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Translation initiation from the ribosomal A site or the P site, dependent on the conformation of RNA pseudoknot I in dicistrovirus RNAs

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SUMMARY

Translation initiation of the second ORF of insect dicistrovirus RNA depends on an internal ribosomal entry site (IRES) in its intergenic region (IGR), and is exceptional in using a codon other than AUG and in not using the canonical initiator methionine tRNA. Studies *in vitro* suggest that pseudoknot I (PKI) immediately preceding the initiation codon occupies the ribosomal P site and that an elongator tRNA initiates translation from the ribosomal A site. Using dicistronic reporters carrying mutations in the initiation codon of the second ORF and mutant elongator or initiator tRNAs capable of reading these codons, we provide direct evidence for initiation from the A site in mammalian cells and, under certain conditions, also from the P site. Initiation from the A but not the P site requires PKI. Thus, PKI structure may be dynamic and optimal IGR IRES-mediated translation of dicistroviral RNAs may require trans-acting factors to stabilize PKI.

INTRODUCTION

Many RNA viral RNAs have structures that are different from those of cellular mRNAs and use distinct modes of translation initiation (Jang et al., 1988; Pelletier and Sonenberg, 1988; Jackson, 2005; Doudna and Sarnow, 2007; Kieft, 2008). The genome organization of invertebrate dicistrovirus, previously called insect picorna-like virus, is unusual in that the viral RNA (Fauquet et al. 2004; Figure 1A) consists of two open reading frames (ORFs), which are separated by approximately 200 nucleotides of intergenic region (IGR). Unlike alphavirus, no subgenomic RNA was detected in infected cells and the two ORFs are translated as a dicistronic mRNA (Sasaki et al., 1998; Wilson et al., 2000b; Figure 1A). The first ORF, which encodes non-capsid proteins, is translated using an internal ribosomal entry site (IRES) located in the 5' untranslated region (UTR), and the canonical AUG as the initiation codon (Terenin et al.,

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SUPPLEMENTAL DATA

Supplemental data includes Experimental Procedures, some of the Results and Discussion, four tables and eight figures, and can be found with this article online.

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2005). The second ORF, which encodes capsid proteins, is translated using a different IRES located in the IGR (Sasaki and Nakashima, 1999) and a non-AUG codon for initiation (CAA in PSIV, *Plautia stali* intestine virus, and GCU/C/A in other insect picorna-like viruses, such as CrPV, the cricket paralysis virus). Translation of the second ORF does not use the initiator methionine tRNA (Sasaki and Nakashima, 2000; Wilson et al., 2000a) and this mode of translation was called initiation without the initiator tRNA (RajBhandary, 2000).

The molecular mechanism of translation without initiator tRNA has so far been investigated almost exclusively *in vitro*, using biochemical and structural approaches mostly on PSIV or CrPV RNAs. The 186 nucleotides of IGR in PSIV are predicted to contain three pseudoknot (PK) structures (Kanamori and Nakashima, 2001; Figure 1B). The 5'-terminal 143 nucleotides, which contain two pseudoknot structures (PKII and PKIII) are required for binding of the IGR IRES to the 40S ribosome (Jan and Sarnow, 2002; Nishiyama et al., 2003; Pfingsten et al., 2006) and this binding does not require any of the initiation factors (Wilson et al., 2000a; Pestova and Hellen, 2003; Pestova et al., 2004). The 3'-terminal pseudoknot structure (PKI) forms a unit distinct from the 5' structure (Spahn et al., 2004; Costantino and Kieft, 2005; Schüller et al., 2006) and is involved in decoding of the non-AUG codon used for initiation (Sasaki and Nakashima, 2000; Wilson et al., 2000b; Jan and Sarnow, 2002). This region of the RNA is thought to occupy the ribosomal P site and is proposed to work as a functional mimic of initiator tRNA and initiation factors necessary for subunit joining (Spahn et al., 2004). Subunit joining does not require initiator tRNA or the initiation factors eIF5 or eIF5B (Pestova et al., 2004), and translation in a reconstituted system starts from the ribosomal A site (Wilson et al., 2000a; Jan et al., 2003; Pestova and Hellen, 2003). Alternatively, the 80S subunit can be directly recruited to the IGR IRES to start translation from the A site (Pestova et al., 2004). However, because virtually all of the work has been done *in vitro* and because of the rather weak activity of IGR IRES-mediated initiation, there have been doubts expressed as to whether the IGR IRES is an IRES and whether translation initiation can really occur from the A site and without the involvement of initiator tRNA^{Met_i} (Kozak, 2001; 2007).

Here, we describe the development of a DNA-based dicistronic reporter system to study PSIV IGR IRES-mediated translation initiation in mammalian cells. The dicistronic reporter contained the *Renilla* luciferase (RLuc) coding sequence upstream and the firefly luciferase (FLuc) coding sequence downstream separated by the PSIV IGR IRES sequence. Investigation of translation products as well as of the dicistronic mRNA revealed that analysis of IRES-mediated translation was hampered both by alternative splicing and read through of the termination codon from the first cistron. These problems were eliminated by mutating the termination codon of the upstream cistron and a splice donor site in the RLuc coding region. The CAA codon used for initiation of FLuc ORF was then mutated to UAG (the amber termination codon) or to AGG and mutant tRNAs derived from elongator serine or methionine tRNAs and initiator methionine tRNA, were co-expressed to determine whether the ribosomal A or the P site is used to decode the non-AUG initiation codons. In the presence of the cognate initiation codon, both serine amber suppressor tRNA and the C35 mutant of elongator methionine tRNA (Met_e) enhanced IGR IRES activity, providing the first direct *in vivo* evidence for translation initiation from the ribosomal A site. Interestingly, the C35 mutant of initiator methionine tRNA (Met_i) also enhanced IGR IRES activity, indicating the potential of IGR IRES to start translation also from the P site. Using mutants of PKI, we show that IGR IRES-mediated translation initiation from the P or the A site depends on different conformations of PKI. Results obtained with IGR IRES mutants carrying AUG or GCU as initiation codons for FLuc confirm these findings. Importantly, results obtained using RNA transfection parallel those obtained using DNA transfection. Thus, IGR IRES, originally shown to initiate translation from the ribosomal A site, has the potential to start translation from either the A site or from the P site.

RESULTS

Development of a DNA-based dual luciferase dicistronic reporter system for monitoring PSIV IGR IRES activity

To recapitulate translation dependent on IGR IRES, the IGR (6007-6192) of PSIV (Sasaki and Nakashima, 2000) was cloned into the intercistronic region of the RLuc-FLuc dual luciferase reporter system (Figure 1A, bottom). The first twenty-one nucleotides of the capsid-coding gene, starting with the CAA initiation codon, were also included because some IRESs require the proximal coding regions for optimal activity. The termination codon of the RLuc gene was changed to UGA because the termination codon of the first ORF of PSIV RNA is UGA (Figure 1A, top) and the last two nucleotides GA of the UGA are predicted to be part of an RNA stem in the secondary structure model of the IRES (underlined in Figure 1B).

Dicistronic mRNA, expressed from a pCI-neo vector (Figure S1A), was analyzed by RNA blot hybridization using firefly luciferase DNA as a probe. A single band corresponding to the length of dicistronic transcript was observed (lane 2, Figure 1C). As expected, the dicistronic mRNA isolated from transfected cells, which has a polyA tail, migrates slightly slower (lane 2) than the RNA synthesized *in vitro* using T7 RNA polymerase (lane 5), and slightly faster than the dicistronic mRNA carrying the 347 nucleotide long hepatitis C virus IRES (lane 1). Importantly, no band corresponding in size to the monocistronic FLuc RNA (lane 4) was observed, even after prolonged autoradiography (data not shown).

When translation products were analyzed by immunoblotting using a polyclonal FLuc antibody, however, in addition to the desired monocistronic FLuc, five other polypeptides with different mobilities were detected (Figure S1B, lane 3). Figure S1A provides a schematic identification of these polypeptides. Intensities of these polypeptide bands were actually higher than that of the 62 kDa FLuc, which was detected only after prolonged exposure. The slowest band corresponded to a fusion of RLuc and FLuc due to read through of the RLuc termination codon UGA (Figure S2). The remaining four bands originate from translation of alternatively spliced mRNAs (Figures S1C, S3 and S4). Mutation of the RLuc termination codon from UGA to UAAga (the reason for adding the sequence ga after UAA is explained in the supplementary data) and of the splice donor site in the RLuc coding sequence from GGU to GGA, led to the synthesis, only of FLuc, the desired product (Figure S1C, lane 6). These and other changes introduced into the dicistronic reporter (Figure S1D) are described more fully under “Results” in the supplementary data section. The sequence of the 7703 bp dicistronic reporter plasmid thus generated is presented in Figure S5.

The dicistronic reporter carrying all these changes synthesized FLuc, independent of RLuc and can, therefore, be used for study of IGR IRES-mediated translation in mammalian cells (Hellen and Sarnow, 2001). In cells starved for amino acids, FLuc/RLuc ratio increased four fold over a five hour time period, whereas there was no such increase in cells grown in media containing amino acids (fed cells) (Figure S6A). This increase is dependent upon the presence of the IGR IRES and is not due to translation termination followed by reinitiation, since mRNA lacking the IGR IRES but containing FLuc coding sequences with AUG as the initiation codon showed only a minimal increase (Figure S6B). Control experiments showed that starvation of cells for amino acids led to phosphorylation of eIF2 α (due to activation of GCN2 kinase) and to dephosphorylation of eIF4E binding protein 1 and S6 kinase 1 (due to inactivation of mTOR kinase) (Figure S6C). These results are similar to those described for the CrPV IGR IRES (Thompson et al., 2001; Fernandez et al., 2002).

An amber suppressor tRNA rescues the IGR IRES activities of mutant reporters with UAG as the initiation codon

Suppressor tRNAs, derived from elongator tRNAs, are transported to the ribosomal A site by the eukaryotic elongation factor 1. The human serine amber suppressor tRNA used in this work suppressed amber mutations in the monocistronic FLuc gene in which an internal serine codon at amino acid 284 or 504 was changed to UAG (Table S1) but not one in which the initiator AUG codon was changed to UAG. Thus, the amber suppressor tRNA can go to the A site but not to the P site. Therefore, for a direct test of whether the initiation codon of PSIV IGR IRES is decoded in the ribosomal A site *in vivo*, the CAA initiation codon was mutated to UAG (UAG6193 mutant) and the effect of expression of the serine amber suppressor tRNA (Capone et al., 1985) was analyzed (Figure 2A). To minimize competition between the eukaryotic release factor and the amber suppressor tRNA (Jan et al., 2003), the base following the UAG codon was also changed from G to C (Brown et al., 1990). Since there are no endogenous tRNAs for translation of UAG in mammalian cells, basal FLuc activity in cells transfected with the UAG6193 mutant goes down significantly, ~10-fold, and FLuc protein was not detected in immunoblots (Figure 2A, left, compare lane 4 to lane 2). When the amber suppressor tRNA was co-expressed, translation initiation from the UAG codon increased to the same level as from the wild type CAA codon (Figure 2A, left, lane 5). Northern blot analyses showed that this was not due to increases in levels of the RLuc-FLuc dicistronic mRNA in cells expressing the amber suppressor tRNA (Figure 2A, right). Enhancement of FLuc synthesis with UAG as the initiation codon is dependent specifically on expression of the amber suppressor tRNA, but not of the ochre suppressor tRNA, which reads the termination codon UAA (Table S2).

The increased FLuc activity in cells expressing the amber suppressor tRNA is not due to translation initiation at an upstream codon followed by read through of the UAG codon by the suppressor tRNA, since there is no upstream AUG codon within the PSIV IGR in frame with the FLuc ORF (Figure S2). Immunoblot analysis, which shows no difference in mobility of FLuc synthesized in presence of the amber suppressor tRNA, is also in agreement with this (Figure 2A, left, compare lanes 2, 3 and 5). To fully rule out the possibility that translation is initiating at an upstream codon nearby, a series of mutants with UAG at codons -1, -2 and -3 were generated (Figure 2B) and effects of expression of the amber suppressor tRNA on FLuc synthesis were investigated. If translation is initiating at an upstream codon, all of these mutants should produce much less FLuc and expression of the amber suppressor tRNA should rescue the IGR IRES activities of these mutants. The results, however, showed that within the four codon positions investigated (+1, -1, -2 and -3), UAG6193 was the only mutant, whose activity in translation was significantly enhanced by expression of the amber suppressor tRNA (Table S2). Since the suppressor tRNA cannot go to the ribosomal P site, initiation must be occurring at the ribosomal A site, providing direct evidence for initiation from the A site for IGR IRES-mediated translation initiation *in vivo*.

Effects of expression of C35 mutants of human elongator (Met_e) and initiator (Met_i) methionine tRNAs on IGR IRES-mediated translation of reporters with AGG as the initiation codon

Activity of mutant tRNAs in initiation and in elongation with monocistronic FLuc mRNAs—The C35 mutant of initiator methionine tRNA, which is most likely aminoacylated with methionine, and which carries a change in the anticodon sequence from CAU to CCU, can initiate protein synthesis using AGG as the initiation codon (Drabkin and RajBhandary, 1998). For the current work, A35 in the anticodon sequence of elongator methionine tRNA was mutated to C35 to obtain a tRNA, which has the potential to read the arginine codon AGG. Monocistronic FLuc reporter genes were used for studies of these mutant tRNAs in initiation and in elongation steps of protein synthesis (Table 1). Mutant Met1 with a change in the initiation codon from AUG to AGG, is used to measure activities of the mutant tRNAs in

initiation. Mutant Met196 with a mutation in codon 196 of the FLuc gene from AUG to AGG, is used to measure the activities of the mutant tRNAs in elongation. The C35 mutant of the Met_e tRNA will read the arginine codon AGG but insert methionine and thereby act as a missense suppressor and correct the defect of a methionine to arginine mutation in a reporter gene (Drabkin et al., 1998). In searching for an appropriate methionine to arginine mutation in the FLuc gene, likely to greatly reduce activity of FLuc, we settled on Met196 within the sequence IMNSSGSTGLPK, which forms part of the AMP binding site. This mutation essentially abolished activity of the mutant FLuc protein (Table 1). With these monocistronic mutant FLuc reporters, we show that the Met_i mutant tRNA worked as an initiator but extremely poorly as an elongator tRNA. In contrast, the Met_e mutant tRNA did not work as an initiator tRNA but worked as an elongator tRNA. Therefore, the C35 mutants derived from these two tRNAs can be used, along with the dicistronic mRNA with AGG as the initiation codon as reporter, to determine whether the PSIV IGR IRES-mediated translation initiation occurs at the ribosomal A site or the P site.

Met_i tRNA is reported to work to some extent as an elongator in mutant strains of yeast deleted of all the elongator methionine tRNA genes (Åstrom et al., 1993) and in a mammalian reconstitution system lacking any of the initiation factors (Pestova and Hellen, 2003). The activity of Met-tRNA_i in yeast is most likely due to the fact that this tRNA, when overproduced, is partly deficient in a modified base known to be critical for preventing its binding to the elongation factor. The possibility that mammalian Met_i mutant tRNA goes to the A site in our *in vivo* system is extremely unlikely, because activity of the Met_i mutant tRNA in translation of the monocistronic M196R luciferase reporter mRNA was only 0.4% (0.62 versus 152) of that of the corresponding Met_e mutant (Table 1).

Activity of the Met_e and Met_i mutant tRNAs in IGR IRES-mediated translation of FLuc—These two mutant tRNAs were expressed in cells along with the dual luciferase dicistronic reporter carrying AGG as the initiation codon. Since cells contain an AGG reading wild type tRNA^{Arg}, even without expression of mutant tRNA, there is some basal synthesis of FLuc (Figure 3, lane 3), although intensity of the FLuc band is much weaker than that with AUG (lane 6) or CAA (not shown) as the initiation codons. This could be because there is less of the AGG decoding arginine tRNA in mammalian cells compared to the CAA decoding glutamine tRNA and the AUG decoding methionine tRNA. Alternatively, proteins initiated with arginine turn over more rapidly in eukaryotic cells (Varshavsky, 1996). In COS-1 cells, co-expression of Met_e and Met_i mutant tRNAs increased IGR IRES dependent synthesis of FLuc by 3.3- and 2.7-fold, respectively (Table 2). Immunoblot analysis also showed similar increases in FLuc protein levels without any increase in RLuc levels (Figure 3, lanes 4 and 5) or activities (data not shown). Since the Met_e mutant can only go to A site of the ribosome (Table 1), enhancement of IGR IRES activities by the Met_e mutant tRNA provides further evidence for translation of FLuc synthesis from the A site in mammalian cells using an elongator tRNA.

Enhancement of FLuc synthesis by the Met_i mutant tRNA suggests that translation can also start from the P site using an initiator tRNA. This enhancement of IGR IRES-mediated translation initiation was also observed in other cell types, yielding fold increases of 3.8- and 6.5- for the Met_e and Met_i mutant tRNAs, respectively, in HeLa cells (Table 2).

A possible explanation for increased FLuc synthesis in the presence of the C35 mutant Met_i tRNA is that the dual luciferase reporter used contains a cryptic promoter within the IGR IRES sequence. This would produce a monocistronic FLuc mRNA, which would be translated in a cap-dependent manner and, thereby, initiate translation using the C35 mutant Met_i tRNA. To evaluate this possibility, the C35 Met_i mutant tRNA plasmid was co-transfected with the mutant dual luciferase reporter plasmid carrying AGG as the initiation codon and with or without CMV

promoter and RLuc and FLuc activities in cell extracts were determined (Figure S7). Expression of FLuc activity from plasmid without promoter was at the most 3.4 % of that with CMV promoter. Thus, cryptic promoter activity within IGR IRES is really low and cryptic transcripts, if any, are negligible and do not contribute to the fold-increase in FLuc activity observed in cells expressing the C35 Met_i mutant tRNA in Table 2 and Figure 3.

Effects of mutations in the IGR IRES on FLuc synthesis

Deletion mutants of IGR IRES—To investigate the importance of sequences within the IGR IRES on activities of the Met_e and Met_i tRNA mutants in initiation of FLuc synthesis, a series of deletion mutants were generated from the 3'-end of the IGR IRES (Figure 4A). These deletion mutants lacked the entire 186 nucleotide long IGR IRES (mutant 6007), all of PKI (mutant 6148), part of PKI (mutant 6163) or only six nucleotides, five of which form the tertiary base pairs of PKI (mutant 6187). Assay for FLuc and RLuc activities in extracts of cells co-transfected with these mutant dicistronic reporter genes and either the C35 Met_e or the C35 Met_i mutant tRNA genes showed that there was essentially no stimulation of FLuc synthesis upon expression of the Met_e mutant tRNA. Background expression of FLuc, which uses wild type arginine tRNA was also reduced in all of the mutants. Thus, the IGR IRES sequence and the tertiary base pairs of PKI play critical roles in initiation of FLuc synthesis using an elongator tRNA.

In contrast to cells expressing the Met_e mutant tRNA, those expressing the Met_i mutant tRNA produced even more FLuc (1.5–2.7 fold more) except for mutant 6148 lacking all of PKI of the IGR IRES. With mutant 6007 in which the AGG initiation codon for FLuc directly follows the UAAga termination sequence of RLuc, FLuc synthesis in presence of the Met_i mutant tRNA is probably a rare example of termination-reinitiation (Jackson et al., 2007), in which some of the ribosomes that have translated the upstream RLuc ORF also translate the FLuc ORF. With the other IGR IRES mutants, which contain the PKII and PKIII regions, however, initiation of FLuc synthesis is most likely due to IGR IRES-mediated recruitment of ribosomes to the mRNA. The lack of stimulation of FLuc synthesis by the Met_i mutant tRNA with the IGR IRES deleted of all of the PKI structure (mutant 6148) could suggest that this deletion, which abuts the end of PKII, destabilizes the PKII structure and thereby affects IGR IRES-mediated recruitment of the 40S ribosome to the mRNA. The activity of mutant 6163 in FLuc synthesis, in cells expressing the Met_i mutant tRNA, means that the Met_i mutant tRNA dependent synthesis of FLuc does not require PKI. Strikingly, with mutant 6187, which has the hairpin loop of PKI but lacks only the tertiary base pairs, there is 2.7 fold more FLuc synthesis in cells expressing the mutant Met_i^{tRNA} compared to cells transfected with the plasmid carrying the full length IGR IRES sequence. Thus, while the PKI structure is essential for FLuc synthesis involving the Met_e mutant tRNA, it is not necessary for FLuc synthesis involving the Met_i mutant tRNA.

Mutations in PKI of the PSIV IGR IRES—To further dissect the role of PKI on Met_e and Met_i mutant tRNA dependent initiation of FLuc synthesis, all five of the tertiary base pairs in PKI were disrupted by mutagenesis (Figure 4B). In the tertiary structure model of PSIV IGR IRES sequence (Figure 1B), the five nucleotides CACUU₆₁₈₈₋₆₁₉₂ immediately preceding the AGG initiation codon are predicted to form tertiary base pairs with AAGUG₆₁₆₃₋₆₁₆₇ in the loop sequence. When the CACUU₆₁₈₈₋₆₁₉₂ sequence is replaced with GUGAA (M.1), these five nucleotides are no longer base paired, as shown by reduced cleavage of phosphodiester bonds around nucleotides 6188-6192 by the double-strand specific RNase VI (Figure S8). Assay for FLuc showed that basal FLuc synthesis and stimulation by Met_e mutant tRNA was reduced significantly in both COS-1 and HeLa cells. On the other hand, FLuc synthesis in cells expressing the Met_i mutant tRNA increased by 1.4-fold in COS-1 cells and stayed at the same level in HeLa cells. Similarly, basal FLuc synthesis and its stimulation by the mutant Met_e

tRNA went down upon mutation of the distal AAGUG to UUCAC (M.2). FLuc synthesis in cells expressing the Met_i mutant tRNA went down slightly especially in COS-1 cells, but expression level of FLuc was still higher (> 3-fold) than basal synthesis for this mutant. In the compensatory mutant (M.1/2), there was partial recovery in background synthesis of FLuc and its stimulation by the Met_e mutant tRNA (68% and 73% activity compared to wild type IRES in COS-1 and HeLa cells, respectively). FLuc synthesis in cells expressing the Met_i mutant tRNA was even higher. Thus, stimulation of IGR IRES-mediated translation initiation by the Met_e mutant tRNA requires tertiary base pairs in PKI whereas stimulation by the Met_i mutant tRNA does not.

Different effects of PKI mutation on FLuc synthesis with mRNAs carrying AUG versus GCU as the initiation codons

Results from DNA transfection—Further evidence that PSIV IGR IRES-mediated translation of FLuc can initiate with the initiation codon decoded in either the A site or, in some cases, in the P site comes from the use of mutants carrying AUG or GCU as the initiation codons. The wild type CAA initiation codon of the FLuc reporter (Figure 1A) was changed to either AUG or GCU and the effect of the same M.1 mutation in PKI (described above in Figure 4B) was investigated in COS-1 cells. The normalized IGR IRES activity of the reporters with AUG or GCU as initiation codons are essentially the same (Figure 4C). However, whereas the PKI mutation led to a significant decrease in IGR IRES activity of the reporter with GCU as the initiation codon (8 to 9-fold), the same mutation led, if anything, to a slight increase in activity of the reporter with AUG as the initiation codon. Results of immunoblot analyses also confirm these findings (data not shown). The difference between GCU and AUG as initiation codons is that while GCU is read exclusively by an elongator alanine tRNA, AUG can be read by either the elongator or the initiator species of methionine tRNAs. The different effects of the same PKI mutation on FLuc synthesis using AUG or GCU as the initiation codons is, therefore, most likely due to the fact that while the elongator tRNA dependent translation of FLuc using AUG or GCU as the initiation codons is affected by the PKI mutation, the initiator tRNA dependent translation of FLuc using AUG as the initiation codon is not.

Results from RNA transfection—To fully rule out the possibility that synthesis of FLuc from the dicistronic reporter involving an initiator tRNA is due to the presence of cryptic promoters in the IGR IRES (Figure S7), we transfected 5'-capped dicistronic RNA coding for RLuc and FLuc into COS-1 cells. Assay for RLuc and FLuc showed that the results of PKI mutation on mRNAs carrying AUG or GCU in the initiation codon for FLuc (Figure 4D) parallel those obtained using DNA transfection (Figure 4C).

DISCUSSION

The dual luciferase reporter described in here has proven to be an excellent system for studying dicistronic IGR IRES-mediated translation initiation in mammalian cells. Mutations of the FLuc initiation codon from CAA to UAG or to AGG reduced basal IGR IRES-mediated translation of FLuc to a very low level and, thereby, allowed a study of whether mutant elongator tRNAs could be used to read these as initiation codons *in vivo*. A serine amber suppressor tRNA and a C35 mutant of elongator methionine tRNA (tRNA^{Met}_{C35}), both of which can only go to the ribosomal A site (Tables 1 and S1), initiated FLuc synthesis using UAG and AGG, respectively, as initiation codons. The 13–17 fold stimulation of FLuc synthesis in COS-1 cells expressing the serine amber suppressor tRNA (Figure 2A and Table S2) leaves no doubt that IGR IRES-mediated translation initiation can use UAG as the initiation codon with UAG positioned at the ribosomal A site. These results provide the first direct evidence for IGR IRES-mediated translation initiation from the ribosomal A site *in vivo*.

Interestingly, expression of a mutant initiator tRNA^{Met_i}, which can only go to the ribosomal P site (Table 1) and which can read AGG as an initiation codon (Drabkin and RajBhandary, 1998), also stimulated FLuc synthesis, showing that IGR IRES-mediated FLuc synthesis can also initiate from the ribosomal P site. The enhancement of FLuc synthesis in the presence of the C35 mutant tRNA^{Met_i} is not due to the presence of cryptic promoters in the plasmid DNA (Figure S7). It is also most likely not due to the presence of spliced mRNAs, which could synthesize FLuc in a cap-dependent manner. Thus, IGR IRES-mediated translation initiation leading to FLuc synthesis using an AGG codon can occur either from the ribosomal A site or, in the presence of an appropriate mutant tRNA^{Met_i}, also from the P site. This conclusion is not in disagreement with the results of earlier studies, *in vitro*, showing that IGR IRES-mediated translation initiation occurs from the ribosomal A site (Wilson et al., 2000a; Jan et al., 2003; Pestova and Hellen, 2003). These earlier studies did not specifically address the question of whether initiation could also occur from the ribosomal P site.

Our finding that IGR IRES-mediated translation initiation can also occur from the ribosomal P site does not, however, mean that this happens in insect cells infected with dicistroviruses such as PSIV and CrPV. Initiation from the ribosomal P site involves the initiator tRNA^{Met_i} species and its specific ability, when aminoacylated with methionine, to form a ternary complex with eIF2 and GTP and bind to the P site of the ribosome (RajBhandary and Chow, 1995; Pestova et al., 2007). Since the initiation codons used for IGR IRES-mediated translation initiation are CAA for PSIV and GCU, GCC or GCA for CrPV and other dicistroviruses, initiation from the ribosomal P site involving these codons would require the presence of mutants of tRNA^{Met_i} capable of reading them in insect cells. The presence of such mutant tRNAs, which differ in all three nucleotides of the anticodon sequence, is extremely unlikely.

The effect of mutations in the IGR IRES on translation initiation using AGG as the initiation codon showed that mutations that deleted PKII and III or those that disrupted the tertiary base pairs (Pleij et al., 1985) that form part of PKI structure essentially abolished initiation from the ribosomal A site (Figure 4, A and B). The same result was obtained with a reporter carrying CAA (Table S2) or GCU (Figure 4C) as the initiation codons. These results are in agreement with biochemical, mutagenic and structural studies *in vitro* showing that while PKII and PKIII are involved in recruiting the 40S ribosome to the IGR IRES, an intact PKI structure is necessary to ensure that initiation occurs from the ribosomal A site (Sasaki and Nakashima, 2000; Wilson et al., 2000b; Jan and Sarnow, 2002). In fact, a recently determined crystal structure of a variant of CrPV IGR IRES PKI shows that part of PKI mimics codon:anticodon interactions between mRNA and initiator tRNA on the ribosomal P site (Selmer et al., 2006; Costantino et al., 2008). The crystal structure supports previous suggestions based on results of X-ray and cryo-electron microscopy studies that IGR IRES PKI occupies the ribosomal P site and by doing so positions the initiation codon, which is immediately downstream, in the ribosomal A site (Kanamori and Nakashima, 2001; Spahn et al., 2004; Pflugsten et al., 2006; Schüler et al., 2006).

In contrast to results showing that an intact PKI structure is essential for decoding of the initiation codon at the ribosomal A site, initiation from the ribosomal P site is tolerant to disruptions of the PKI structure (Figures 4A and 4B). The strikingly different effects of an identical disruption of PKI structure on FLuc synthesis initiated by AUG versus GCU (Figure 4C) provide further support to this conclusion. Importantly, results obtained using RNA transfection parallel those obtained using DNA transfection (Figures 4C and 4D). The finding that disruption of PKI structure has little effect on initiation of FLuc synthesis from an AUG codon (Figures 4C and 4D) is particularly noteworthy in that initiation in this case involves the canonical AUG initiation codon and the cellular initiator tRNA instead of an exogenously introduced mutant tRNA.

How can the mutant dicistronic mRNA carrying AGG as the initiation codon for FLuc synthesis be translated with the initiation codon being decoded either at the P site using a mutant initiator tRNA or at the A site using a mutant elongator tRNA? The same question can be raised with the mutant carrying the canonical AUG as the initiation codon. A plausible hypothesis is that PKI in the mRNA exists in two conformations, and that the one with an intact PKI structure initiates from the A site, whereas the one with a hairpin loop structure initiates from the P site (Figures 4B, 4C and 4D and Figure 5). The mechanism, in the latter case, may be similar to that of hepatitis C virus IRES-mediated translation initiation in which the IRES binds to the 40S ribosomal subunit in the absence of most of the initiation factors and positions the initiation codon in the ribosomal P site (Hellen and Sarnow, 2001; Otto and Puglisi, 2004; Doudna and Sarnow, 2007; Kieft, 2008; Fraser et al., 2009).

Pseudoknots exist in dynamic equilibrium with hairpin loop structures (Wyatt et al., 1990). In particular, the tertiary base pairs of PKI of both PSIV and CrPV IGR IRES are known to be quite mobile (Jan and Sarnow, 2002; Nishiyama et al., 2003; Costantino and Kieft, 2005; also confirmed in Figure S8 for PSIV IGR IRES). Because of this, the variant of CrPV IGR IRES PKI used for crystal structure determination contained multiple changes in sequence to stabilize the PKI structure (Costantino et al., 2008). Our hypothesis, along with the dynamic nature of PKI structure in PSIV and CrPV IGR IRES, suggest that efficient IGR IRES-mediated translation of PSIV or CrPV RNAs in infected cells, which initiates from the ribosomal A site, may require trans-acting factors to stabilize the PKI structure. Trans-acting factors have been implicated before in several IRES-mediated translation processes (Pelletier and Sonenberg, 1988; Doudna and Sarnow, 2007).

Assembly and propagation of a virus require the synthesis of much more of structural proteins such as capsid proteins than other virally encoded proteins such as replicases, helicases, etc. For example, *Drosophila* cells infected with CrPV synthesize much more of the viral structural proteins than non-structural proteins (Moore et al., 1980). The genomic RNAs of dicistroviruses are organized in such a way that synthesis of capsid proteins is all IGR IRES-mediated (Jan, 2006). Therefore, trans-acting factors, which stabilize PKI structure, may be necessary to ensure maximal IGR IRES activity. Such trans-acting factors could also be involved in temporal regulation of IGR IRES-mediated translation to ensure capsid protein synthesis only during certain stages of the viral life cycle.

Finally, the results described in this work have relied almost exclusively on work based on DNA transfection into mammalian cells. Importantly, in the one case examined, results obtained using RNA transfection essentially replicate those obtained using DNA transfection. As pointed out before (Kozak, 1999; Wilson et al., 2000b; Kozak, 2001; van Eden et al., 2004), experiments using DNA transfection to establish IRES function require rigorous controls to rule out the possibility of mRNA splicing or cryptic promoters, which produce monocistronic transcripts and allow cap-dependent translation of the reporter protein instead of IRES-mediated translation. Our work shows that Northern blot analysis alone is not enough to rule out the possibility of the presence of shorter mRNAs that lead to cap-dependent translation of the reporter protein. Besides mutation of the leaky UGA termination codon of the upstream RLuc ORF to prevent its read through, it was necessary to also remove a splice donor site in the RLuc ORF (Figure S1). In addition to assays for FLuc activity, use of immunoblot analysis to monitor FLuc synthesis revealed the nature of the problem. The final constructs, carrying mutations in the initiation codon for the FLuc reporter protein, allowed us to establish IGR IRES-mediated decoding of the initiation codon in the ribosomal A site *in vivo* and to demonstrate the potential, in the presence of an appropriate mutant initiator tRNA, for initiation also from the ribosomal P site.

EXPERIMENTAL PROCEDURES

Plasmids

The dicistronic reporters were cloned downstream of CMV immediate early promoter in the pCI-neo mammalian expression vector (Promega). Mutations were introduced by PCR using mutagenic primers listed in Tables S3 and S4.

Transfection of Cells With DNA

Cells maintained in D-MEM/10% calf serum were plated into 24-well plate the day before the transfection. A mixture of 0.25 µg of mRNA expression plasmid and 0.08 µg of tRNA expression plasmid was incubated with 0.5 µl PLUS reagent and 1 µl Lipofectamine (Invitrogen) and were transfected into 80–90% (COS-1) or 50–60% (HeLa) confluent cells, according to manufacturer's protocol. After 36 hours of incubation at 34°C, cells were washed with 1× PBS (–) and lysed with 120 µl of 1× Passive Lysis Buffer (Promega) supplemented with 1mM PMSF. Luciferase activities were quantified using Dual-Luciferase Reporter Assay (Promega) in the Sirius Luminometer (Berthold). IRES activity in a given sample is expressed as the ratio of firefly to *Renilla* luciferase activity. The ratio thus obtained was normalized to the standard IRES activity in each transfection. These are specified in individual Figures and Tables. Unless otherwise specified, averages of normalized activities from three independent transfections are shown with standard error of the mean (SEM) in all figures and tables including Supplemental data.

The amount of plasmid DNA and transfection reagent was proportionally increased according to the manufacturer's recommendation when the transfection was scaled up from 3.5 to 10 cm dishes.

Transfection of Cells With RNA—The RLuc-FLuc dicistronic RNA to be used for transfection was prepared using T7 RNA polymerase (see Supplemental data). The 5' end was capped by including 3'-O-Me-m⁷GpppG cap analogue (New England Biolabs #S1411) in a 5:1 molar excess over GTP. RNA (0.75 µg) was mixed with 0.75 µl of DMRIE-C (Invitrogen) and transfected into 80–90% confluent COS-1 cells in 24-well plate as described by supplier. Cells were incubated for 12 hours at 34°C and luciferase activities were determined.

RNA Analyses—Total RNA for Northern blot analysis was prepared from COS-1 cells transfected in 10 cm dishes using total RNA isolation kit (Promega). 10 µg of total RNA was separated on a 1% agarose/2.2M formaldehyde gel and blotted onto Hybond-N nylon membrane (Amersham-Pharmacia). The RNA in the blot was hybridized to radiolabeled DNA probes prepared using the Nick-translation Kit (Promega) and [α -³²P]-dCTP (Perkin Elmer).

Immunoblot Analysis—COS-1 cells transfected in 3.5 cm dishes were lysed with Triton X-100 lysis buffer [10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100] supplemented with 37 µg/ml TLCK, 70 µg/ml TPCK and 1 mM PMSF. Protein amounts were determined using the modified Lowry method (Bio-Rad, Dc Protein Assay). 8 µg of protein in the lysate was fractionated on a 10% SDS-PAGE gel, blotted onto Immobilon-P PVDF membrane (Millipore) and treated with anti-FLuc goat polyclonal antibody or anti-RLuc mouse monoclonal antibody followed with appropriate HRP-conjugated secondary antibodies. Re-Blot Plus (Chemicon, 60513) was used for the stripping of antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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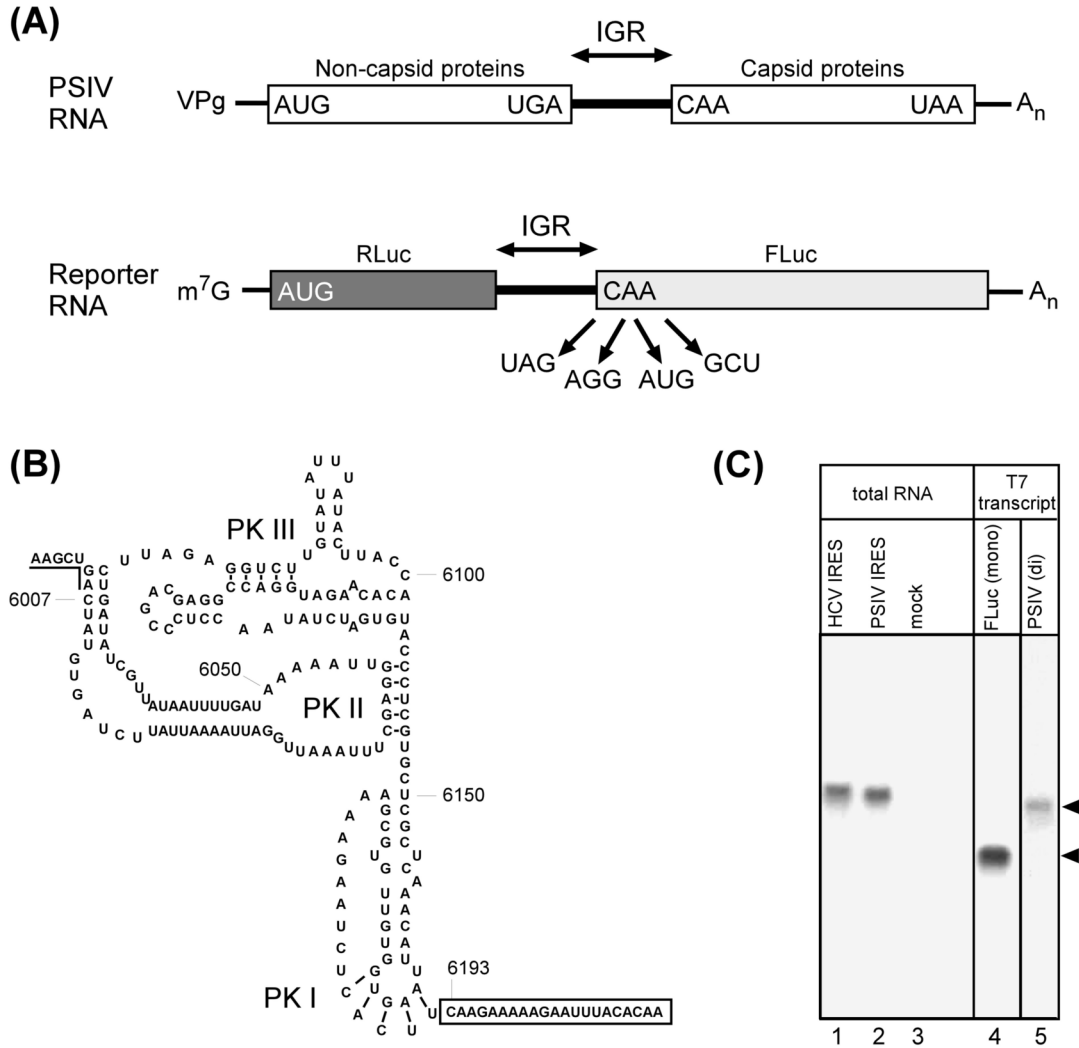


Figure 1. Expression of an RLuc-FLuc dicistronic reporter mRNA with the PSIV internal ribosomal entry site (IRES) in the intergenic region (IGR).
 A. Schematic representation of PSIV RNA (top) and a dicistronic reporter mRNA containing RLuc and FLuc (bottom). Open reading frames (ORFs) are boxed. Arrows indicate mutations introduced into the initiation codon of FLuc.
 B. Predicted RNA structure of PSIV IGR IRES (Kanamori and Nakashima, 2001). The end of the first ORF and the beginning of the second ORF are underlined and boxed, respectively. Tertiary base pairs in pseudoknots I, II and III are indicated by lines.
 C. Northern blot analysis of total cellular RNA, in which the dicistronic mRNA was expressed from the pCI-neo vector (lanes 1–2). Positions of monocistronic FLuc (lane 4) and dicistronic (lane 5) T7 RNA polymerase transcripts are indicated by carets. The DNA probe used was complementary to FLuc coding sequences. Lane 3, mock transfection.

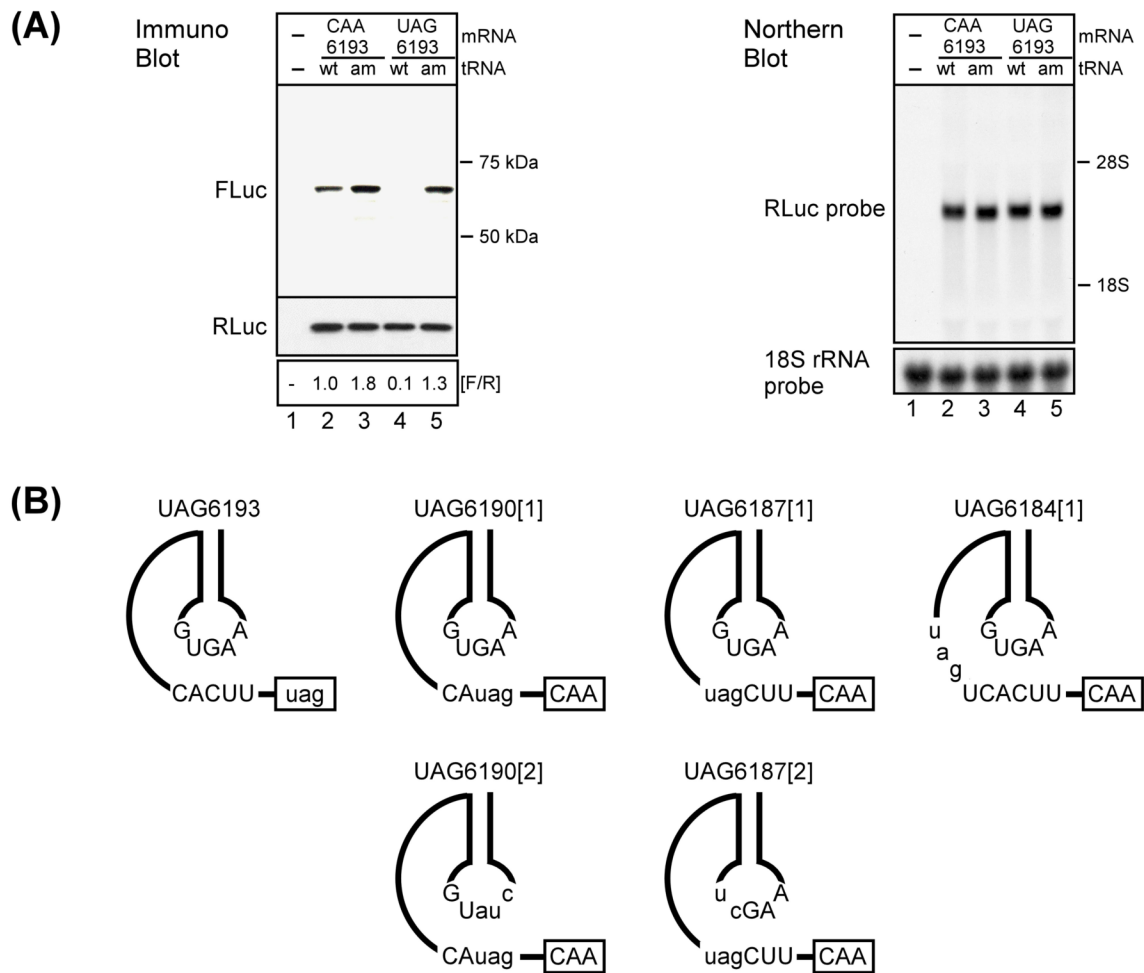


Figure 2.

Translation initiation from the ribosomal A site in the presence of serine amber suppressor tRNA in COS-1 cells.

A. Effect of expression of serine tRNA, wild type (wt) or amber suppressor (am), on activity of IGR IRES carrying the CAA to UAG mutation in the initiation codon (left) and on levels of the RLuc-FLuc dicistronic mRNA (right). The numbers inside the boxed region (left) are FLuc/RLuc ratios normalized to that of the reporter carrying CAA as the initiation codon and expressing the wt serine tRNA.

Northern blot analysis of RNA isolated from cells transfected with the wild type (CAA 6193) (lanes 2 and 3) and mutant (UAG6193) reporters (lanes 4 and 5) and either serine wild type (wt) or amber (am) suppressor tRNA genes (right). The blot was probed with DNA complementary to the RLuc coding region or oligonucleotides complementary to human 18S rRNA (Chappell et al., 2004). Lane 1, mock transfection.

B. PSIV IGR IRES mutants carrying the UAG mutation at different positions upstream and in frame with the FLuc initiation codon. The sites of UAG mutations and the compensatory mutations to maintain PKI structure are indicated by lowercase letters (Details in Supplementary Results Section and Table S2).

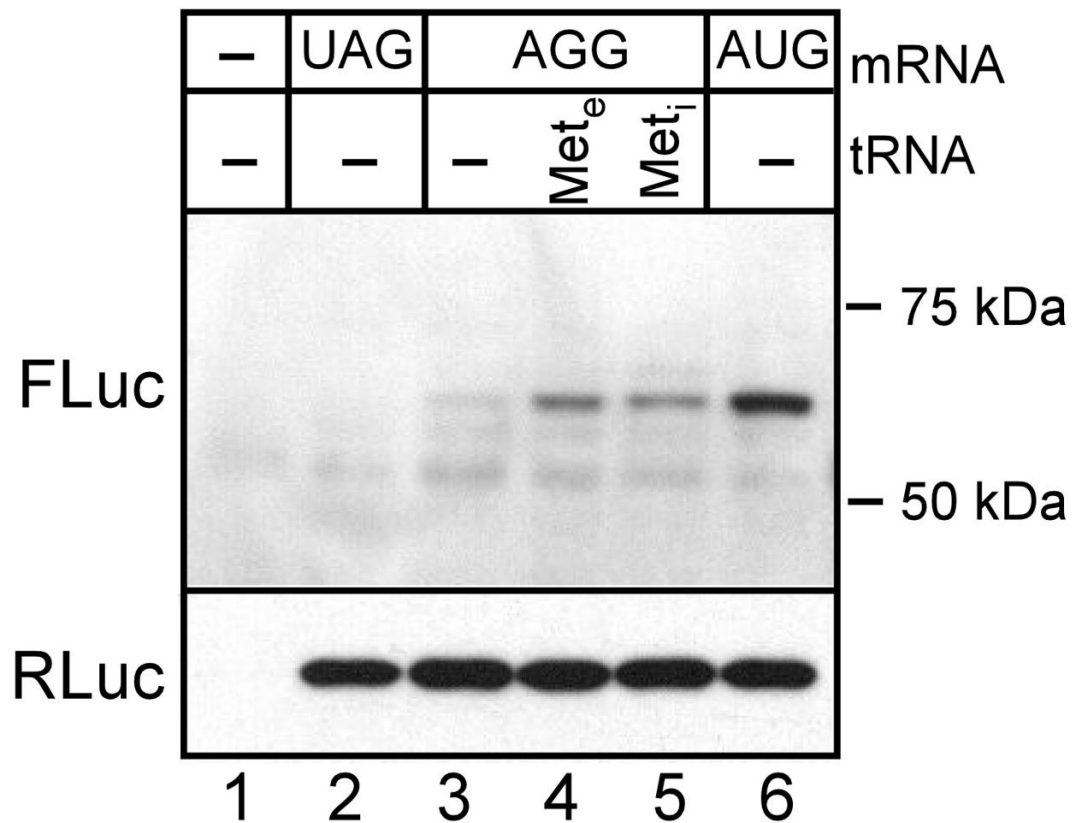


Figure 3.

IGR IRES directs translation from the ribosomal A site using elongator methionine tRNA (Met_e) and from the P site using initiator methionine tRNA (Met_i).

Immunoblot analysis of FLuc and RLuc synthesis in lysates of COS-1 cells transfected with mutant reporters carrying UAG, AGG or AUG as the initiation codons as indicated. Lanes 2, 3 and 6, no tRNA; lane 4, (+Met_e mutant tRNA) and lane 5, (+Met_i mutant tRNA). Lane 1, mock transfection.

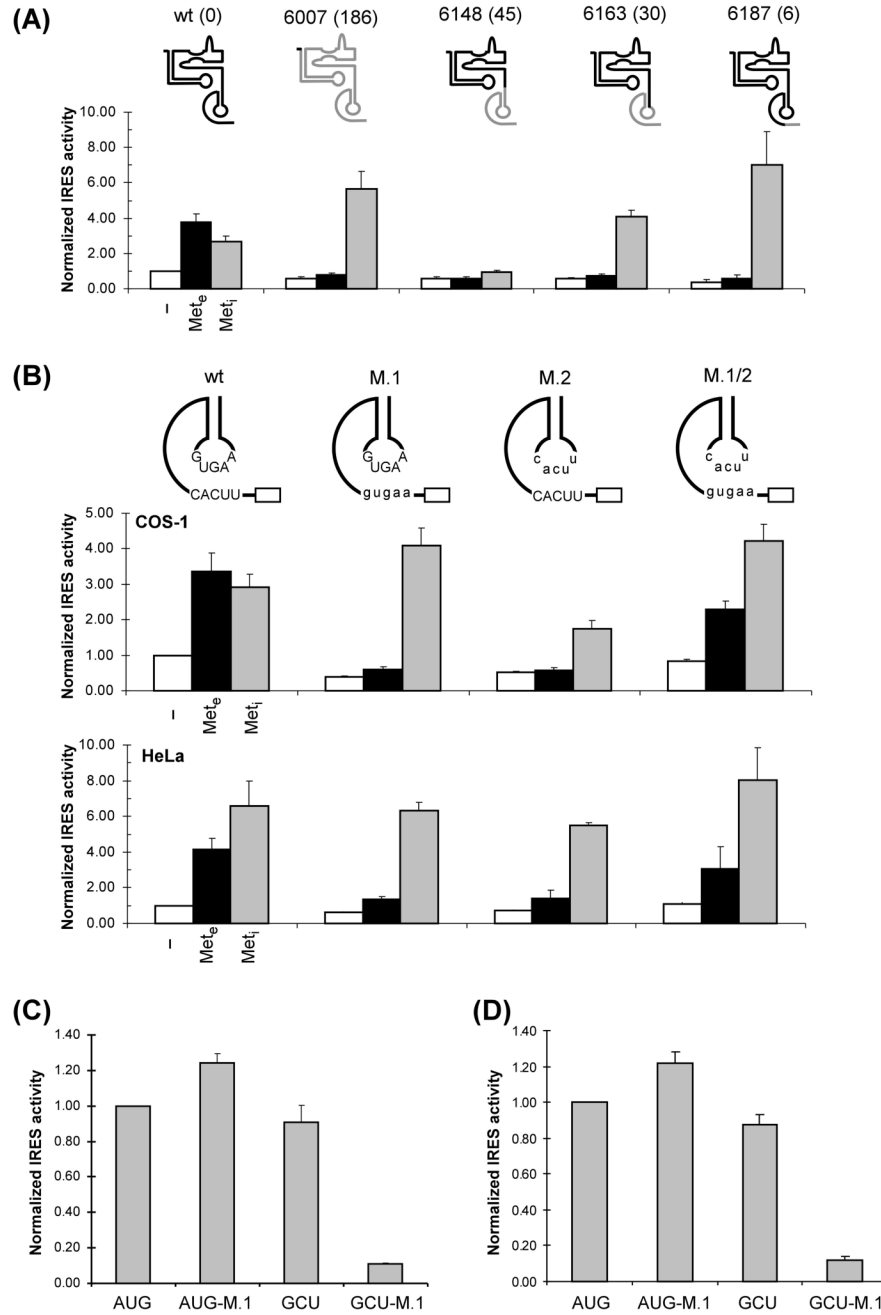


Figure 4.

Effects of mutations in IGR IRES on initiation from the AGG codon in cells expressing the C35 mutants of Met_e or Met_t tRNA.

A. (Top) Deleted regions are indicated by shading, numbers inside parenthesis show the number of nucleotides deleted. (Bottom) Effects of expression of mutant tRNAs in COS-1 cells are shown as FLuc/RLuc ratios normalized to those in the absence of expression of mutant tRNAs for wild type IRES and AGG as the initiation codon.

B. (Top) Nucleotides of PKI that are mutated are shown in lowercase letters. (Bottom) Effects of expression of mutant tRNAs in initiation in COS-1 and in HeLa cells are shown as FLuc/RLuc ratios normalized as in Figure 4A.

- C. The effect of PKI mutation on IGR IRES activities of mutant reporters carrying AUG or GCU as the initiation codon for FLuc synthesis in COS-1 cells. FLuc/RLuc ratios are normalized to that of the reporter with wild type IRES and AUG as the initiation codon.
- D. As in C except that COS-1 cells were transfected with RNA instead of DNA.

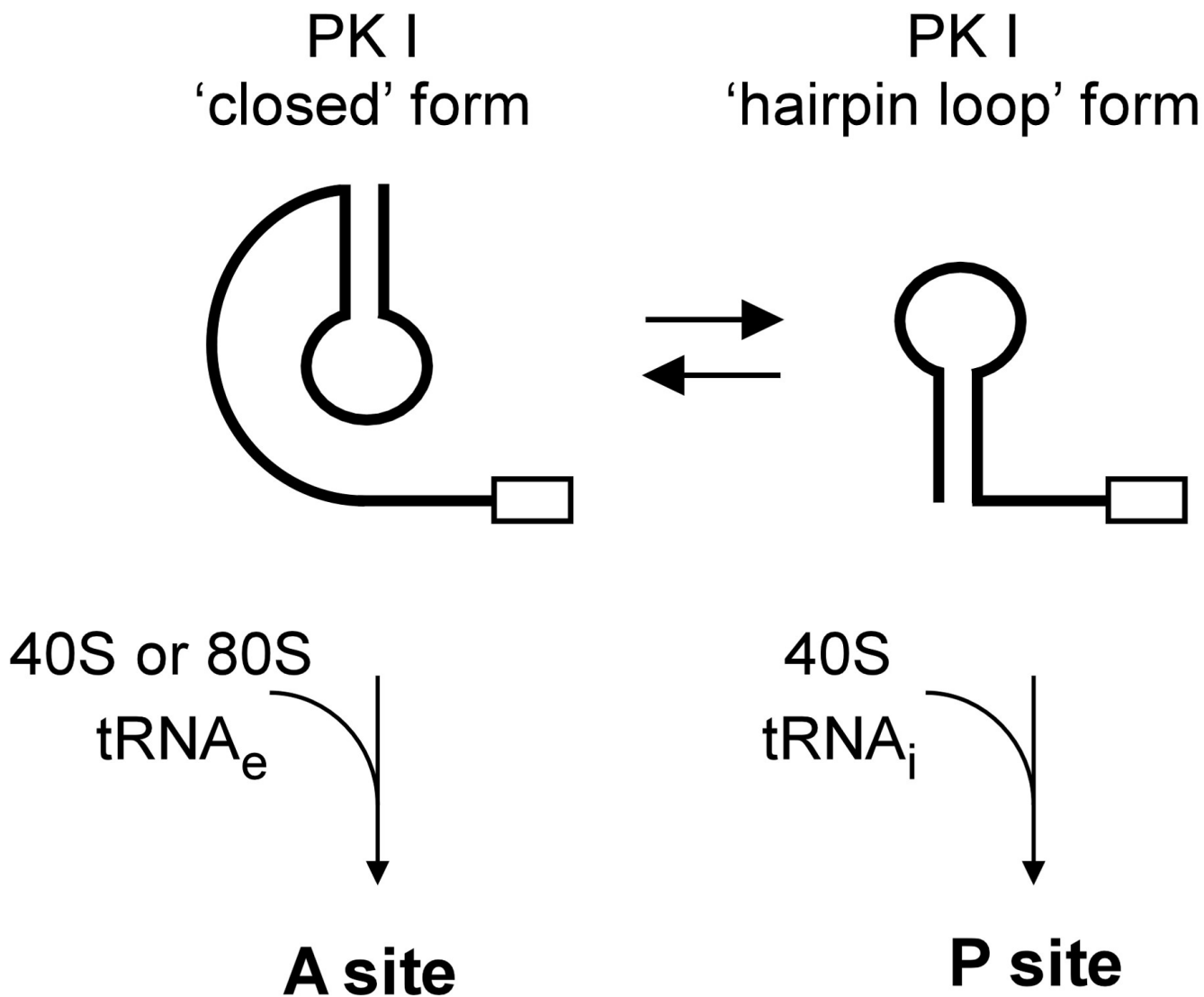


Figure 5.

A plausible hypothesis for IGR IRES-mediated translation initiation from either the ribosomal A site or the P site.

Left, When PKI is formed (closed form), 40S or 80S ribosome recruited to IGR IRES initiates translation from the ribosomal A site using an elongator tRNA, whose anticodon sequence is complementary to that of the initiation codon.

Right, When PKI is not formed (hairpin loop form), ribosome recruited to IGR IRES initiates translation from the P site using an initiator tRNA, if its anticodon sequence is complementary to that of the initiation codon.

Table 1
Activity of mutant methionine tRNAs in translation of mutant monocistronic FLuc mRNAs^a.

FLuc mutant	no tRNA	Met _e	Met _f
Met 1 ^b	0.57±0.15 (0.0)	0.95±0.11 (0.0)	162.0±5.43 (3.7)
Met 196 ^c	0.19±0.02 (0.0)	152.4±22.6 (3.5)	0.62±0.26 (0.0)
wt FLuc	4344.4±479.6 (100)	ND	ND

^a Average and standard deviations of FLuc luminescence (10³ RLU/μg) after 36 hours of triplicate transfections into COS-1 cells are shown. 0.25 μg monocistronic humanized RLuc and 0.25 μg monocistronic mutant or wild type FLuc plasmid were transfected with 0.16 μg tRNA plasmid as described in Experimental Procedures. FLuc activities shown are normalized for transfection efficiency using RLuc activity as control. Percent activity compared to wild type FLuc activity is shown in parenthesis.

^b Initiation codon is changed to AGG..

^c Internal methionine codon at amino acid position 196 is changed to the arginine codon AGG..

ND, not determined.

Table 2

IGR-IRES directs translation initiation from the ribosomal A site using elongator methionine tRNA (Met_e) and from the P site using initiator tRNA (Met_i)^a.

	no tRNA	Met _e	Met _i
COS-1	1.00	3.28±0.28	2.70±0.21
HeLa	1.00	3.82±0.24	6.48±0.46

^a Activities of IGR-IRES with AGG as an initiation codon in COS-1 and HeLa cells in the presence of the C35 mutant of Met_e and Met_i tRNA, respectively.