-IRES complexes reveal a toeprint ~14 nucleotides downstream of the P-site AUG, suggesting that reverse transcriptase can penetrate the leading edge of the 40S ribosome a few nucleotides further when the P site is not occupied by a tRNA molecule (Pestova et al., 1998b). The predominant toeprints observed in 40S/IGR-IRES complexes at C_{6226} and A_{6227} are 13 and 14 nucleotides downstream of the $CCU_{6214–6216}$ triplet, suggesting that in binary 40S/IGR-IRES RNA complexes the $CCU_{6214–6216}$ is positioned in the P site and $GCU_{6217–6219}$ in the A site. However, unlike in 40S/HCV-IRES complexes (Pestova et al., 1998b), inclusion of Met-$tRNA_i$ and translation initiation factors eIF2, 3, 4A, 4B, 4F, 1, or 1A did not alter the pattern of arrest of primer extension on 40S/IGR-IRES complexes; in fact, addition of any or all of these factors in the binding reaction significantly reduced 40S subunit binding to the IGR (Figure 2B, lanes 3–5).

Effect of eIF2 on IGR-IRES Translation

To further examine the inhibitory effects of initiation factors on IGR-IRES-mediated translation, we performed cell- free translation assays in the presence of various amounts of eIF2. Addition of eIF2 to the rabbit reticulocyte lysate (RRL) had no effect on the translational efficiency of the first Rluc-encoding cistron, indicating that eIF2 is not limiting in the RRL; in contrast, addition of eIF2 diminished IGR-dependent translation of Fluc (Figure 2C). Together with the toeprinting data in Figure 2B, this result suggests that translation initiation mediated by the IGR-IRES has no or only a low requirement for eIF2.

Assembly of 80S Monosomes on the IGR-IRES from Purified 40S and 60S Subunits

Formation of 80S initiation complexes requires the participation of eIF2-GTP/Met-$tRNA_i$, eIF3, eIF5, and eIF5B

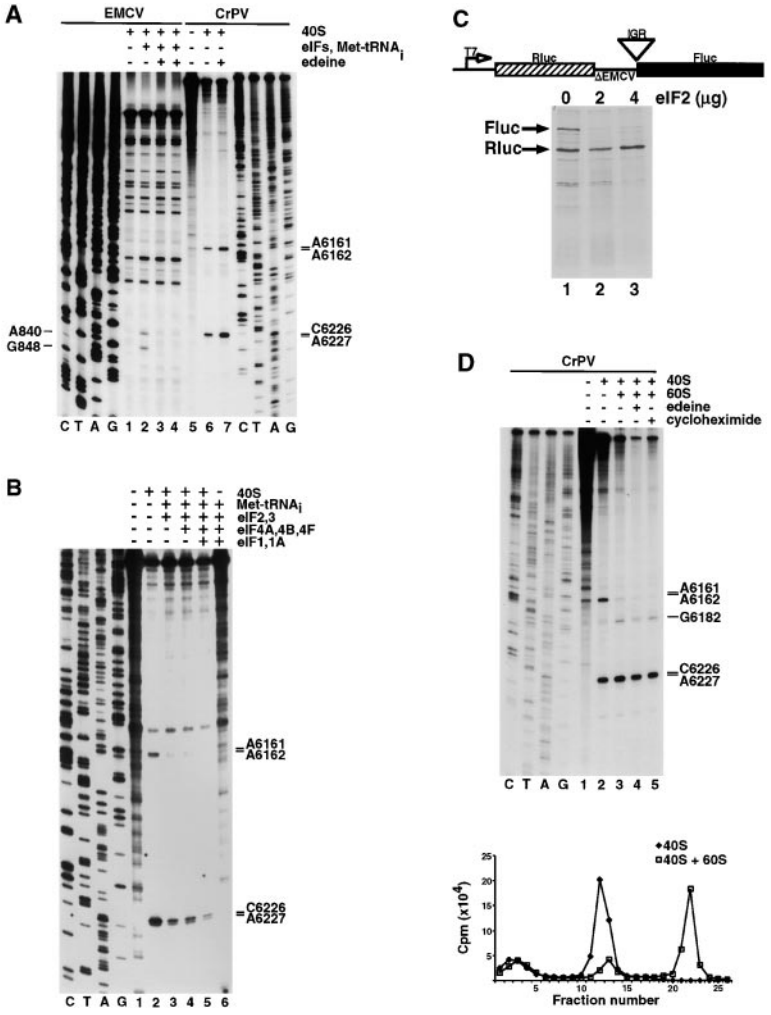

Figure 2. Analysis of Ribosomal Subunit Binding to IRES Elements

(A) 40S subunit binding to EMCV- (lanes 1–4) and IGR-IRES (lanes 5–7) RNAs. The positions of 40S-induced toeprints on EMCV-IRES or IGR-IRES in the absence or presence of eIFs (eIF2, eIF3, eIF4A, eIF4B, eIF4F, eIF1, and eIF1A) and 10 μM edeine (lane 3, added to the reaction without preincubation; lane 4, added after preincubation for 5 min at 37°C) are shown. Sequencing ladders of the EMCV- and IGR-IRESs are shown at far left and right, respectively. The positions of 40S-dependent, major toeprints are indicated.

(B) Effects of initiation factors on 40S subunit binding to IGR-IRES RNA. Shown are patterns of arrest of primer extension on IGR-IRES RNA in the presence of various translation components. The positions of the major toeprints seen upon 40S subunit binding to IGR-IRES RNA are indicated to the right. A sequencing ladder is shown in the four lanes at left.

(C) Factor eIF2 inhibits translation mediated by the IGR-IRES. ^{35}S-labeled products of in vitro translated dicistronic RNAs. Increasing amounts of eIF2 were added to 15 μl translation reactions each containing 0.3 μg of RNA. Diagram of dicistronic RNA is shown at the top: promoter for T7 RNA polymerase (T7), coding regions for *Renilla* luciferase (Rluc) and firefly luciferase (Fluc), a landscape of RNA structures (ΔEMCV), and the location of IGR-IRES insertion (IGR) are indicated. Autoradiographs of polyacrylamide gels are shown in this figure.

(D) Assembly of IGR-IRES/80S monosomes from purified subunits. Patterns of arrest of primer extension on IGR-IRES RNA in the presence of 40S or 60S subunits are shown. Edeine (10 μM) or cycloheximide (500 μg/mL) were added as indicated. The positions of the major toeprints at nucleotides 6161–6162, 6182, and 6226–6227 are indicated on the right. A sequencing ladder is shown at left. At the bottom: sucrose gradient analysis of IGR-IRES RNA/ribosome complexes. Sedimentation profiles of radiolabeled IGR-IRES RNA incubated with 40S subunits alone (solid diamonds) or with 40S and 60S subunits (open boxes) are shown. The top of the gradient is at left, and the cpm in each gradient fraction is displayed.

(Pestova et al., 2000) and hydrolysis of GTP (Merrick and Hershey, 1996). Because of the inhibitory effects of eIF2 on IGR-IRES-mediated translation, we examined the possibility that 80S monosomes could form on the IGR-IRES in the absence of any initiation factor. Purified 40S subunits or a combination of 40S and 60S subunits were mixed with the IGR-IRES and their binding sites on the IGR-IRES examined by toeprinting. Figure 2D shows that the 40S/IGR-IRES toeprint at $AA_{6161–6162}$ was weakened by the addition of 60S subunits, concomitant with the appearance of a new toeprint at G_{6182}. However, the toeprint at $CCU_{6214–6216}$, which is indicative of $CCU_{6214–6216}$ occupying the ribosomal P site, was not affected by the addition of 60S subunits, edeine or the elongation inhibitor cycloheximide. Because the position of arrest of primer extension at the leading edges of 40S/mRNA and 80S/mRNA complexes is indistinguishable (Anthony and Merrick, 1992) (see also Figure 5A, lanes 2 and 3), sucrose gradient centrifugation was used to examine whether 80S monosomes were formed upon incubation of purified 40S and 60S subunits with the IGR-IRES. Remarkably, Figure 2D (bottom panel) shows that 80S monosomes were assembled on the IGR-IRES from purified subunits. The subunit preparations were not contaminated with eIFs, as 80S/mRNA complexes were not formed on the EMCV IRES (data not shown). These findings indicate that the IGR-IRES can assemble 80S monosomes from purified subunits in the absence of any eIFs, tRNAs or GTP, with $CCU_{6214–6216}$ in the ribosomal P site. To further substantiate this notion, we tested more rigorously the requirements for GTP and initiator tRNA molecules in formation of 80S/IGR-IRES complexes in the RRL.

Assembly of 80S Complexes on the IGR-IRES in the RRL

The factor requirements for the assembly of 80S complexes on the EMCV-IRES and the IGR-IRES were examined by sedimentation analysis. Both radiolabeled EMCV-IRES and IGR-IRES elements migrated in the top fractions of the gradient in the absence of translation extract (Figures 3A and 3F). Incubation of the RNAs in the RRL in the presence of cycloheximide resulted in the

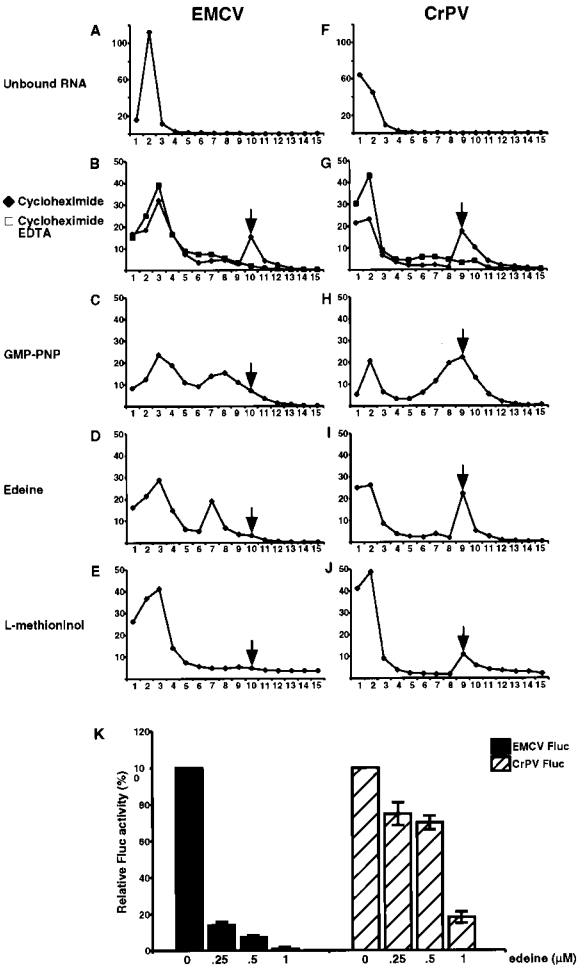

Figure 3. Sucrose Gradient Analysis of Translation Initiation Complexes Formed with IRES Elements and Translation Assays in the RRL

(A and F) Sedimentation profiles of radiolabeled EMCV- or IGR-IRES RNAs without incubation in the RRL.

(B and G) RNAs incubated in the RRL pretreated with cycloheximide, then treated with either 15 mM $MgCl_2$ (solid diamonds) or 10 mM EDTA (open boxes).

(C and H) RNAs incubated in RRL pretreated with 5 mM GMP-PNP and cycloheximide.

(D and I) RNAs incubated in the RRL pretreated with 2.5 μM edeine and cycloheximide.

(E and J) RNAs incubated in the RRL pretreated with 5 mM L-methioninol and cycloheximide. The cpm ($\times 10^3$) in each gradient fraction is displayed. In all panels, the top of the gradient is at left. The position of the 80S peak is indicated with an arrow.

(K) Quantitation by enzymatic assay of the translation products of in vitro-transcribed, monocistronic IRES-containing luciferase RNAs. These results are the average of three or more independent experiments; bars indicate standard error.

formation of 80S monosomes that readily dissociated in the presence of EDTA (Figures 3B and 3G). The slightly slower sedimentation of 80S/IGR-IRES complexes was reproducible, possibly reflecting a structural difference of the 80S/IGR-IRES complex relative to other 80S/mRNA complexes.

GTP is required for the delivery of initiator tRNA/eIF2 complexes to the ribosome, the joining of 60S to the 48S complex (Merrick and Hershey, 1996; Gingras et al., 1999), the release of eIF5B to form an active 80S ribosome (Pestova et al., 2000), and in translation elongation (Merrick and Hershey, 1996). Addition of GMP-PNP to the translation reactions abolished the formation of 80S/EMCV IRES complexes (Figure 3C), as predicted (Gray and Hentze, 1994), and the EMCV-IRES sedimented as a 48S initiation complex. In contrast, most of the IGR-IRES was detected in 80S/IGR-IRES complexes in the presence of GMP-PNP (Figure 3H). Thus, 60S can join 40S/IGR-IRES complexes without GTP hydrolysis; or the IGR-IRES directly recruits preformed 80S ribosomes.

The compound edeine has been shown to interfere with AUG codon recognition by 40S-eIF2-GTP/Met-$tRNA_i$ complexes (Kozak and Shatkin, 1978; Odom et al., 1978). Thus, edeine should have minimal effect on 80S assembly on the IGR-IRES. As expected, edeine severely inhibited the formation of 80S complexes on the EMCV-IRES (Figure 3D). In contrast, edeine had no effect on the assembly of 80S complexes on the IGR-IRES (Figure 3I); a finding predicted by the data shown in Figures 2A and 2D. Addition of EDTA to the reaction mixture abolished formation of the 80S complexes (not shown), indicating that this fraction contained 80S monosomes and not dimers of 40S subunits. This finding suggests that a P-site codon-anticodon interaction is not involved in the formation of 80S/IGR-IRES complexes, further supporting that initiation on the IGR-IRES occurs without Met-$tRNA_i$ and eIF2.

Finally, the pool of Met-$tRNA_i$ in the RRL was depleted by addition of L-methioninol, a substrate analog inhibitor of Met-tRNA synthetase (Kozak and Shatkin, 1978; Murali et al., 1997). Clearly, the assembly of 80S complexes was largely abolished on the EMCV-IRES in the presence of L-methioninol (Figure 3E). In contrast, 80S assembly on the IGR-IRES was only slightly reduced under this condition (Figure 3J).

Formation of 80S monosomes on the IGR-IRES in the presence of GMP-PNP, edeine, and L-methioninol raised the question of whether the observed 80S complexes were functional in translation. Thus, we tested the effects of edeine on EMCV-IRES– and IGR-IRES–mediated translation of luciferase reporter mRNAs edeine (Figure 3K). The translation of luciferase mRNAs mediated by an EMCV IRES leader was inhibited by 90% in the presence of 0.5 μM; in contrast, IGR-IRES luciferase mRNAs were translated in the presence of 0.5 μM edeine at ~70% of the level seen in the absence of edeine. Even in the presence of 1 μM edeine, the IGR-IRES was translated ~10-fold more efficiently than the EMCV IRES. However, edeine displayed a general inhibitory effect at 1 μM, a concentration at which edeine is known to inhibit elongation (Szer and Kurylo-Borowska, 1970; Carrasco et al., 1974). These findings indicate that translation-competent 80S monosomes can assemble on the IGR-IRES in the absence of eIF2-GTP/Met-$tRNA_i$ complexes.

Sequence Analysis of IGR-IRES Translation Products

The CCU triplet in the ribosomal P site could either be decoded by $tRNA^{pro}$ or serve some other function. Sequence analysis of translation products initiated at the IGR-IRES revealed that the N-terminal residue of the ORF2 was alanine, encoded by $GCU_{6217–6219}$ (Wilson

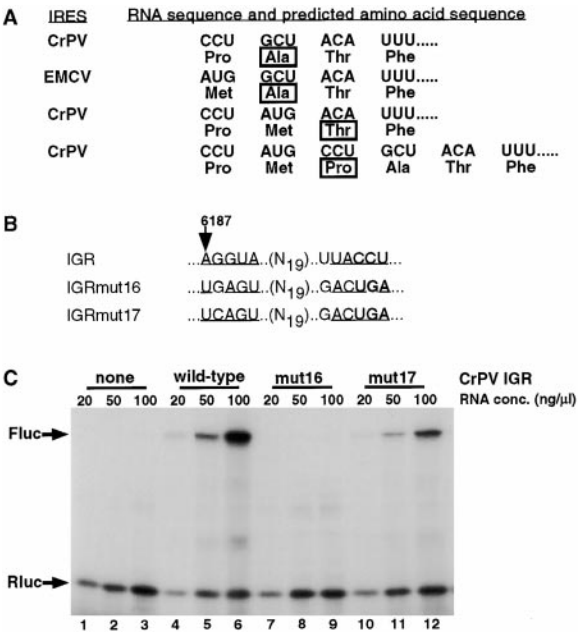

Figure 4. Translation Initiation from the Ribosomal A Site

(A) Proteins were translated in the RRL and purified using an anti-flag M2 affinity matrix (Sigma). N-terminal sequences were determined by mass spectrometry. The N-terminal amino acids are boxed.

(B) Description of IGR-IRES mutants in which the CCU P-site triplet has been replaced with a UGA stop codon. P-site triplets are indicated in bold, complementary sequences are underlined.

(C) Translation of uncapped dual luciferase RNAs (see Figure 2C) in the presence of ^{35}S-methionine in the wheat germ extract. RNAs' concentrations were as follows: 20 ng/μl (lanes 1, 4, 7, and 10); 50 ng/μl (lanes 2, 5, 8, and 11); and 100 ng/μl (lanes 3, 6, 9, and 12). An autoradiograph of the gel is shown.

et al., 2000) (Figure 4A). To test for the presence of methionine N-aminopeptidase or proline N-iminopeptidase (Walter et al., 1980) activities in the RRL, proteins were synthesized from various IGR-IRES mRNAs, and their N-terminal amino acids determined (Figure 4A). Protein products translated from control EMCV IRES-ORF2 mRNAs, which initiate at a P-site AUG, contained an N-terminal alanine, indicating that methionine N-aminopeptidases were active in the RRL. Proteins synthesized from IGR-IRES mRNAs in which the GCU in the A site was replaced with an AUG triplet contained an N-terminal threonine. This result suggests two hypotheses: either the A-site-encoded-methionine was the N-terminal amino acid and was removed by methionine N-aminopeptidases, or the translated sequence was N-proline-methionine-threonine, and both the proline and methionine were removed. To distinguish between these possibilities, IGR-IRES mRNAs were translated in which CCU was in the P site and AUG in the A site, followed by a proline-encoding CCU and an alanine-encoding GCU triplet. N-terminal sequence analysis revealed that proline and alanine were the first two amino acids in the peptide, indicating that proline N-iminopeptidases were not active in the RRL. These results suggest that the P-site CCU triplet is not decoded, and furthermore that the N-terminal amino acid was provided by the tRNA that entered the ribosomal A site.

Translation Initiation with a Stop Codon in the P Site

If the P site is not decoded, the presence of a stop codon in the P site should not abolish IRES activity, provided that the complementarity of the inverted repeat (Figure 1), which has been shown to be required for IRES activity (Wilson et al., 2000), is maintained. To test this idea, IGRmut17 was constructed in which sequences $UUACCU_{6211–6216}$, containing the P-site CCU, were replaced by $GACUGA_{6211–6216}$, containing a UGA stop codon in the P site. To preserve complementary between the inverted repeat, nucleotides $AGGUA_{6187–6191}$ were also changed to $UCAGU_{6187–6191}$ (Figure 4B). IGRmut16 was identical to IGRmut17 except for one nucleotide in the upstream half of the inverted repeat (G_{6188}; Figure 4B), to disrupt the complementarity of the inverted repeat. As shown in Figure 4C, IGRmut17 was a functional IRES, mediating translation of the second Fluc cistron in a dicistronic reporter mRNA, while IGRmut16-containing mRNAs were unable to synthesize Fluc. Quantitation of these results by enzymatic assay revealed that the IGRmut17 IRES activated second cistron translation 52-fold relative to a the IGRmut16 IRES and was approximately one-third as active as the wild-type IGR at the highest RNA concentration tested (data not shown). Moreover, toeprinting analysis revealed that the UGA in the IGRmut17 IRES occupied the ribosomal P site, but no toeprint was detected at this position with IGRmut16 (not shown). Therefore, the IGRmut17 can direct translation initiation with a stop codon in the ribosomal P site, strongly suggesting that the P site in the IRES is not decoded.

Translocation of the Initiating 80S Ribosome on the IGR-IRES

The finding that the IGR-IRES mediates translation initiation with nondecoding $CCU_{6214–6216}$ in the P site and alanine-encoding $GCU_{6217–6219}$ in the A site implies that the first 80S translocation event occurs in the absence of peptide bond formation. To examine this initial translocation event in more detail, we compared the first ribosomal translocation step on EMCV-IRES and IGR IRES elements in the RRL by toeprint analyses.

Edeine interferes with AUG codon recognition by 40S-eIF2-GTP/Met-$tRNA_i$ complexes (Kozak and Shatkin, 1978; Odom et al., 1978). Thus, not surprisingly, incubation of EMCV-IRES-containing RNAs in the RRL in the presence of edeine failed to reveal a specific toeprint indicative of start codon AUG_{834} occupation of the ribosomal P site (Figure 5A). Cycloheximide inhibits the function of elongation factor EF2 which mediates translocation of peptidyl-tRNA from the A site to the P site, thereby stalling translation elongation (Merrick and Hershey, 1996). Addition of cycloheximide to the translation reaction led to formation of EMCV-IRES/80S complexes (Figure 3B) that displayed a toeprint pattern, indicating that the $AUG_{834–836}$ start codon, located 15 to 17 nucleotides upstream of the leading edge of the toeprint, was positioned in the P site (Figure 5A, lane 2) (Pestova et al., 1996). A similar, weaker toeprint was noted when reactions were performed in the presence of GMP-PNP (Figure 5A, lane 3), suggesting that the $AUG_{834–836}$ start codon was positioned in the P site in EMCV-IRES/48S complexes (Figure 3C). Thus, these compounds can be used to determine which mRNA sequences occupy ribosomal P and A sites in 48S and 80S initiation complexes.

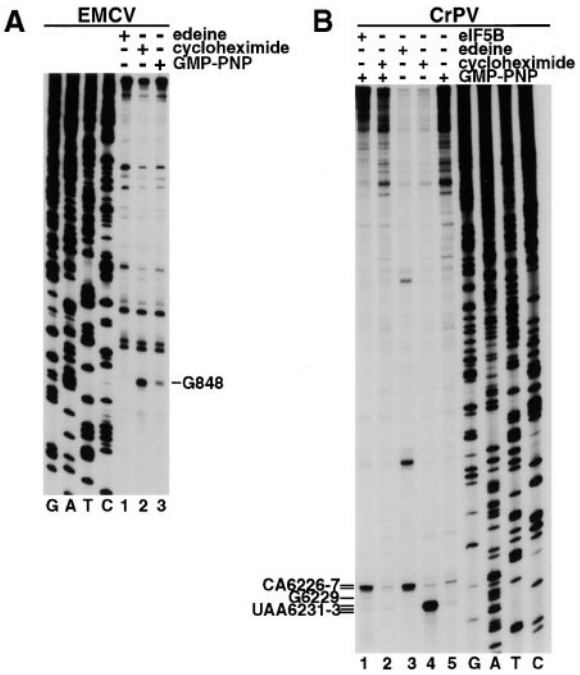

Figure 5. Toeprint Analyses of Translation Initiation Complexes Formed on EMCV- and IGR-IRES Elements in the RRL

(A) Patterns of arrest of primer extension on the EMCV-IRES in the presence of 10 μM edeine (lane 1), 500 μg/mL cycloheximide (lane 2), or 1 mM GMP-PNP (lane 3) are shown. The toeprint at position 848 (numbering as in Figure 2A) is indicated.

(B) Patterns of arrest of primer extension on the IGR-IRES in the presence of 1 mM GMP-PNP plus 3 μg eIF5B (lane 1), 1 mM GMP-PNP plus 500 μg/mL cycloheximide (lane 2), 10 μM edeine (lane 3), 500 μg/mL cycloheximide (lane 4), or 1 mM GMP-PNP (lane 5). In addition to the toeprints at positions 6226–6227 previously detected in experiments with purified components (see Figures 2A and 2D), a strong toeprint was observed at nucleotides 6231–6233 in the presence of cycloheximide (lane 4). Weak toeprints at these latter positions were also seen in the presence GMP-PNP (lanes 1, 2, and 5). An additional weak toeprint at position 6229 was detected in the presence of GMP-PNP and cycloheximide (lanes 1, 4, and 5). The positions of the toeprints are indicated at left. A sequencing ladder is shown at right. Autoradiographs of polyacrylamide gels are shown.

As seen with purified ribosomal subunits (Figure 2D), a prominent toeprint was observed at $CA_{6226–6227}$ when IGR-IRES RNA was incubated in the RRL in the presence of edeine (Figure 5B, lane 3). Because 80S complexes form on the IGR-IRES in the presence of edeine (Figure 3I), this toeprint indicates that $CCU_{6214–6216}$ was positioned in the ribosomal P site of 80S complexes (Figure 7). Edeine at high concentrations, as used in these experiments, has been shown to inhibit the interaction of amino-acylated tRNA with the ribosomal A site (Szer and Kurylo-Borowska, 1970; Carrasco et al., 1974); thus, these 80S complexes likely contain an empty A site which may explain the extent of penetration of the leading edge of the ribosome by reverse transcriptase during the primer extension reaction. Two additional toeprints in the 5′ region of the IGR-IRES were noted in the edeine-containing translation reactions; one of which corresponds exactly to the G_{6182} toeprints seen on the IGR in the presence of purified 40S and 60S subunits (Figure 2D). Very surprisingly, three sets of toeprints were observed when translation reactions contained cycloheximide (Figure 5B, lane 4). A weak toeprint at $CA_{6226–6227}$ corresponds to the one seen in the presence of edeine (see above), and in the reconstituted system where formation of 80S monosomes from purified 40S and 60S subunits was examined (Figure 4A). A very prominent toeprint was also observed at $UAA_{6231–6233}$. Because the codon located in the P site is typically 15–17 nucleotides upstream of the toeprint when tRNA is present in both the ribosomal A and P sites (Anthony and Merrick, 1992), this result indicates that, in the RRL in the presence of cycloheximide, $GCU_{6217–6219}$ primarily occupies the P site. Thus, the toeprint at $UAA_{6231–6233}$ is likely to reflect an arrested ribosome which has undergone a pseudotranslocation event, i.e., translocation without peptide bond formation (Figure 7). In addition, a very weak toeprint was seen at G_{6229} (Figure 5B, lanes 1 and 4). Because of its position, this toeprint likely reflects 80S/IGR-IRES complexes with $CCU_{6214–6216}$ in the P site and an ala-tRNA bound to the A site $GCU_{6217–6219}$. This complex is transient, suggesting that once a tRNA occupies the A site, pseudotranslocation occurs rapidly. An analogous "processive" toeprint was not observed with EMCV-IRES RNA or any other mRNA tested so far (Figure 5A and data not shown).

Translation reactions with the IGR-IRES in the presence of GMP-PNP or GMP-PNP and cycloheximide yielded toeprints at $CA_{6226–6227}$, G_{6229}, and $UAA_{6231–6233}$ (Figure 5B, lanes 5 and 2). The $UAA_{6231–6233}$ toeprints were much less prominent than those seen without GMP-PNP (Figure 5B, lane 4). Together with results from sucrose gradient analysis (Figure 3H), these data suggest that 80S ribosomes can assemble on the IGR-IRES and undergo, at a reduced extent, an initial translocation event in the presence of GMP-PNP (Figure 7). Furthermore, the toeprints on the IGR-IRES seen in the presence of GMP-PNP or cycloheximide were strikingly different (Figure 5B, lanes 4 and 5). This was not the case for the EMCV IRES, on which the toeprints were identical under these conditions (Figure 5A, lanes 2 and 3). Thus, in the presence of cycloheximide, 80S ribosomes can move on the IGR IRES in a way that has not been observed for any other examined mRNA.

It was predicted that the pseudotranslocation step could be inhibited by excess eIF5B in the presence of GMP-PNP, because in the presence of GMP-PNP, eIF5B binds stably to 80S ribosomes (most likely to the ribosomal A site) (Pestova et al., 2000) and, therefore, could compete with EF1, which delivers aminoacylated tRNA to the A site. Indeed, supplementing the translation reaction with both GMP-PNP and excess eIF5B produced a strong toeprint at $CA_{6226–6227}$, and toeprints at G_{6229} and $UAA_{6231–6233}$ were weaker than the corresponding toeprints in the presence of GMP-PNP alone (compare Figure 5B, lanes 1 and 5), suggesting that pseudotranslocation was impaired. Finally, addition of sparsomycin, an inhibitor of peptide bond formation which strengthens the interaction of aminoacylated tRNA in the A site with the ribosome (Lazaro et al., 1991), produced a predominant toeprint at $CA_{6226–6227}$, indicating that pseudotranslocation was inhibited (data not shown).

Translation during the Unfolded Protein Response

Translation initiation from the A site of the ribosome without requirement for known eukaryotic initiation factors, including initiator tRNA, suggests that the IGR-IRES should function at times when translation initiation factors are limiting. Thus, we examined the translational

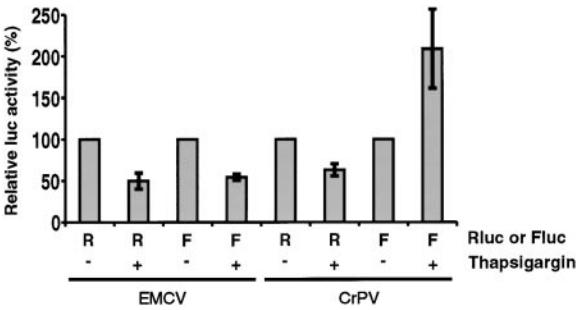

Figure 6. IGR-IRES Mediated Translation in Mammalian Cells Is Enhanced by Thapsigargin Treatment

Quantitation by enzymatic assay of Rluc (R) and Fluc (F) activities in extracts from untreated cells (−) or cells pretreated with 1 μM thapsigargin (+), followed by transfection with capped dual luciferase RNAs (see Figure 2C) containing either the EMCV- or IGR-IRES as indicated. Results are the average of three independent experiments; bars indicate standard error.

activity of the IGR-IRES in cultured cells at times when translation initiation was inhibited as a result of phosphorylation of eIF2 (reviewed in Kaufman, 1999). Phosphorylation of eIF2, which results in the rapid depletion of eIF2-GTP/Met-tRNA$_i$ complexes and cessation of protein synthesis, can be induced by accumulation of incorrectly folded proteins in the endoplasmic reticulum, resulting in the activation of the eIF2-kinase PERK (Shi et al., 1998; Harding et al., 1999). An unfolded protein response in COS7 cells was induced by treatment with thapsigarin (Wong et al., 1993; Harding et al., 1999), and the effects on 5′ cap-dependent and IRES-mediated translation were monitored after transfection of various dicistronic mRNAs (Figure 6). 5′ cap-dependent translation of the first cistron, Rluc, was reduced by thapsigargin treatment to ~50%–60% of the levels seen in untreated cells. Similarly, EMCV IRES-mediated translation of the second cistron, Fluc, was reduced by thapsigargin treatment to ~55% of the level seen in untreated cells. In contrast, IGR-IRES-mediated translation of Fluc was enhanced more than 2-fold after thapsigargin treatment. Similar results were obtained when the unfolded protein response was induced with tunicamycin or DTT (data not shown). Therefore, The IGR-IRES mediates efficient translation in cells when eIF2-GTP/Met-tRNA$_i$ complexes are limiting (Shi et al., 1998; Harding et al., 1999).

Discussion

Phylogenetic and mutational analyses of the IGR-IRESs of a group of related insect viral RNA genomes have identified a pseudoknot-like structure, diagrammed in Figure 1B, whose integrity is required for IRES activity (this study, Sasaki and Nakashima, 1999; Domier et al., 2000; Wilson et al., 2000). In the present study, toeprint analysis has shown that ribosomes bind the IGR-IRES directly at the pseudoknot, such that a CCU is positioned in the ribosomal P site and GCU in the A site. Change of the P-site CCU triplet to a UGA stop codon did not abolish IRES activity if the pseudoknot-like structure is maintained, indicating that the P-site CCU codon is not decoded and that P-site codon-tRNA anticodon interactions are not involved in translation initiation. These findings predict that a novel, unusual mechanism operates in translation initiation mediated by the IGR-IRES.

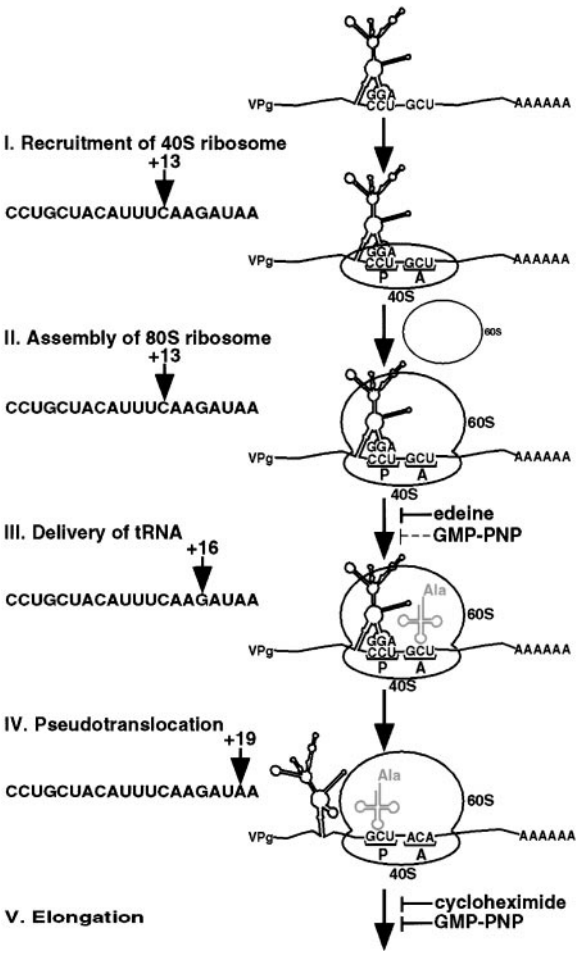

Figure 7. Model for IGR-IRES-Mediated Translation Initiation

In this model, ribosomal subunits are recruited directly to the IGR-IRES. Tertiary interactions between the CCU P-site triplet and upstream sequences in the IGR substitute for the usual interaction between Met-tRNA$_i$ and the P-site initiator codon, and no charged tRNA occupies the P site. A charged tRNA^{ala} is recruited to the A site, presumably through standard codon:anticodon interactions. Subsequently, a pseudotranslocation event occurs in the absence of peptide bond formation, placing the GCU codon and tRNA^{ala} in the P site and an ACA triplet into the ribosomal A site. As determined by toeprint analyses (see Figures 2 and 5), the positions of the ribosome on the IGR-IRES at each step in the pathway is summarized at left. For simplicity, only nucleotides 6214–6233 of the IGR-IRES are shown (numbering as in Figure 1), and only the strongest toeprint defining each step in this pathway is indicated, with the number above each arrow showing the position of the toeprint relative to C_{6214}. The steps which are blocked by various inhibitors are also indicated. The viral mRNA with genome-linked protein, VPg, 40S and 60S ribosomal subunits, and ribosomal P and A sites are indicated.

Mechanism of Formation of 80S/IGR-IRES Complexes

In the first step (Figure 7), the IGR-IRES binds, without eIFs or initiator tRNA, purified 40S subunits to form a binary complex. Although the toeprint at position +13 does not fit the expected spacing of +15 to +17 for positioning of the CCU in the ribosomal P site, we suggest that the absence of initiator tRNA leads to further penetration of the leading edge of the 40S subunit by reverse transcriptase. Such a scenario was observed

in toeprinting assays with binary 40S/HCV complexes (Pestova et al., 1998b).

In the second step, purified 60S subunits join the binary 40S/IGR-IRES complexes to form 80S complexes displaying the same +13 toeprint seen in binary complexes. In contrast to other 80S-mRNA complexes, IGR-IRES/80S complexes form without factors eIF3, eIF5, and eIF5B. So far, we have not found any compound that blocks this step in ribosome assembly, consistent with the notion that accessory factors are not required. An important but unresolved question is whether preformed 80S complexes can be recruited directly to the IGR-IRES or whether 40S and 60S subunits bind successively.

The third step involves delivery of tRNA to the empty ribosomal A site, and is indicated by the faint +16 toeprint at G_{6229}. It is generally thought that ternary EF1/tRNA/GTP complexes bind the ribosomal A site, leaving the tRNA in the A site with concomitant hydrolysis of GTP and release of EF1-GDP (Merrick and Hershey, 1996). Thus, it is expected that this step would be inhibited by GMP-PNP. However, this step was only somewhat inhibited by GMP-PNP as +16 and +19 toeprints were observed in translation assays containing GMP-PNP. It is possible that the requirement for the hydrolysis of GTP by EF1 for tRNA delivery to the ribosomal A site depends on whether the P site is empty or occupied by a tRNA molecule. In any case, compounds such as edeine, which block interaction of tRNAs with the A site (Carrasco et al., 1974; Szer and Kurylo-Borowska, 1970), inhibited the +16 and +19 toeprints on IGR-IRES/80S complexes, as did excess GMP-PNP and eIF5B, which can compete with the EF1/tRNA/GMP-PNP complex for binding to the ribosomal A site. The +16 toeprint was very faint, suggesting that once the A site is occupied by tRNA, the ribosome undergoes efficient pseudotranslocation.

The fourth step is a pseudotranslocation event, indicated by a toeprint at $UAA_{6231–6233}$, that moves the GCU into the ribosomal P site without peptide bond formation. If this step were an enzymatic translocation event requiring EF2, it would be inhibited by cycloheximide, which blocks EF2 activity. That pseudotranslocation can occur in the presence of cycloheximide, and to a lesser extent in the presence of GMP-PNP, suggests that the first translocation event without peptide bond formation has different requirements from a first translocation event that results in the formation of a peptide bond.

From studies with prokaryotic ribosomes, it is clear that occupancy of both the P and A sites by tRNAs is essential for in vitro ribosome translocation reactions (Holschuh et al., 1980): while the A site can be occupied by a minimal anticodon stem-loop analog, the P site must be occupied by a full-length tRNA which, however, need not be acylated (Joseph and Noller, 1998). A precedent for tRNA-like RNA molecules that can replace the function of authentic tRNAs exists. 10Sa is a 363 nucleotide tRNA-like molecule in *Escherichia coli* that can be aminoacylated with alanine (reviewed in Muto et al., 1998). 10Sa RNA can enter ribosomal A sites when truncated mRNAs lacking a stop codon are present, and 10Sa-attached alanine is incorporated into the growing polypeptide chain. Ribosomes then decode, by a co-translational switch, additional bases of 10Sa RNA which functions as mRNA.

Translation Initiation without eIF2-GTP/Met-tRNA$_i$ Complex

Control of translation initiation in eukaryotic mRNAs occurs primarily by two mechanisms. One is through the reversible phosphorylation of the eIF4E binding proteins 4E-BPs or of eIF4E itself, which controls the efficiency by which ribosomal subunits can be recruited onto mRNAs (Gingras et al., 1999). Second, through the phosphorylation of eIF2 by several kinases, the amount of eIF2-GTP/Met-tRNA$_i$ complex is controlled; as is, consequently, the number of functional 40S preinitiation complexes (Merrick and Hershey, 1996). It has been well documented that activation of various eIF2 kinases during nutritional deprivation, heat shock, apoptosis, hypoxia, endoplasmic reticulum stress, hemin deficiency, or viral infection (reviewed in Kaufman, 1999) results in inhibition of translation due to eIF2 phosphorylation. We have provided evidence for efficient translation of the IGR-IRES in thapsigargin-treated cells when eIF2 is phosphorylated by PERK kinase (Harding et al., 1999), resulting in inhibition of both cap-dependent translation and EMCV IRES-mediated translation. In the case of the IGR-IRES, viral structural proteins are predicted to be synthesized during times when cells have undergone an antiviral response leading to eIF2 phosphorylation. Clearly, such a scenario would be greatly beneficial for virus propagation.

Finally, we have shown that the IGR-IRES directs translation of a protein whose N-terminal residue is not methionine. This result has important implications for genome sequence analysis as certain cellular gene products may also be initiated at codons other than conventional initiation codons with tRNAs other than Met-tRNA$_i$.

Experimental Procedures

DNA Constructs

The dicistronic luciferase plasmid containing the IGR IRES cDNA has been described (Wilson et al., 2000). Dicistronic plasmids containing the EMCV IRES were generated by PCR amplification of the EMCV IRES cDNA (Chen and Sarnow, 1995) using gene-specific primers encoding EcoRI and NcoI restriction sites. EcoRI/NcoI DNA fragments were ligated into the intercistronic region of the dual luciferase plasmid (Johannes et al., 1999). IGRmut16 and IGRmut17 were generated by PCR using primers carrying the desired mutations, digested with EcoRI and NcoI, and ligated into the dicistronic plasmid as above. Monocistronic luciferase plasmids were generated by inserting EcoRI-BamHI IRES-firefly luciferase containing fragments (Wilson et al., 2000) into the polylinker of pGEM3 (Promega Biotec.).

For amino acid sequence analysis, various IRES-containing cDNAs, carrying 5′ BamHI and 3′ XbaI restriction sites, linked to 18 nucleotides of wild-type or altered ORF2 coding sequences, were generated by PCR and inserted upstream of sequences encoding a Flag tag in pGEM3.

In Vitro Transcription and Translation

Monocistronic and dicistronic luciferase plasmids used in in vitro transcription assays were previously described (Wilson et al., 2000). Uncapped RNAs were translated in the RRL or wheat germ extract in the presence of 154 mM or 130 mM potassium acetate, respectively (Wilson et al., 2000). In edeine inhibition studies, the RRL was preincubated with the indicated concentrations of edeine for 5 min at 30°C before addition of RNA. For eIF2 inhibition studies, 15 μl RRL was preincubated for 5′ at 30°C with 2 or 4 μg eIF2 before the addition of mRNA.

Purification of Factors and Ribosomal Subunits

Ribosomal subunits eIF2, eIF3, eIF4F, and eIF5B were purified from rabbit reticulocyte lysate as described (Pestova et al., 1996, 1998a,

2000). Recombinant eIF1, eIF1A, eIF4A, and eIF4B were purified as described (Pestova et al., 1996, 1998b).

Assembly and Analysis of Ribosomal Complexes

For toeprinting analysis, ribosomal complexes were assembled essentially as described (Pestova et al., 1998b). 0.5 μg aliquots of IGR-IRES RNA were incubated for 5 min at 30°C in a 40 μl reaction volume that contained buffer A (2 mM DTT, 100 mM potassium acetate, 20 mM Tris [pH 7.6], 2.5 mM magnesium acetate, 1 mM ATP, 0.1 mM GMP-PNP, and 0.25 mM spermidine), and eIF2 (3 μg), eIF3 (6 μg), eIF4A (2 μg), eIF4B (0.3 μg), eIF4F (1.5 μg), 6 pmol 40S subunits, 8 pmol 60S subunits, 6 pmol Met-tRNA$_i$, eIF1 (0.3 μg), and eIF1A (0.3 μg), as indicated. Incubation was continued for 3 min at 30°C following the addition of 4 pmol of primer 5′-GAAAGATTAAC GATATCAG-3′ (complementary to IGR nt 6341–6359). Ribosomal and RNP complexes were analyzed by primer extension (Pestova et al., 1996) using avian myeloblastosis virus reverse transcriptase (Promega Biotec.) in the presence of α-^{32}P dATP (~6000 Ci/mmol; ICN Radiochemicals, Irvine, CA). cDNA products were compared with appropriate dideoxynucleotide sequence ladders.

For toeprinting analysis of ribosomal complexes assembled in RRL, 0.5 μg RNA was incubated in 15 μl RRL (Promega) in the presence of 1 mM GMP-PNP, 500 μg/ml cycloheximide, 10 μM edeine, 10 μM sparsomycin or 3 μg eIF5B as described, for 5 min. at 30°C. The reaction mixture was diluted with buffer A to 40 μl final volume prior to the addition of 4 pmol of the extension primer (see above). Toeprint analysis was carried out as described above.

Sucrose Gradient Analysis

For sucrose gradient analysis, monocistronic luciferase constructs containing either the EMCV or the IGR IRES were digested with NarI and transcribed in the presence of ^{32}P-CTP (3000 Ci/mmol). For experiments with purified components, 100 μl reactions contained 10 pM 40S subunits, 12 pM 60S subunits and 1.5 μg RNA. For experiments in crude extracts, RRL was treated prior to the addition of RNA as follows: 500 μg/ml cycloheximide (5 min., 30°C); 5 mM GMP-PNP (5 min., 30°C), then 500 μg/mL cycloheximide for an additional 5 min at 30°C; 2.5 μM edeine (5 min, 30°C), then 500 μg/ml cycloheximide for 5 min at 30°C; 5 mM L-methioninol (30 min, 30°C), then 500 μg/ml cycloheximide for 5 min at 30°C. RNAs (20 μg/μl; 1–2x10^6 cpm) were then added, and the reactions incubated for an additional 20 min at 30°C. Reactions were stopped by the addition of 4 volumes of ice-cold gradient buffer (15 mM Tris-Cl [pH 7.4], 15 mM $MgCl_2$, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/mL cycloheximide, 1 mg/mL heparin, and 0.05% glutaraldehyde) or gradient buffer without 15 mM $MgCl_2$ and with 10 mM EDTA as indicated. After a 5 min incubation on ice, the reactions were layered over 10%–50% sucrose gradients and sedimented by ultracentrifugation at 200,000 × g for 2 hr and 45 min at 4°C. Fractions were then collected using an ISCO fraction collection system, and the amount of RNA in each fraction determined by scintillation counting.

Thapsigargin Treatment and RNA Transfections of Cos7 Cells

Approximately 10^7 Cos7 cells were mock treated or incubated with 1 μM thapsigargin for 30 min as described (Harding et al., 1999), then transfected with 400 ng capped RNA using the lipofectin reagent (GibcoBRL) (Wilson et al., 2000). Extracts were made 1 hr post-transfection in 50 μl passive lysis buffer (Promega Biotec.) and assayed using the Dual Luciferase Reporter Assay System (Promega Biotec.) as recommended.

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