INTRODUCTION

The 5’ ends of eukaryotic mRNAs are generally capped by 7-methylguanosine. In the presence of eukaryotic initiation factors, recruitment of ribosomes to mRNAs is cap-dependent and translation initiation occurs via the ribosome scanning mechanism (Kozak, 1989). In contrast, viral RNAs, which do not have a 5’ 7-methylguanosine cap, usually have a long 5’ untranslated region (UTR) with tertiary structure that recruits ribosomes independently of the 5’ termini. Such cis-acting RNA elements are known as internal ribosome entry sites (IRES) (Hellen & Sarnow, 1989).

*Plautia stali* intestine virus (PSIV, *Cripavirus, Dicistroviridae*) is a member of the genus *Cripavirus* in the family *Dicistroviridae*. *Rhopalosiphum padi* virus (RhPV), *Triatoma virus* (TrV), *Cricket paralysis virus* (CrPV), *Drosophila C virus* (DCV) and *Taura syndrome virus* (TSV) also belong to the family *Dicistroviridae* (Christian et al., 2005). Dicistroviruses are characterized by a monopartite positive-stranded RNA genome with two non-overlapping open reading frames (ORFs). The 5’ and 3’ ORFs encode non-structural and structural protein precursors, respectively. The intergenic region (IGR) between the two ORFs contains an IGR-IRES (Czibener et al., 2005; Domier et al., 2000; Sasaki & Nakashima, 1999, 2000; Wilson et al., 2000). Because dicistroviral IGR elements have a common secondary structure with only minor variations, the mechanism for initiation in IGR-IRES-mediated translation is believed to be essentially the same in all dicistroviruses (Kanamori & Nakashima, 2001; Nishiyama et al., 2003; Hatakeyama et al., 2004). Of the IGR-IRES elements that have been tested, all function in numerous eukaryotic lysate systems, including mammal (Domier et al., 2000; Sasaki & Nakashima, 1999), plant (Wilson et al., 2000; Shibuya et al., 2003) and yeast (Thompson et al., 2001).

Translation of a truncated 5’ ORF under the control of the 5’ UTR of PSIV was previously examined in rabbit reticulocyte lysate (RRL), but no products were detected (Sasaki et al., 1998). Recent reports, however, show that the 5’ UTRs of other dicistroviruses, such as RhPV and CrPV, function as an IRES: the 5’ UTR of RhPV functions in plant, mammal and insect cell lysate systems (Royall et al., 2004; Woolaway et al., 2001), whereas the 5’ UTR of CrPV functions in RRL, but not in wheatgerm extract (WGE) (Wilson et al., 2000). These reports suggest that the 5’ UTR of PSIV may function as an IRES, but that its activity is dependent on which cell-free system is used. Data presented here show that the 5’ UTR of PSIV contains an IRES that is functional in insect cell lysates, but not in RRL or WGE, suggesting that the requirements for translation initiation mediated by the 5’ UTR IRES vary among dicistroviruses.

METHODS

Plasmid construction. A cDNA fragment containing the 5’ UTR of PSIV (nt 1–736 of the PSIV genome) was amplified by RT-PCR.
from viral RNA template using primers 5'-gaagtcgaccaatggtgagtagata-3' (forward) and 5'-tacagttgctatgataataagga-3' (reverse). The underlined regions of the forward and reverse primer sequences represent recognition sequences for PstI and NcoI, respectively. The 736 nt PCR fragment was digested with PstI and NcoI and gel-purified for ligation with PstI/NcoI-cut pT7BFluc, Novagen’s pT7Blue plasmid with the cDNA sequence for firefly luciferase (Fluc) from pSP-luc (Promega) inserted at KpnI and EcoRI. The resulting plasmid, which carries a Flu direction gene under the control of the 5' UTR of PSIV, was named pT7B5PSIVFluc. pT7B5PSIVFluc was digested with PstI and blunted with T4 DNA polymerase and the vector was then further digested with EcoRI to isolate the 2.5 kb PSIV-Fluc fragment. A plasmid containing the Renilla luciferase gene (RLuc) (pRLuc; Hatakeyama et al., 2004) was digested with XbaI, treated with Klenow fragment and then cut with EcoRI for ligation with the 2.5 kb PSIV-Fluc fragment to generate pT7Rluc-1IRES286-Fluc. Construction of pT7Rluc-5950IRES6240-Fluc, pRLuc and pRLuc-Fluc has been described previously (Hatakeyama et al., 2004; Shibuya et al., 2004).

Mutants to determine the initiation AUG triplets and the 3' and 5' borders of the 5' IRES were constructed by PCR-based mutagenesis using pT7B5PSIVFluc as template. The mutated PSIV sequence was recovered by digestion with PstI and NcoI and then ligated into those sites of pT7B5PSIVFluc to replace wild-type PSIV sequence with mutated sequences. Mutations were confirmed by DNA sequencing. Dicistronic constructs carrying these mutations were prepared according to the methods for pT7Rluc-1IRES736-Fluc described above.

For structure analysis, a partial cDNA of PSIV (nt 225–736) was amplified by RT-PCR with forward and reverse primers that incorporate PstI and NcoI recognition sequences, respectively, into their 5' sequences. The obtained fragment was digested with PstI and NcoI and ligated into those sites of pT7B5PSIVFluc. The resultant recombinant plasmid was linearized with NcoI prior to in vitro transcription.

**In vitro transcription.** In general, plasmids were linearized with EcoRI. Capped RNAs were transcribed using mMESSAGE mMACHINE (Ambion) and uncapped RNAs were transcribed using T7-RiboMAX Express Large Scale RNA Production System (Promega) according to the manufacturers’ recommendations. Transcribed RNAs were extracted with phenol and precipitated with 2-propanol in the presence of ammonium acetate.

**In vitro translation and detection of translation products.** In vitro translations using insect Spodoptera frugiperda 21 (SF21) cell lysate (Transdirect insect cell; Shimadzu), WGE (Promega) and RRL (Promega) were conducted according to the manufacturers’ recommendations. To examine the effect of a cap analogue, mGppG (Promega) was added to reaction mixtures at a final concentration of 0.5 mM. Rluc and Fluc enzymic activities were measured as described previously (Shibuya et al., 2004). To determine their relative translation efficiencies, translation products were labelled with 1 μl biotinylated lysyl-tRNA (Transcend tRNA; Promega) per 2 μl reaction mixture containing 3 pmol template RNA. After incubation for 60 min, an aliquot of each reaction mixture was separated by SDS-PAGE (9% polyacrylamide) and products were detected by chemiluminescence as described previously (Shibuya et al., 2003). To detect translation products by fluorescence, FluoroTect Green Lys-tRNA (Promega) was used instead of biotinylated lysyl-tRNA and translation products were visualized using Typhoon 9410 (Amersham Bioscience).

**Chemical and enzymic structure probing analysis.** Modifications of refolded RNAs after heat treatment using dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate (CMCT) and RNase T1 (Ambion) were carried out as described previously (Shibuya et al., 2003). Primers corresponding to nt 342–327, 428–412, 510–493, 590–573 and 666–650 in the PSIV sequence were used for targeting reverse transcriptase to the template.

**RESULTS AND DISCUSSION**

**The 5' UTR of PSIV contains an IRES**

To test internal initiation mediated by the 5' UTR of PSIV, a dicistronic plasmid pT7Rluc-1IRES736-Fluc (Fig. 1a) was constructed that expresses Rluc as the first cistron, nt 1–736 of the PSIV sequence and Fluc as the second cistron, all under the control of a T7 promoter. Transcripts from the plasmid were translated in vitro in the presence or absence of cap analogue using lysate prepared from SF21 insect cells. In a comparison of luciferase activities derived from different translation mixtures, Rluc activity decreased 5-fold in the presence of 0.5 mM cap analogue compared with its activity in the absence of analogue (Fig. 1b). In contrast, Fluc activity did not decrease in the presence of cap analogue, indicating that translation of the second cistron of the RNA was cap-independent.

The efficiency of translation mediated by the 5' UTR of PSIV was examined by comparing Fluc activity levels generated using the following RNA templates: capped monocistronic Fluc RNA, uncapped dicistronic Rluc–1IRES736–Fluc RNA, Rluc–5950IRES6240–Fluc RNA and Rluc–Fluc RNA that has no inserted sequence between the two cistrons. Because translation efficiencies in cell-free systems are sensitive to ions, the concentrations of potassium and magnesium were adjusted in the SF21 cell lysate. Since preliminary tests showed that adding salts increased IGR-IRES activity, but decreased the activity of other RNAs, translation of RNAs other than IGR-IRES was done without any adjustment in ion concentration. PSIV 5' UTR-mediated translation showed Fluc activity [0.55 × 10^6 relative light units (RLU)] that was half that of IGR-IRES-mediated translation (Fig. 1c). Because Fluc activity originating from the Rluc–Fluc dicistronic RNA that lacked the intervening sequence between the two cistrons was 14-fold lower than activity from Rluc–1IRES736–Fluc RNA, it is concluded that the 5' UTR of PSIV functions as an IRES in the insect cell-free system.

**The first in-frame AUG triplet is the initiation site for PSIV ORF1**

In sequence databases, the ORF1 start site of dicistroviruses has been assigned in various ways. ORF1 in TrV (GenBank accession no. AF178440) opens with the triplet that immediately follows an in-frame stop codon, probably because dicistroviral IGR-IRES do not use an AUG triplet for initiation. In both DCV (AF014388) and TSV (AF277675), the second in-frame AUG triplet has been assigned as the ORF1 start site because it is present in a suitable context for invertebrate initiation (Cavener & Ray, 1991). In other dicistroviruses, the first in-frame AUG triplet has been assigned as the initiation site. In PSIV, the 5'
UTR (nt 1–570) contains nine AUG triplets and the 5’ part in the 5’ ORF of PSIV also encodes four in-frame AUG triplets (at nt 571–573, 574–576, 628–630 and 697–699).

**Fig. 1.** IRES-mediated translation by the PSIV 5’ UTR in Sf21 insect-cell lysate. (a) Plasmids used for T7 polymerase-mediated transcription. Open triangles indicate the T7 promoter. Open boxes indicate ORFs for firefly luciferase (Fluc), Renilla luciferase (Rluc), truncated PSIV non-structural (NS) or capsid (CP) coding sequences that have been fused with the Fluc coding sequence. Bold lines indicate the PSIV 5’ UTR or IGR, with nucleotide positions in the PSIV genome shown below. (b) Cap-independent translation by the 5’ UTR of PSIV. Capped transcripts from pT7Rluc-1RES736-Fluc were translated in the presence or absence of cap analogue. Enzyme activity in the absence of cap analogue was set to a relative value of 1.0. Error bars represent the SD of triplicate assays. (c) Translation of the second cistron mediated by the 5’ UTR of PSIV. Capped transcripts from pFluc or uncapped transcripts from pT7Rluc-1RES736-Fluc, pT7Rluc-5950RES6240-Fluc or Rluc-Fluc were translated and Fluc activity was measured. Error bars represent the SD of triplicate assays.

UTR (nt 1–570) contains nine AUG triplets and the 5’ part in the 5’ ORF of PSIV also encodes four in-frame AUG triplets (at nt 571–573, 574–576, 628–630 and 697–699).

**Fig. 2.** Identification of the initiator AUG for the PSIV non-structural protein precursor. (a) PSIV genomic sequence of nt 551–736. In-frame AUG triplets in the 5’ region of PSIV ORF1 are underlined. An in-frame UAG triplet preceding the first AUG is shown in bold. (b) Translation products visualized by chemiluminescent detection. Arrowheads point to endogenous biotinylated proteins in Sf21 insect cell lysate. (c) Translation products from uncapped transcripts of wild-type pT7Rluc-1RES736-Fluc and AUG variants visualized by fluorescence imaging.
(Fig. 2a). Among these, AUG571–573 and AUG697–699 are most likely to be suitable for translation initiation in invertebrates, but their functionality has not been examined.

To identify the translation initiation site for ORF1 of PSIV, the relative molecular masses of the 5′ IRES-mediated translation products were compared by SDS-PAGE. Transcripts from pT7Rluc-1IRES736-Fluc produced an Fluc fusion protein that migrated more slowly (Fig. 2b, lane 4) than authentic Fluc (Fig. 2b, lane 3), suggesting that initiation occurred within the upstream PSIV sequence. To compare the relative electrophoretic mobilities of NS–Fluc fusion proteins that could initiate at AUG697–699, AUG628–630, AUG574–576 or AUG571–573 with known standards, monocistronic PSIV-Fluc RNAs that are initiated by ribosome scanning were constructed. The mobilities of products initiated at AUG628–630 and AUG697–699 were faster than those of products of Rluc-1IRES736-Fluc (Fig. 2b, lanes 1, 2 and 4), indicating that the initiation site is likely to be either AUG571–573 or AUG574–576. To distinguish between these two sites, AUG574–576 was changed to AUA by PCR-based mutagenesis and fluorescence was used as a measure of initiation because of the many endogenous biotinylated proteins in insect cell lysates (Fig. 2b). Mutation of AUG574–576 did not affect the amount of product from the second cistron (Fig. 2c, lane 4); however, there was a significant decrease in the amount of product from the second cistron in a mutant in which AUGAUG571–576 was replaced by AUAAUA (Fig. 2c, lane 5). Translation of this mutant resulted in a very faint band that migrated to the same position as NS–Fluc (Fig. 2b, lane 5). It is hypothesized that this was a non-AUG-initiated product from the mutated AUA codon because a UAG stop codon is located five triplets upstream of AUG571–573 (Fig. 2a) and there are no AUG-like triplets between the UAG triplet and AUAAUA571–573 in the mutated RNA. These results indicate that AUG571–573 is the initiation site for viral protein production.

**The 5′ IRES of PSIV is composed of approximately 350 nt**

To examine the 5′ boundary of the 5′ IRES of PSIV, deletion mutants were constructed from pT7Rluc-1IRES736-Fluc (Fig. 3a). When uncapped transcripts from the mutants were translated, luciferase activity from 5′-Δ25 and 5′-Δ225 did not decrease (Fig. 3b). This indicates that the 5′-terminal 225 nt of PSIV are not necessary for IRES-mediated translation. However, the luciferase activity of p5′-Δ300 and p5′-Δ375 transcripts was two-thirds and one-third of that of the wild-type, respectively. Although these results do not define a clear 5′ boundary for the IRES, they suggest that a 5′ UTR that includes a sequence downstream of position 225 would be necessary for a fully functioning IRES.

In assays defining the 3′ boundary of the IRES, transcripts truncated at p696-Fluc and p627-Fluc promoted more than 75 % of the wild-type luciferase activity, but luciferase levels from p570-Fluc and p573-Fluc were only 6 and 11 % of the wild-type, respectively (Fig. 3b). These results are in contrast to similar assays of RhPV; the RhPV 5′ IRES does not require any viral coding sequence (Woolaway et al., 2001). The G+C content of the PSIV 5′ UTR is unusually high (43 %) relative to RhPV (34 %). Based on previous experiments with the IGR-IRES of PSIV, in which a G+C-rich introduced nucleotide sequence inhibited Fluc from p570-Fluc and p573-Fluc were only 6 and 11 % of the wild-type, respectively (Fig. 3b). These results are in contrast to similar assays of RhPV; the RhPV 5′ IRES does not require any viral coding sequence (Woolaway et al., 2001). The G+C content of the PSIV 5′ UTR is unusually high (43 %) relative to RhPV (34 %). Based on previous experiments with the IGR-IRES of PSIV, in which a G+C-rich introduced nucleotide sequence inhibited Fluc
(Shibuya et al., 2003), it is predicted that the high G+C content downstream of the 5′ IRES might also be inhibitory to translation. The requirement for the viral coding sequence, on the other hand, was conditional, and there was a positive correlation between high IGR-IRES activity and low G+C content within the 5′ regions of coding sequences (Shibuya et al., 2003).

To test whether the same correlation existed within the 5′ IRES, silent mutations that lowered the G+C content of Fluc at positions 570-Fluc and 573-Fluc were constructed as described previously (Shibuya et al., 2004). Luciferase activity from 570-Fluc(mut) and 573-Fluc(mut) templates was 4- and 11-fold higher, respectively, than from the wild-type (Fig. 3b), suggesting that the high G+C content of wild-type Fluc decreases translation efficiency. The 573-Fluc(mut) showed 5-fold higher translation activity compared with 570-Fluc(mut). The difference in nucleotide sequences in the two constructs is the presence of an additional AUG triplet after the initiator AUG in the 573-Fluc(mut). Because other constructs were not examined, the reason for this difference in translation activity is difficult to explain; however, efficiency of translation initiation is sometimes affected by nucleotide sequences downstream of the initiation codon. Although 570-Fluc(mut) showed lower translation activity of the second cistron compared with 573-Fluc(mut), detected Fluc activity from 570-Fluc(mut) was apparently higher than 570-Fluc containing the wild-type Fluc sequences (Fig. 3b). These results suggest that sequences downstream of the initiation codon affect translation efficiency, but the viral coding sequence is not an absolute requirement for 5′ IRES-mediated translation of PSIV.

Structure of the 5′ IRES elements in PSIV and RhPV is distinct

Chemical modifications that probe the structure of 5′ IRES have been utilized with RhPV, but not other dicistron viruses. Among the compounds tested, DMS modifies unpaired adenine and cytosine residues, CMCT modifies unpaired uridines and guanines and RNase T1 cleaves unpaired guanines. Subsequent reverse transcription of modified RNA templates with 32P-labelled primers produces bands at position −1 relative to any modified nucleotide.

In this study, renatured RNA molecules encoding nt 225–736 of PSIV were probed with DMS, CMCT and RNase T1 (Fig. 4). The resulting data were analysed with the mathematical predictions that operate in the program MFOLD (Zuker, 2003) to construct a secondary structure model of the 5′ IRES (Fig. 5). The resulting models suggest that the 5′ IRES of PSIV forms several stem–loop structures. In addition, a pseudoknot formation was suggested between nt 323–326 (ggcc) and 344–341 (cggg) (Fig. 5), because these nucleotides were not modified by probing reagents (Fig. 4). To examine the effect of these base-pair interactions on 5′ IRES-mediated translation, nt 323–326 (ggcc) in

![Fig. 4. Chemical and enzymic probing to determine PSIV 5′ IRES structure. Gels show primer extension analyses of RNAs treated with DMS (lane 5), CMCT (lane 7) or RNase T1 (lane 9). Lanes 6, 8 and 10 are untreated controls. Lanes 1–4 are marker lanes that mark the positions of modified residues. Nucleotide positions corresponding to the PSIV genomic sequence are shown to the left.](http://vir.sgmjournals.org)
the Rluc-1IRES$_{368}$-Fluc construct were changed to cggg. Translation efficiency of the second cistron in this construct was estimated to be 54% of that of the authentic Rluc-1IRES$_{368}$-Fluc by measuring Fluc activity of the reaction mixture of the Sf21 insect cell lysate. Additional mutations restoring base-pair interaction between 323–326 (cggg) and 344–341 (gccc) recovered translation efficiency of the second cistron to 84% of that of the authentic Rluc-1IRES$_{368}$-Fluc construct. Because disruption of base-pair interactions between nt 322–326 and nt 345–341 moderately inhibited Fluc translation, formation of the pseudoknot may not be an absolute requirement for 5' IRES activity in vitro. However, the recovery of luciferase activity in the construct containing the compensatory mutation, 323–326 (cggg) and 344–341 (gccc), indicates that base-pair interactions in this region would affect IRES-mediated initiation of translation.

In previous studies with RhPV, uracil-rich sequences were reported within the 5' UTR of RhPV; however, no such sequences were recognized in PSIV. Furthermore, the accessibility of 74% of the 200 RhPV nt proximal to the initiation codon to DMS, CMCT and RNase T1 indicated that the 3' region of the 5' IRES is single-stranded (Terenin et al., 2005). In contrast, only about 50% of the 200 PSIV nt proximal to the initiation codon were accessible to DMS, CMCT and RNase T1 (Fig. 5). These data demonstrate that the PSIV sequence upstream of the initiation site has a different structure from that of RhPV and that the 5' IRES elements of PSIV and RhPV are distinct from one another.

The 5' IRES of PSIV does not function in plant or mammalian cell-free translation systems

When Fluc activity generated by PSIV 5' IRES-mediated translation was measured in WGE, the extremely low RLU values of the experimental template and the negative control (with no inserted sequence between Rluc and Fluc coding sequences) were nearly identical (Fig. 6a). In contrast, the activity mediated by the PSIV IGR-IRES measured 2.09 x 10$^6$ RLU, which is 275-fold higher than that of the negative control. These data indicate that the 5' IRES of PSIV does not function in WGE. The activity measured for translation of a PSIV 5' IRES template using RRL was 1.82 x 10$^5$ RLU (Fig. 6b), which was only one-twentih of the activity of the IGR-IRES template, but was 2-fold higher than that of a negative control template with no inserted sequence (Fig. 6b).

In general, RRL-based in vitro translation was carried out at salt concentrations endogenous to the lysate: 79 mM
Characterization of the 5' IRES of PSIV

There are two IRES elements within the monopartite positive-stranded RNA genome of dicistroviruses. Whereas 5' IRES elements control translation of non-structural protein precursors, IGR-IRES elements control translation of capsid protein precursors.

Characterization of dicistroviral IGR-IRES elements indicates that the structure and function of these elements are conserved among dicistroviruses. In contrast, the 5' IRES of dicistroviruses do not appear to be conserved. To date, three 5' IRES elements have been reported in dicistroviruses: in CrPV (Wilson et al., 2000), RhPV (Woolaway et al., 2001) and TrV (Czibener et al., 2005). The 5' IRES of RhPV can function in RRL, WGE and insect cell lysate systems (Royall et al., 2004; Woolaway et al., 2001). Similarly, the 5' IRES of TrV functions in Xenopus oocytes, baby hamster kidney cells and insect cells, suggesting a simplified mode of internal ribosome entry, since host-specific trans-acting factors are unlikely to be required (Czibener et al., 2005). These data indicate that the 5' IRES elements in RhPV and TrV work independently of the translational specificity of the host apparatus.

In contrast, the 5' IRES of CrPV functions in RRL, but not in WGE (Wilson et al., 2000), and that of PSIV functions only in insect cell lysate. These differences demonstrate that individual dicistroviral 5' IRES elements have distinct requirements for mediating internal initiation and that, unlike IGR-IRES elements, initiation mechanisms mediated by 5' IRES elements vary among dicistroviruses. Furthermore, the stem–loop structures suggested by the structure probing analysis of PSIV 5' IRES do not appear to be conserved in the 5' UTR of other dicistroviruses. Contrary to our expectations, these facts imply that translation strategies for the non-structural protein precursor in dicistroviruses are diverse.

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REFERENCES


