The 5′ untranslated region of Perina nuda virus (PnV) possesses a strong internal translation activity in baculovirus-infected insect cells

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Abstract A bicistronic baculovirus expression vector and fluorescent protein-based assays were used to identify the sequences that possess internal translation activity in baculovirus-infected insect cells. We demonstrated that the 5′ untranslated region (5′UTR; 473 nucleotides) of Perina nuda virus (PnV) and the 5′UTR (579 nucleotides) of Rhopalosiphum padi virus (RpHV), but not the IRES sequence of Cricket paralysis virus, have internal translation activity in baculovirus-infected Sf21 cells. In addition, we found that including the first 22 codons of the predicted PnV open reading frame (ORF; a total of 539 nucleotides) enhanced internal translation activity in baculovirus-infected Sf21 cells. Although the genus Iflaviruses is currently unassigned to any family, the members of this genus share many characteristics with the Dicistroviridae family of insect-infecting viruses. All of these viruses are icosahedral or spherical in shape, and their ssRNA genomes contain three major capsid protein genes. In terms of the size of their capsid proteins and the biophysical properties of their RNA, all of these insect-infecting viruses are similar to the mammalian picornaviruses and dicistroviruses, the iflaviruses also have no 5′ cap structure [3]. Recently, the 5′UTRs of two iflaviruses, Varroa destructor virus 1 (VDV-1) and Ectropis oblique picorna-like virus (EoPV), have been shown to have IRES activity in insect cell lines [8,9]. However, some 5′UTRs of the iflaviruses are smaller than the 5′UTRs of the picorna-viruses and dicistroviruses, leading Christian et al. [3] to suggest that the IRES-like element may be absent. In this study, we have shown that the 5′UTR of PnV (nucleotides (nt) 1–473 of the PnV genome) has internal translation activity, and that this activity can be enhanced by the first 22 codons of the PnV open reading frame (ORF).

1. Introduction

The picorna-like virus Perina nuda virus (PnV) is a small RNA-containing virus that infects the figus transparent wing moth [1]. Its complete genome has been sequenced, and it has been assigned to the genus Iflaviruses, which is named for its type species Infectious flacherie virus (IFV) [2]. Although the genus Iflaviruses is currently unassigned to any family, the members of this genus share many characteristics with the Dicistroviridae family of insect-infecting viruses. The 5′UTR of two iflaviruses, Varroa destructor virus 1 (VDV-1) and Ectropis oblique picorna-like virus (EoPV) [4–7], it might be expected that the 5′UTRs of iflavivirus genomes would also include an IRES that is used in protein synthesis. This expectation is further supported by the fact that, like the picornaviruses and dicistroviruses, the iflaviruses also have no 5′ cap structure [3]. Recently, the 5′UTRs of two iflaviruses, Varroa destructor virus 1 (VDV-1) and Ectropis oblique picorna-like virus (EoPV), have been shown to have IRES activity in insect cell lines [8,9]. However, some 5′UTRs of the iflaviruses are smaller than the 5′UTRs of the picorna-viruses and dicistroviruses, leading Christian et al. [3] to suggest that the IRES-like element may be absent. In this study, we have shown that the 5′UTR of PnV (nucleotides (nt) 1–473 of the PnV genome) has internal translation activity, and that this activity can be enhanced by the first 22 codons of the PnV open reading frame (ORF).

2. Materials and methods

2.1. Cells, viruses, and transfection

The Spodoptera frugiperda cell line IPL-BS21AE (S21AE) was cultured in TNM-FH insect medium containing 8% heat-inactivated fetal bovine serum [10]. S21AE monolayers were used for virus propagation. All viral stocks were prepared and titers were determined according to the standard protocols described by Summers and Smith [11]. For transfection, cellfectin (Invitrogen) was used according to the protocol provided by the manufacturer.

2.2. Construction of plasmid transfer vectors

DNA preparations and manipulations were performed using standard methods as described by Sambrook et al. [12] or by the manufacturers of the reagents. To construct a baculovirus transfer vector with dual fluorescence protein genes to monitor IRES activity in S21AE cells, we first digested the pHES-EGFP plasmid (ClonTech) with EcoRI and SalI and subcloned the 2.2 kb IRES-EGFP DNA fragment into AcMNPV transfer vector pBlueBac4.5 (Invitrogen). The resulting plasmid was named pBacIRE. The DsRed gene from the plasmid pDsRed1-N1 (ClonTech) was PCR amplified to produce a DNA fragment containing an Nhel restriction site on the 5′ end and an EcoRI restriction site on the 3′ end. The sequences of the primers used were: 5′Nhel ATCGG CTAGC (the Nhel site is underlined), and 3′EcoRI GTAGG CACCT GTACT (the EcoRI site is underlined). The PCR amplified DNA fragment was cloned into the Nhel and EcoRI sites of the transfer vector pBacIRE and the resulting plasmid was named pBacDreIRE. A plasmid containing the IGR IRES sequence of the Cricket paralysis virus (CrPV; 247 nucleotides, GenBank No. AF218039) was
constructed. CrPV was chosen because it is the type species of the genus Cripavirus, which is the only genus in the family Dicistroviridae [4,5,13]. Chemical synthesis (MDBio Inc., Taiwan) was used to add an EcoRI restriction site at the 5′ end and a BamHI restriction site at the 3′ end of the CrPV IRES sequence, and the whole sequence was then subcloned and inserted into the BamHI sites of pBacD-CriE. The resulting plasmid was named pBacD-CriE.

To obtain plasmids containing the putative 5′UTR IRES region of PnV, genomic RNA was extracted from purified PnV particles using TRIzol reagent (Invitrogen) according to the method described by Wu et al. [1]. The entire 5′UTR of PnV was amplified by RT-PCR (reverse transcription-polymerase chain reaction) from PnV genomic RNA using the forward primer 5′-GGCAGG TCTT TTAA TATCG GTGAC AGGG TTATAC C-3′ (nucleotides 1–29 of the PnV genome), and the reverse primer 5′-GGCGA TCCCT ATGCG TTGCG CTCTT GATCT C-3′ (complementary to nucleotides 451–473). Both primers contained a BamHI site (underlined). RT-PCR was performed using an RT-PCR Kit (Superscript One-Step RT-PCR for Long Templates; Invitrogen), and the RT-PCR products were cloned directly into the pGEM®-T Easy Vector (Promega). Sequences of the inserted cDNA were verified by sequencing. The PnV 5′UTR cDNA (473 nt) was then released by BamHI digestion and inserted between the two reporter genes at the BamHI site of the bicistronic plasmid pBacD-CriE. Orientation of the inserted cDNA was determined by sequencing with the primer 5′-CTACG TGGAC TCCAA GCTGG-3′ (derived from the downstream sequence of the DsRed gene), and only those colonies of the plasmid that contained the PnV 5′UTR in the sense (genomic) orientation were selected. The resulting plasmid was named pBacD-Pn5′539-E.

For the pBacD-Pn5′539-E plasmid, a 539 nt sequence containing the PnV 5′UTR and the first 22 codons of the PnV ORF [1] were amplified by RT-PCR from PnV genomic RNA using the forward primer 5′-GGCGA TCCCT TTAAATATCG GTGAC AGGGTTTATAC C-3′ and the reverse primer PnPV-R539 5′-GGTTGATCC TGCGA TGGCA AAGTTC GTCAG-3′ (complementary to nt 514–539), each of which contained a BamHI site (underlined). The RT-PCR product was inserted into the BamHI site of the bicistronic plasmid pBacD-CriE as described above, and the resulting plasmid was named pBacD-Pn5′539-E with sense orientation and pBacD-Pn5′539r-E with antisense orientation.

2.3. Recombinant virus production and titer determination
Using cellfectin (1 μl), S21AE cells (2 × 105 cells per well, in a 24-well plate) were co-transfected with the linearized viral DNA Bac-N-Blue (0.25 μg, Invitrogen) and 0.8 μg of one of the AcMNVP transfer vectors pBacD-CriE, pBacD-Pn5′539-E, or pBacD-Pn5′539r-E. Green fluorescent protein (EGFP) fluorescence was detected using an FITC channel with a 450/490 filter set (Nikon), while DsRed fluorescence was detected using a conventional rhodamine channel with a 510/560 filter (Nikon). Thus, the successful recombinant viruses were easily distinguished by the red fluorescence emitted under a fluorescence microscope (Nikon). The recombinant viruses were then selected and purified by a series of three end-point dilutions. The resulting viruses were named vAcD-Cri-E, vAcD-Pn5′539-E, vAcD-Pn5′539r-E, and vAcD-Pn5′539r-E, respectively. A fifth recombinant baculovirus, vAcD-Rhir-E, was also used in this study. vAcD-Rhir-E contains the same red and green fluorescent protein genes flanking the RhPV 5′UTR IRES, and its construction was described by Chen et al. [14]. vAcD-Rhir-E can simultaneously produce dual fluorescence in infected S21AE cells, and was used here as a positive control for vAcD-Cri-E, vAcD-Pn5′539-E, vAcD-Pn5′539r-E and vAcD-Pn5′539r-E. Titers (50% tissue culture infectious dose, TCID50) of the progeny viruses were determined by end-point dilution of emitted red fluorescence in a 96-well plate [11].

2.4. Western blot analysis
After S21AE cells had been infected with the recombinant viruses for 4 days, the proteins in the cell extracts were separated by SDS-PAGE according to the procedure of Laemmli [15] on a mini-Protein-III system (Bio-Rad). The separated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), which was then blocked with Tris-buffered saline (TTBS: 100 mM Tris, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) containing 5% BSA (Sigma) at room temperature for 1 h with gentle shaking on an orbital shaker. Subsequently, membranes were incubated overnight at 4°C with PBS-diluted (1:2000) anti-EGFP or anti-DsRed antibody (Clontech). Unbound antibodies were removed by three 5-min washes in TTBS buffer at room temperature with gentle shaking. Membranes were then incubated with 1:2500 diluted alkaline phosphatase (AP) secondary antibodies (Jackson) for 1 h at room temperature. The AP on the membrane was detected by an enhanced chemiluminescence kit (Pierce) following the protocol provided by the manufacturer.

2.5. Northern blot analysis
To check and confirm the integrity of the RNA transcripts, extracts from infected S21AE cells were analyzed by Northern blot using a probe specific for the EGFP sequence. Briefly, an EGFP gene fragment (366 bp) was amplified by PCR from the plasmid pBacD-Pn5′539-E using the primer set EGFP-F 5′-ACGAGTTTCTTTAAGGTTG-3′ and EGFP-R 5′-TGCTCTAGGTAAGGTGTGTGGCG-3′. The fragment was then cloned into pGEM-T Easy Vector (Promega), which contains T7/SP6 promoter-promoters. DIG-RNA probes were prepared by in vitro transcription with a commercial kit (DIG-RNA Labeling Kit, Roche) as described by the manufacturer. Total RNA transcripts were extracted from vAcD-Pn5′539-E, vAcD-Cri-E, and vAcD-Rhir-E-infected S21AE cells at 4 days postinfection and also from uninfected S21AE cells. Extracts were electrophoresed in a 1% agarose gel containing formaldehyde, blotted onto a nylon membrane (Hybond-N, Amersham), and probed with the EGFP probe according to the standard procedure of Sambrook et al. [12]. Standard chemiluminescent detection was performed according to the manufacturer’s instructions (Roche), and the blot was exposed to X-ray film (Kodak XAR-5).

2.6. Fluorescence measurement
At 4 days postinfection, S21AE cells (2 × 103 cells in a 24-well plate) infected with vAcD-Cri-E, vAcD-Pn5′539-E, vAcD-Pn5′539r-E, or vAcD-Rhir-E were lysed for 10 min in 300 μl of culture cell lysis reagent containing 100 mM potassium phosphate (pH 7.8), 1 mM EDTA, 10% Triton X-100, and 7 mM β-mercaptoethanol. After centrifugation at 15200 × g for 30 min, the lysate supernatant (100 μl) was taken for fluorescence measurement. The fluorescence intensities of EGFP and DsRed were measured using a Cary Eclipse Fluorescence spectrophotometer.

2.7. Secondary structure prediction
An RNA secondary structure for the PnV 5′UTR was predicted using the Dynalign program [16], and the resulting images were modified for the figures using RNAViz 2.0 software [17].

3. Results
In previous studies, we have demonstrated that RhPV IRES functions well in baculovirus-infected S21AE cells and can be used to construct bicistronic baculovirus expression vectors [14]. To identify more IRES elements that can mediate cap-independent translation in a baculovirus expression system, we examined whether the PnV 5′UTR can act as an IRES element in a baculovirus expression system. Furthermore, in viruses such as hepatitis C virus, some of the downstream codons in the IRES may contain inherent frameshift mutations to allow for downstream translation of the IRES sequence [18,19]. To investigate whether this is also true for PnV, we examined whether the PnV 5′UTR can act as an IRES element in baculovirus-infected insect cells.

To facilitate monitoring of internal translation or IRES-mediated activity, we constructed EGFP and DsRed fluorescent protein genes containing recombinant viruses (Fig. 1). If the
PnV 5'UTR can act as an IRES element, then the virus-infected Sf21AE cells will simultaneously emit green as well as red fluorescence, and can be easily identified by fluorescence microscopy. Fig. 2C shows that in vAcD-Pn5'0473-E-infected Sf21AE cells, both the cap-dependent fluorescent marker (DsRed; left column) and the cap-independent fluorescent marker (EGFP; right column) were being translated. By contrast, in the vAcD-Pn5'473-E-infected cells, IRES-mediated fluorescent signals were present but substantially weaker (Fig. 2B, right column). Figs. 2A and D show that neither the CrPV IGR IRES nor the antisense sequence of the PnV 5'UTR fragment could mediate EGFP expression in Sf21AE cells. From these results we conclude that the 5'0UTR of PnV, in the sense but not antisense orientation, is able to initiate translation internally, and further, that this ability is enhanced by the first 22 codons of the ORF. Conversely, the IGR IRES element of CrPV cannot mediate cap-independent translation in baculovirus-infected Sf21AE cells.

Western blotting analysis produced similar results: DsRed protein was detected in all of the Sf21AE cells, including those infected by vAcD-Crir-E and vAcD-Pn5'473-E, whereas EGFP protein was found only in the cell lysates of the positive control and the vAcD-Pn5'473-E- and vAcD-Pn5'539-E-infected Sf21AE cells. From these results we conclude that the 5'UTR of PnV, in the sense but not antisense orientation, is able to initiate translation internally, and further, that this ability is enhanced by the first 22 codons of the ORF. Conversely, the IGR IRES element of CrPV cannot mediate cap-independent translation in baculovirus-infected Sf21AE cells.

Fig. 1. Schematic representation of bicistronic constructs of the recombinant baculoviruses used to measure PnV IRES functionality. (A) vAcD-Crir-E, in which the IGR-IRES of CrPV is inserted between the DsRed and EGFP genes. (B) vAcD-Pn5'473-E and (C) vAcD-Pn5'539-E, the experimental viruses that contain the dual fluorescent protein genes flanking the PnV 5'UTR (473 nucleotides) and the PnV 5'UTR plus the first 22 codons of the ORF (539 nucleotides), respectively. (D) vAcD-Pn5'539-r-E, the experimental viruses that contain the dual fluorescent protein genes flanking the PnV 5'UTR plus the first 22 codons of the ORF (539 nucleotides) in antisense orientation. (E) Positive control virus, vAcD-Rhir-E, in which the RhPV 5'UTR-IRES is located between the DsRed and EGFP genes. PPH, polyhedrin promoter; DsRed, red fluorescent protein gene; EGFP, enhanced green fluorescent protein gene.

PnV 5'UTR can act as a IRES element, then the virus-infected Sf21AE cells will simultaneously emit green as well as red fluorescence, and can be easily identified by fluorescence microscopy. Fig. 2C shows that in vAcD-Pn5'539-E-infected Sf21AE cells, both the cap-dependent fluorescent marker (DsRed; left column) and the cap-independent fluorescent marker (EGFP; right column) were being translated. By contrast, in the vAcD-Pn5'473-E-infected cells, IRES-mediated fluorescent signals were present but substantially weaker (Fig. 2B, right column). Figs. 2A and D show that neither the CrPV IGR IRES nor the antisense sequence of the PnV 5'539-nt fragment could mediate EGFP expression in Sf21AE cells. From these results we conclude that the 5'UTR of PnV, in the sense but not antisense orientation, is able to initiate translation internally, and further, that this ability is enhanced by the first 22 codons of the ORF. Conversely, the IGR IRES element of CrPV cannot mediate cap-independent translation in baculovirus-infected Sf21AE cells.

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Fig. 5 shows that in the cell lysates of the vAcD-Crir-E-, vAcD-Rhir-E- and vAcD-Pn5'539-E-infected Sf21AE cells, a species of RNA with a size of about 2 kb was detected. This is consistent with the predicted size of a bicistronic RNA transcript containing the DsRed gene (680 bp), the 5'UTR of PnV (473 bp) and the EGFP gene (798 bp). If the 5'UTR of the PnV sequence either contained a cryptic promoter or was able to induce RNA cleavage, then monocistronic EGFP
transcripts would have been generated. However, no smaller RNA band was detected in the vAcD-Crir-E- and vAcD-
Pn5’539-E-infected Sf21AE cells (Fig. 5, lanes 1 and 3, respectively). We also found that a parallel Northern blot assay produced exactly corresponding results with the vAcD-Pn5’473-E construct (data not shown). We therefore conclude that there is no cryptic promoter, nor any ability to induce RNA cleavage either in the 5’UTR of PnV or in the 5’UTR +22 codons. Interestingly, an additional transcript with a size of about 1 kb was present in the cell lysates of vAcD-Rhir-E-infected cells (Fig. 5, lane 2). This transcript appears to be a putative mRNA transcribed within the RhPV
IRES and its presence implies that RhPV IRES may contain a cryptic promoter. Interestingly, there are six conserved TAAG (see Fig. 6A) transcriptional initiation motifs of the baculovirus late promoter [21] in the DNA sequence of the RhPV IRES, which may correspond to this unusual promoter activity. On searching the entire nucleotide database of GenBank with the blastn program, we found that the sequence of the 5′ UTR of PnV has 80.0% homology with the 5′ UTR of EoPV and 45.0% homology with the 5′ UTR of RhPV (Fig. 6A). Furthermore, this implies that we can propose a PnV 5′ UTR secondary structure by comparison with the 5′ UTR of the EoPV sequence from co-variations or compensated base changes in stem regions. Fig. 6B indicates that the predicted secondary structure of the PnV 5′ UTR has seven dominant structural features, most of them stem-loops. Interestingly, the stem-loop labeled 6 was branched and the stem-loops labeled 3, 4, 6 and 7 were similar to stem-loops H, I, J, K, and L in the 5′UTRs of cardiovirus and aphthovirus [22]. In addition, the 5′ UTR of EoPV containing the IRES element has been identified [9]. Therefore, an IRES in the 5′ UTR of PnV may be the most plausible explanation for the internal translation of EGFP in baculovirus-infected Sf21AE cells.

4. Discussion

The efficiencies of IRES-containing expression vectors vary in different host cells [23,24]. Finkelstein et al. [25] suggested that insect cells may lack at least some of the trans-acting factor(s) that are necessary for protein expression to be initiated by an IRES originating from the mammalian picornaviruses. In in vitro translation systems that used insect cell-free lysates, IRES elements originating from insect viruses, such as the IRES in the 5′ UTR and IGR of CrPV or RhPV, have been shown to mediate cap-independent translation [6,26,27]. The RhPV IRES has also been used as an element of a conven-
tional bicistronic baculovirus transfer vector [14,28]. In the present study, we have shown that the translation efficiency of the 539 nucleotide fragment at the 5′ end of PnV is approximately 2–3 times higher than that of RhPV IRES. We have also demonstrated here that, like hepatitis C virus [12,13], the IRES of PnV requires part of the coding region for greatest activity, although the predicted secondary structure does not fold like the RNA pseudoknot containing HCV IRES. This distinguishes PnV from the mammalian picornaviruses and dicistroviruses, because in the latter two families coding sequences are not required for optimal activity [4,6,7]. Further studies of the structure of PnV IRES, e.g. deletion and mutational analysis to define the minimal sequence for IRES activity and RNA structure determination, will be important to learn why the 66 nucleotides can enhance internal translation activity. It will also be of interest to know whether this sequence of 66 nt can enhance the activity of other IRES, as with RhPV IRES or CrPV IRES.

In conclusion, we have shown that the 5′UTR of PnV can be used to construct a bicistronic transfer vector. In the future, this may facilitate the development of polycistronic baculovirus transfer vectors that can be used in BEVS for the production of multiple protein complexes. Furthermore, the different expression abilities of the 473 and 539 nt fragments of PnV may provide convenient alternatives for different applications. For example, if two proteins are to be co-expressed, where one is a selectable marker and the other is the gene of interest, then the 473 nt fragment might be the optimal choice. Conversely, if the aim is to express a functional heterogeneous protein composed of two different polypeptides, such as an immunoglobulin, then a construct based on the 539 nt PnV fragment would be more suitable for the simultaneous expression of the two different genes.

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References


