Methionine-independent initiation of translation in the capsid protein of an insect RNA virus

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Protein synthesis is believed to be initiated with the amino acid methionine because the AUG translation initiation codon of mRNAs is recognized by the anticodon of initiator methionine transfer RNA. A group of positive-stranded RNA viruses of insects, however, lacks an AUG translation initiation codon for their capsid protein gene, which is located at the downstream part of the genome. The capsid protein of one of these viruses, *Plautia stali* intestine virus, is synthesized by internal ribosome entry site-mediated translation. Here we report that methionine is not the initiating amino acid in the translation of the capsid protein in this virus. Its translation is initiated with glutamine encoded by a CAA codon that is the first codon of the capsid-coding region. The nucleotide sequence immediately upstream of the capsid-coding region interacts with a loop segment in the stem–loop structure located 15–43 nt upstream of the 5′ end of the capsid-coding region. The pseudoknot structure formed by this base pair interaction is essential for translation of the capsid protein. This mechanism for translation initiation differs from the conventional one in that the initiation step controlled by the initiator methionine transfer RNA is not necessary.

Plautia stali intestine virus (PSIV) is an insect RNA virus that was isolated from the brown-winged green bug *P. stali* (1). PSIV is physicochemically similar to mammalian picornaviruses (1), but its genome organization is different from that of picornaviruses (2). The positive-sense RNA genome of PSIV has two nonoverlapping ORFs (Fig. 1*A*). Several viruses with a genome organization similar to that of PSIV, including cricket paralysis virus (3), *Drosophila* C virus (DCV) (4), *Rhopalosiphum padi* virus (RhPV) (5), and Himetobi P virus (HiPV) (6), have been found in various species of insects (7). Recently, these viruses have been classified into a novel group, the "cricket paralysis-like viruses," which is distinct from the family *Picornaviridae* (8).

The capsid protein precursor of PSIV, which is encoded in the downstream ORF (Fig. 1*A*), is synthesized by internal ribosome entry site (IRES)-mediated translation from the genomic RNA (9). IRES-mediated translation first was identified in mammalian picornaviruses (10, 11). The IRES consists of a highly structured RNA sequence located in the upstream region of the coding sequence. The tertiary structure of IRES recruits ribosomes onto the mRNA, and translation starts independently of the 5′ cap (12).

An unusual feature concerning PSIV capsid translation is that the capsid gene lacks an AUG initiation codon (9). The capsid protein genes of the other "cricket paralysis-like viruses" also share this feature (4–6). The codon used for translation initiation is almost always AUG in eukaryotes, although several non-AUG initiation codons, such as CUG, GUG, and ACG, have been identified (13–16). The previously reported non-AUG initiation codons differ from AUG by a single nucleotide. Because non-AUG initiation codons also are recognized by the anticodon of the initiator methionine transfer RNA ($tRNA_i^{Met}$), it is believed that a codon that is completely unrelated to AUG cannot support initiation (17–20). Therefore, the initiating amino acid is always methionine in a natively synthesized protein even though non-AUG codons are used as an initiation codon. We previously searched for the translation initiation site of the capsid protein gene of PSIV by using various site-directed mutants and identified the CUU triplet that is located just upstream of the capsid-coding region as the initiation site (9) (Fig. 1*C*). CUU differs from AUG by 2 nucleotides, and when translation was conducted by the conventional ribosome-scanning mechanism in a deletion mutant that lacked the 5′ part of the IRES, CUU did not function as the initiation site (9). This suggested that translation initiation directed by the PSIV IRES does not require a stable base pair interaction between the initiation site and the anticodon of $tRNA_i^{Met}$.

Here, we report that methionine is not the initiating amino acid for the translation of the PSIV capsid protein. An RNA pseudoknot structure formed just upstream of the capsid-coding region is required for this unusual translation initiation event.

Materials and Methods

Plasmids and Site-Directed Mutagenesis. To evaluate the effect of the pseudoknot structure on translation, mutations were introduced into pT7CAT-5375 (9) by using a Transformer Site-Directed Mutagenesis Kit (CLONTECH).

The monocistronic plasmids for the methionine incorporation assay were constructed by using pT7CAT-5375 (Fig. 1*B*) and pT7Blue (Novagen). To construct a truncated capsid-coding region containing a single AUG triplet (IRES-7072Met, Fig. 3*A*), we first amplified nucleotides 5800–6264 of the PSIV sequence from pT7CAT-5375 by PCR using the forward primer 5800-P (5′-CCG TCG ACT CTG GAG AGG ATG AAG GA-3′), which has a *Sal*I site at the 5′ end, and the reverse primer 6264-M (5′-CGG GAT CCT GCC TGC GCT CCT GGT A-3′), which has a *Bam*HI site at the 5′ end. This amplified product was blunt-ended, digested with *Bam*HI, and then ligated into pT7Blue that had been digested with *Hin*dIII, blunt-ended, and then digested with *Bam*HI. Next, we amplified a fragment containing nucleotides 6622–7096 of the PSIV sequence using the forward primer 6622-P (5′-CGG GAT CCG GGG CTC TGC CAT CCA AAG-3′), which has a *Bam*HI site at the 5′ end and the 3′ vector-specific reverse primer (9). The amplified fragment was digested with *Bam*HI and *Eco*RI and then ligated into those sites of pT7Blue into which nucleotides 5800–6264 of the PSIV sequence had been inserted, generating pIRES-7072Met (Fig. 3*A*). The mutagenesis of AUG to AGG at nucleotides 7072–7074 was performed by using pT7CAT-5375 as a template. The fragment nucleotides 6622–7096 containing this mutation was amplified by PCR using the 6622-P and the 3′ vector-specific primers and then digested with *Bam*HI and *Eco*RI. This result-

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Abbreviations: PSIV, *Plautia stali* intestine virus; IRES, internal ribosome entry site; $tRNA_i^{Met}$, initiator methionine transfer RNA; RRL, rabbit reticulocyte lysate; eIF, eukaryotic initiation factor.

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ant fragment was ligated into those sites of pT7Blue into which nucleotides 5800–6264 had been inserted, generating pIRES-ΔMet. A fragment of nucleotides 5800–6264 containing two mutations, AAG to CAT at nucleotides 6163–6165 and CTT to ATG at nucleotides 6190–6192, was amplified by PCR with the 5800-P and the 6264-M primers, using p6163cat-6190atg (Fig. 2*A*) as a template. This PCR product was digested with *Sal*I and *Bam*HI and ligated to pIRES-ΔMet, from which a 0.4-kb *Sal*I-*Bam*HI fragment had been removed, yielding pIRES-pkAUGΔMet. To allow translation to start at the AUG codon by the conventional scanning mechanism, we constructed pΔIRES-6190Met. PCR was performed with a forward primer (nucleotides 6173–6202) that contained a CUU-AUG substitution at nucleotides 6190–6192 and the 3′ vector-specific primer by using pIRES-pkAUGΔMet as a template. The amplified fragment was blunt-ended, digested with *Eco*RI, and then ligated into pT7Blue that had been digested with *Hin*dIII, blunt-ended, and then digested with *Eco*RI. We confirmed the sequences of the plasmids that should be transcribed with T7 RNA polymerase by DNA sequencing.

***In Vitro* Transcription and *in Vitro* Translation.** Before *in vitro* transcription, all of the plasmids were linearized by digestion with *Eco*RI. The uncapped RNAs were transcribed by T7 RNA polymerase by using a Ribomax large-scale RNA production system (Promega). Twenty micrograms per milliliter of RNA was used in the *in vitro* translation assay. The *in vitro* translation assay of dicistronic RNAs was carried out in rabbit reticulocyte lysate (RRL) by using an enhanced chemiluminescence (ECL) *in vitro* translation system (Amersham) as described (9). Monocistronic RNAs were translated in a reaction mixture containing 20 mM Hepes buffer (pH 7.6), 1 mM ATP, 20 μM GTP, 2 mM DTT, 5 mM creatine phosphate, 0.05 mg/ml creatine kinase, 0.1 mM spermine tetrahydrochloride (Sigma), 40% RRL (type I; Boehringer Mannheim), 0.5 mM magnesium acetate, 110 mM potassium chloride, biotinylated lysyl-tRNA (Amersham), and 20 μM of each amino acid except lysine and methionine. For each RNA, a master reaction mixture without methionine was divided into two aliquots: 2.2 MBq of [^{35}S]methionine (Amersham) was added to one aliquot, and 20 μM unlabeled methionine was added to the other aliquot. The final volume of each reaction mixture was 25 μl, and the samples were incubated at 30°C for 90 min. The translation products of the reaction with unlabeled methionine were detected by the ECL system. To concentrate the translation products of the reaction with [^{35}S]methionine, 5 μl of Tetralink Tetrameric Avidin Resin (Promega), and 0.5 ml of PBST buffer (80 mM Na_2HPO_4/20 mM NaH_2PO_4/100 mM NaCl/0.1% Tween 20, pH 7.5) were added to each reacted sample and agitated for 90 min. The absorbed resins were washed with PBST three times and were suspended in a SDS loading buffer. The concentrated translation products were electrophoresed on an SDS/15% polyacrylamide gel and then visualized by autoradiography.

Results and Discussion

A Pseudoknot Structure Is Required for IRES-Dependent Translation Initiation of the PSIV Capsid Protein. Translation of the PSIV capsid protein is mediated by IRES (9). Analysis of the secondary structure of the PSIV IRES suggested a pseudoknot formation by the 5-bp interaction between the loop segment (nucleotides 6163–6167) and the sequence immediately upstream of the capsid-coding region (nucleotides 6188–6192) (Fig. 1*C*). Similar pseudoknot structures seem to have been conserved in viruses related to PSIV, including DCV, RhPV, and HiPV (6, 9). The capsid protein gene of these three viruses also lacks an AUG initiation codon. These observations suggested that the predicted pseudoknot structure plays an important role in initiating translation of the capsid gene.

To evaluate the importance of the predicted pseudoknot

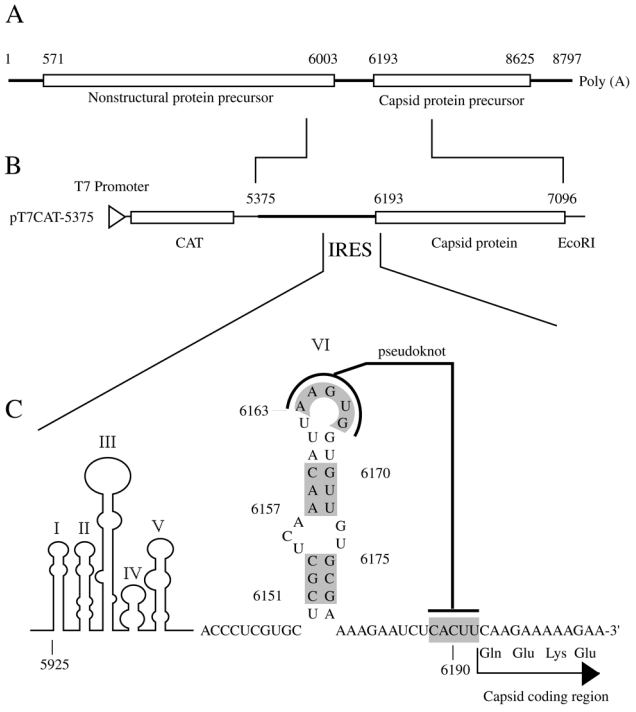

Fig. 1. Schematic diagram of the PSIV genome, pT7CAT-5375, and the IRES region of pT7CAT-5375. (*A*) Organization of the PSIV genome. The open boxes and bold lines indicate coding and noncoding regions, respectively. The numbers on the line represent nucleotide positions in the PSIV sequence. (*B*) Organization of the dicistronic plasmid, pT7CAT-5375, which contains the chloramphenicol acetyltransferase (CAT) gene and the 5′ portion of the capsid protein gene of PSIV. The thin line indicates the vector sequence, and the triangle on the line represents the location of the T7 promoter sequence. The thick line is the PSIV sequence. The numbers on the lines represent nucleotide positions in the PSIV sequence. (*C*) The predicted stem–loop structures (I–VI) upstream of the capsid-coding region of PSIV. Mutations were introduced into the shaded regions in stem–loop VI and in nucleotides 6190–6192. The base pair interactions in the predicted pseudoknot between the loop of the stem–loop structure (nucleotides 6163–6167) and the sequence just upstream of the capsid-coding region (nucleotides 6188–6192) are connected with a bold line.

structure for translation of the capsid protein, we first examined whether the stem–loop structure that includes base pair interactions between nucleotides 6151–6153 and 6175–6177 and between nucleotides 6157–6159 and 6170–6172 is actually necessary for translation mediated by PSIV IRES. Various mutations were introduced into the two stem segments of stem–loop VI of the dicistronic plasmid, pT7CAT-5375 (Fig. 1 *B* and *C*), and RNAs transcribed from these mutants were translated in RRL. Mutations that disrupted the base pair interaction of a stem segment (at nucleotides 6151–6153, 6157–6159, 6170–6172, or 6175–6177) abolished the capsid protein translation (Fig. 2*B*, lanes 1, 2, 4, and 5). When base pairing of the stem segments was restored, the translation products reappeared (Fig. 2*B*, lanes 3 and 6). These results confirm the existence of these stem segments and their functional importance in the translation of the capsid protein. Next, we tested the base pair interaction between the previously identified CUU triplet (9), nucleotides 6190–6192, and the loop segment, nucleotides 6163–6165 (AAG). The CUU triplet was mutated to AUG, UGC, and UAG. These three mutations were designed to represent a normal triplet for initiation, a triplet that is not related to either CUU or AUG, and a stop codon, respectively. In pT7CAT-5375, in which the CUU triplet was mutated to AUG, UGC, or UAG, the level of capsid protein translation was reduced when mutated to AUG or UGC (Fig. 2*B*, lanes 8 and 11) or abolished when

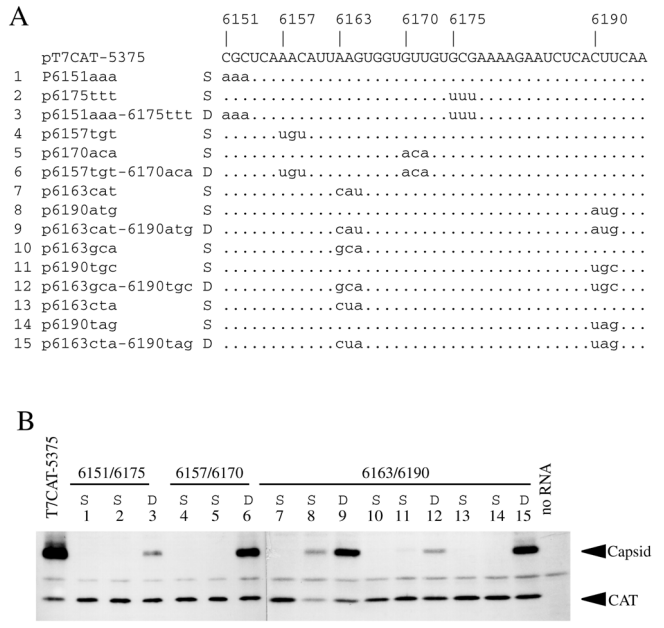

Fig. 2. Effect of mutations in the pseudoknot structure on IRES-mediated translation of PSIV. (*A*) Nucleotide sequence of a part of the transcript from the pT7CAT-5375 (Fig. 1*B*) and its site-directed mutants used for the translation assay. The numbers on the sequence represent nucleotide positions on the PSIV genome. (*B*) ECL detection of the translation products of the RNAs shown in *A*. The characters S and D preceding the sequences in *A* and above the lanes in *B* indicate mutations of a single triplet to disrupt base pair interactions and double mutations of the triplet along with its corresponding triplet to restore the base pair interactions, respectively.

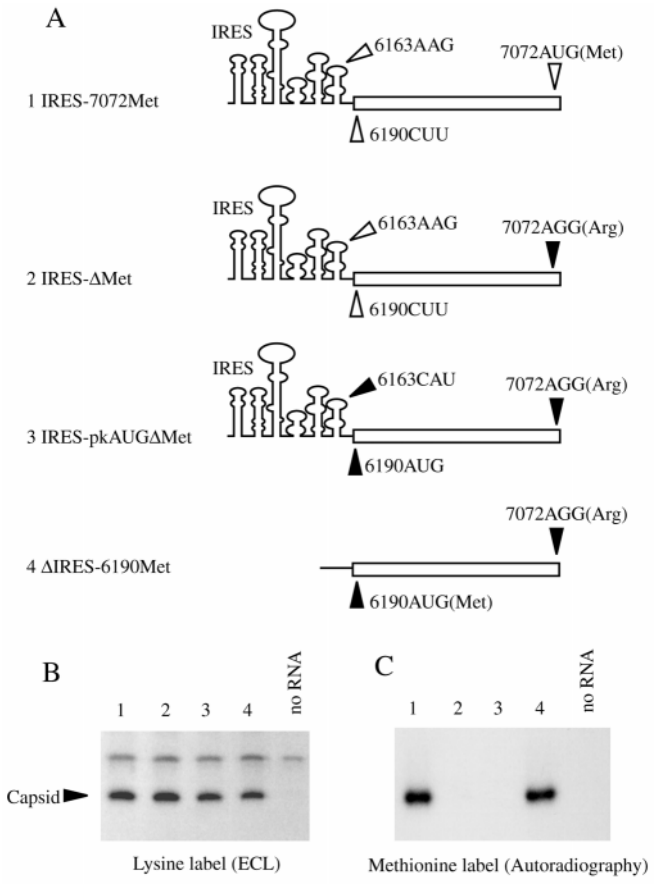

Fig. 3. Evidence that methionine is not the initiating amino acid in IRES-mediated translation of PSIV. (*A*) The RNA constructs used in the methionine-incorporation assay. The white and black arrowheads indicate wild-type and mutated codons, respectively. Each of the RNAs was translated in RRL in the presence of biotin-lysyl-tRNA and unlabeled or [^{35}S]methionine. (*B*) ECL detection of the translation products labeled with biotinylated lysine. (*C*) Autoradiography of the translation products labeled with [^{35}S]methionine.

mutated to UAG (Fig. 2*B*, lane 14). Similarly, in pT7CAT-5375, where AAG at nucleotides 6163–6165 were mutated to CAU, GCA, or CUA, there was no capsid translation (Fig. 2*B*, lanes 7, 10, and 13). When double mutations were introduced to restore the base pair interactions between nucleotides 6163–6165 and nucleotides 6190–6192, efficient translation occurred again in 6163cau-6190aug and 6163cua-6190uag (Fig. 2*B*, lanes 9 and 15). In the case of 6163 gca-6190ugc (Fig. 2*B*, lane 12), the efficiency of translation was lower than that of the other two double mutations (Fig. 2*B*, lanes 9 and 15); however, the yield of translation product was higher than that of the two single mutations (Fig. 2*B*, lanes 10 and 11). Recovery of the translation also was observed in a similar compensatory mutational analysis to test the base pair interaction between nucleotides 6167 and 6188 (data not shown). These results indicate that the structure of the region just upstream of the capsid-coding region is a pseudoknot structure and that this structure is important for translation of the capsid protein.

Methionine Is Not the Initiating Amino Acid in PSIV Capsid Translation. As shown in p6163cua-6190uag (Fig. 2*B*, lane 15) as well as in the wild-type pT7CAT-5375, an AUG codon at the initiation site is not necessary for translation initiation mediated by the PSIV IRES. This strongly indicates that a base pair interaction between the initiation site and the anticodon of $tRNA_i^{Met}$ is not essential for the IRES-dependent translation initiation. It has been shown that the 40S ribosomal subunit binds to a ternary complex composed of $tRNA_i^{Met}$, eukaryotic initiation factor 2 (eIF2), and GTP, before binding to mRNA (21). Therefore, our results raise the question of whether methionine is used in translation initiation mediated by PSIV IRES even though the initiation site does not interact with the anticodon of $tRNA_i^{Met}$.

To resolve this question, we synthesized four monocistronic RNAs (Fig. 3*A*). IRES-7072Met is composed of nucleotides 5800–7096 with a deletion of nucleotides 6265–6621, so that the capsid-coding region contained only one in-frame AUG codon at nucleotides 7072–7074. In comparison with IRES-7072Met, IRES-ΔMet has a mutation of AUG to AGG, at nucleotides 7072–7074, to leave out the in-frame AUG codon from the capsid-coding region. IRES-pkAUGΔMet has the same sequence as IRES-ΔMet except for two mutations in which the CUU triplet was changed to AUG and the corresponding triplet was changed to preserve the pseudoknot structure. Translation of these three RNAs is directed by the IRES. ΔIRES-6190Met has the same sequence as IRES-pkAUGΔMet except for a deletion of the 5′ part of the IRES (nucleotides 5800–6171), so that translation of ΔIRES-6190Met would be initiated at AUG at nucleotides 6190–6192 by the ribosome-scanning mechanism. We employed two methods for detection of translation products. The first method was the ECL system to detect proteins labeled with biotinylated lysine. The second method was autoradiography to detect proteins labeled with [^{35}S]methionine. ECL detection showed expression of the capsid protein from all four RNAs (Fig. 3*B*, lanes 1–4). The product of IRES-7072Met and ΔIRES-6190Met also was detected by autoradiography (Fig. 3*C*, lanes 1 and 4); however, that of IRES-ΔMet and IRES-pkAUGΔMet was not (Fig. 3*C*, lanes 2 and 3). These results indicate that the capsid proteins are translated from IRES-ΔMet and IRES-pkAUGΔMet; however, the products of the two RNAs

do not contain methionine. The N-terminal methionine of nascent polypeptides is known to be removed cotranslationally by a methionine aminopeptidase, and its removal is dependent on the penultimate residue (22). If methionine was used as the first amino acid of the products from IRES-ΔMet and IRES-pkAUGΔMet, the penultimate amino acid should be glutamine encoded by CAA at nucleotides 6193–6195 (Fig. 1*C*). Because the methionine–glutamine pair is known to be poorly cleaved by the methionine aminopeptidase (22), it is unlikely that this enzyme removes the N-terminal methionine from the products of these two RNAs. Indeed, autoradiography showed that the product from ΔIRES-6190Met keeps the N-terminal methionine (Fig. 3*C*, lane 4). These results demonstrate that the translation mediated by the PSIV IRES does not use methionine as the initiating amino acid.

In previous mutational analyses, we showed that a +1 frameshift by the insertion of cytosine immediately upstream of the CAA codon (nucleotides 6193–6195) of pT7CAT-5375 abolished translation of the capsid protein and that an additional −1 frameshift at nucleotide 6255 rescued the translation (9). This indicated that the reading frame of the capsid protein gene is determined upstream of the CAA codon. The triplet that is immediately upstream of the CAA codon is the CUU triplet (Fig. 1*C*). When the CUU triplet was changed to UAG, a universal stop codon, translation occurred if the pseudoknot structure was maintained (Fig. 2*B*, lane 15). Stop codons are recognized by protein molecules, not by tRNAs (23). This implies that the triplet immediately upstream of the CAA codon, i.e., the CUU triplet, does not interact with any tRNAs. Therefore, we concluded that PSIV capsid translation starts immediately downstream of the pseudoknot structure and that the CAA codon is the first codon that should be translated in this translation mechanism. The pseudoknot structure probably plays a key role in determining the first codon of translation directed by PSIV IRES.

A Putative Mechanism for Methionine-Independent Translation Initiation. In eukaryotes, it has been believed that the codon–anticodon interaction between an initiation codon and $tRNA_i^{Met}$ is essential for initiating translation in both the scanning mechanism and internal ribosome entry; however, this interaction is not essential for the translation initiation mediated by PSIV IRES. In the scanning mechanism, in addition to the codon–anticodon interaction, ancillary features such as flanking sequences of the initiation codon and secondary structure of the downstream sequence of the initiation codon also play roles in initiating translation (24, 25). These ancillary features are considered to slow the scanning of the 40S ribosome subunit to support the selection of the initiation codon (25). Although IRES-mediated translations of hepatitis C virus and two pestiviruses require the codon—anticodon interactions, the IRESs of these viruses have the ability to set the 40S ribosome subunit at the precise position without eIF2 and $tRNA_i^{Met}$, i.e., the peptidyl-tRNA site (P site) of the 40S ribosome subunit is placed at the authentic AUG initiation codon of the RNAs (26, 27). The binding of ribosomes to these IRESs does not require eIF3, 4A, 4B, and 4F, suggesting that a prokaryotic-like mode of ribosome binding is possible in these IRESs (26). In the case of prokaryotes, Phe-$tRNA^{Phe}$ can bind to the aminoacyl-tRNA site (A site) of poly(U)-programmed 70S ribosome (28). In addition, translocation and synthesis of oligophenylalanine are possible (28). These observations would explain why PSIV IRES can initiate translation with an amino acid other than methionine. The tertiary structure of PSIV IRES would set the ribosome at the proper position, whereas the pseudoknot structure occupies the ribosomal P site. Then, Gln-$tRNA^{Gln}$ entry to the A site would occur and polypeptide synthesis would begin. Although it is not known which eIFs and host proteins are involved in the initiation mediated by PSIV IRES, the stable positioning of ribosomes on PSIV RNA would allow protein synthesis to begin at the elongation step, skipping the conventional translation initiation step.

Capsid protein genes of the other cricket paralysis-like viruses, DCV, RhPV, and HiPV, also lack AUG initiation codons (4–6), and a pseudoknot structure seems to be formed just upstream of their capsid-coding regions (6, 9). These observations suggest that translation of their capsid genes also is initiated with an amino acid other than methionine by a mechanism similar to that used in PSIV.

Translation initiation other than methionine in native protein synthesis recently has been reported in cryptic peptides presented by MHC class I molecules (29). This initiation decodes the CUG initiation codon not as the canonical methionine but as a leucine residue (29). Translation activity of the cryptic peptide is low, whereas that of the PSIV capsid protein is relatively high. As far as we examined the secondary structure of the region immediately upstream of the coding sequence of the cryptic peptide using computer programs, a pseudoknot structure similar to that of PSIV was not formed. This means that the translation initiation mechanism of the cryptic peptide is different from that of the PSIV capsid protein. In conclusion, data of PSIV and the cryptic peptide show that the rule that methionine is the initiating amino acid is not absolute in eukaryotic protein synthesis.

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