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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 (RhPV), 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 by approximately 18 times. This is the first report of internal translation activity for a baculovirus expression system (BEVS) in the iflavirus 5′ sequence and may facilitate the development of polycistronic baculovirus transfer vectors that can be used in BEVS for the production of multiple protein complexes.**

Keywords: AcMNPV; Baculovirus; IRES; PnV; *Perina nuda* virus

1. Introduction

The picorna-like virus *Perina nuda* virus (PnV) is a small RNA-containing virus that infects the ficus transparent wing moth [1]. Its complete genome has been sequenced, and it has been assigned to the genus *Iflavirus*, which is named for its type species *Infectious flacherie* virus (IFV) [2]. Although the genus *Iflavirus* 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 [3,4]. However, the genome of viruses in the genus *Iflavirus* is monocistronic; containing a 5′ untranslated region (5′UTR); in contrast, the genome of *Dicistroviridae* viruses is bicistronic, containing a 5′UTR and an intergenic region (IGR) [3,4].

Because there is an IRES element in the 5′UTR of all genomes of the mammalian picornaviruses and dicistroviruses [4–7], it might be expected that the 5′UTRs of iflavirus 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 picornaviruses 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 IPLB-Sf21AE (Sf21AE) was cultured in TNM-FH insect medium containing 8% heat-inactivated fetal bovine serum [10]. Sf21AE 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 Sf21AE cells, we first digested the pIRES-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 NheI restriction site on the 5′ end and an EcoRI restriction site on the 3′ end. The sequences of the primers used were: 5′Nhe1 ATCGG CTAGC GGTCG CCACC ATGGT GCGCT CT (the NheI site is underlined), and 3′EcoR1 GTAGG AATTC GCTAC AGGAA CAGGT GGTGG (the EcoRI site is underlined). The PCR amplified DNA fragment was cloned into the NheI and EcoRI sites of the transfer vector pBacIRE and the resulting plasmid was named pBacDirE. A plasmid containing the IGR IRES sequence of the *Cricket paralysis* virus (CrPV; 247 nucleotides, GenBank No. AF218039) was

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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 into the EcoRI and BamHI sites of pBacDirE. The resulting plasmid was named pBacD-Crir-E.

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′-GCGGA TCCTT TTAAA TATCG GGTAC AGGGT TTTAAC C-3′ (nucleotides 1–29 of the PnV genome), and the reverse primer 5′-GCGGA TCCTT ATGGG TTGCC CCTCT GTACT 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 pBacDirE. 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′473-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′-GCGGA TCCTT TTAAA TATCG GGTAC AGGGT TTTAA CC-3′ and the reverse primer PnPV-R539 5′-GGTGGATCCG TGCGA TCGCA AAGTTC GTCAG-3′ (complementary to nt 514–539), each of which contains a BamHI site (underlined). The RT-PCR product was inserted into the BamHI site of the bicistronic plasmid pBacDirE as described above, and the resulting plasmid was named pBacD-Pn5′539-E with sense orientation and pBacD-Pn5′539r-E with anti-sense orientation.

2.3. Recombinant virus production and titer determination

Using cellfectin (1 μl), Sf21AE cells (2×10^5 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 AcMNPV transfer vectors, pBacD-Crir-E, pBacD-Pn5′473-E, 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 selected and purified by a series of three end-point dilutions. The resulting viruses were named vAcD-Crir-E, vAcD-Pn5′473-E, vAcD-Pn5′539-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 Sf21AE cells, and was used here as a positive control for vAcD-Crir-E, vAcD-Pn5′473-E, vAcD-Pn5′539-E and vAcD-Pn5′539r-E. Titers (50% tissue culture infectious dose, $TCID_{50}$) of the progeny viruses were determined by end-point dilution of emitted red fluorescence in a 96-well plate [11].

The construction of the five recombinant baculoviruses used in this study is summarized in Fig. 1.

2.4. Western blot analysis

After Sf21AE 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 polyvinyldiene 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 shaking. Membranes were then incubated with 1:2500 diluted alkaline phosphate (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 Sf21AE 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′473-E using the primer set EGFP-F 5′-ACGAC TTCTT CAAGT CCGCC-3′ and EGFP-R 5′-TGCTC AGGTA GTGGT TGTCG-3′. The fragment was then cloned into pGEM-T Easy Vector (Promega), which contains T7/SP6 opposed 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-Crir-E- and vAcD-Rhir-E-infected Sf21AE cells at 4 days postinfection and also from uninfected Sf21AE 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, Sf21AE cells (2×10^5/well in a 24-well plate) infected with vAcD-Crir-E, vAcD-Pn5′473-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 $15200 \times g$ 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 Sf21AE 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 coding sequence must also be present for the IRES to function efficiently [18,19]. To investigate whether this is also true for PnV, a plasmid containing the PnV 5′UTR and the first 22 codons of the PnV ORF was constructed (Fig. 1). In addition, it has previously been shown that IGR IRES of CrPV is active in mammalian cells [20], therefore we also tested the translational activity of the CrPV IRES 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

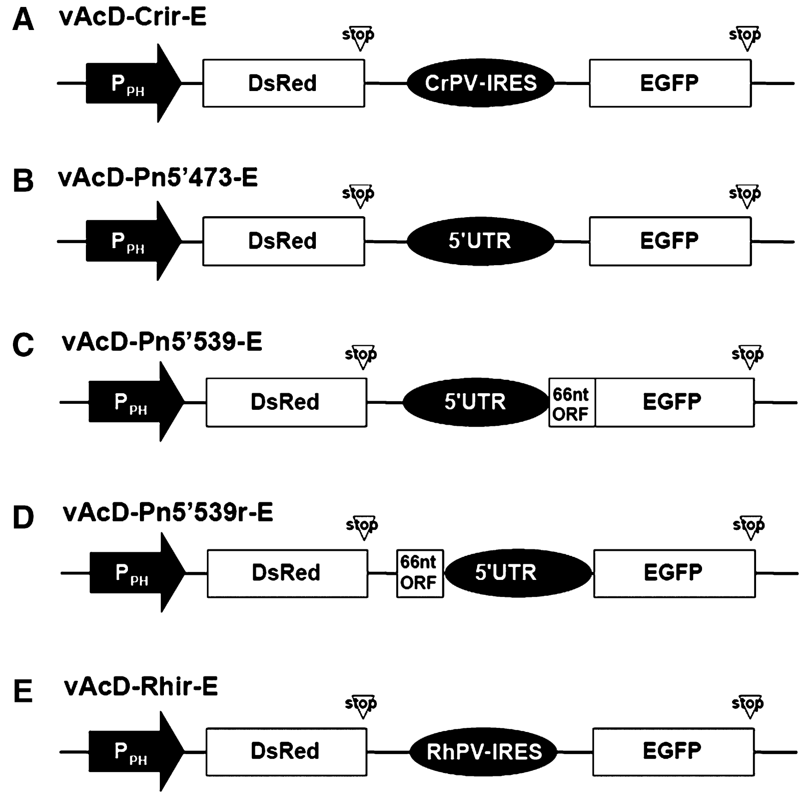

Fig. 1. Schematic representation of bicistronic constructs of the recombinant baculoviruses used to measure PnV IRES functionality. (A) vAcD-Crir-E, in which the IGR-IRES of CrPV is inserted between the DsRed and EGFP genes. (B) vAcD-Pn5′473-E and (C) vAcD-Pn5′539-E, the experimental viruses that contain 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′539r-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. P_{PH}, 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.

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 (Fig. 3). Fig. 3 also suggests that the molecular weight of the EGFP protein produced in the vAcD-Pn5′539-E-infected Sf21AE cells was slightly higher than the EGFP in the vAcD-Rhir-E-infected Sf21AE cells, and this was confirmed using a second Western blot with lower quantities of protein (data not shown). This suggests that one of the AUGs in this 66 nt coding region (see Fig. 6A) is being used as the start codon instead of the AUG in the EGFP gene. These results show that the 5′UTR of PnV, nucleotides 1–473 of the PnV genome, has internal translation activity, and that this activity is enhanced by the first 22 codons of the PnV ORF, shown quantitatively in Fig. 4. The efficiency of PnV 5′UTR mediated translation of EGFP is about seven times weaker than that of RhPV IRES, while the efficiency of PnV 5′UTR +22 codons is two or three times stronger. The expression of EGFP observed in our experiments may arise from a cryptic promoter, RNA cleavage, ribosome reinitiation or IRES. So, we performed Northern blot experiment and RNA secondary structure prediction to clarify this question.

We performed Northern blots to analyze the RNA species with a DIG-labeled probe specific for the EGFP gene. 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

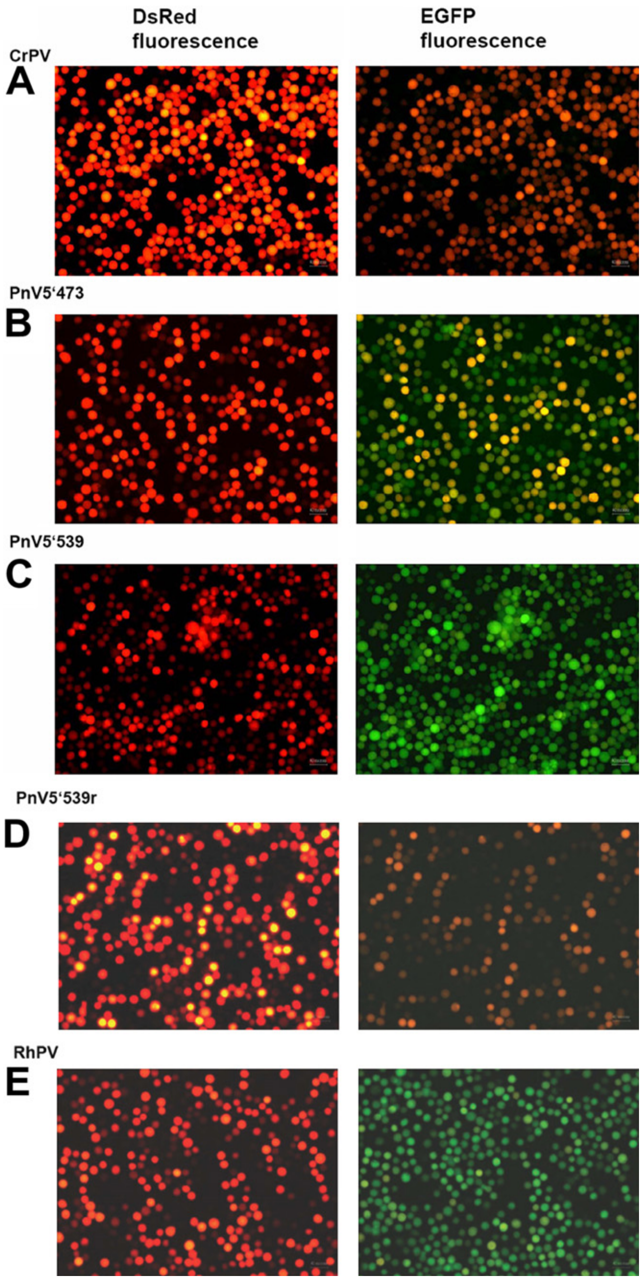

Fig. 2. Fluorescence micrographs of Sf21AE cells infected with (A) vAcD-Crir-E, (B) vAcD-Pn5′473-E, (C) vAcD-Pn5′539-E, (D) vAcD-Pn5′539r-E and (E) vAcD-Rhir-E (positive control) at a multiplicity of infection of 1 at 4 days postinfection. The left-hand column shows cap-dependent DsRed fluorescence, and the right-hand column shows cap-independent IRES-mediated EGFP fluorescence. Exposure time = 260 ms; magnification = 100×.

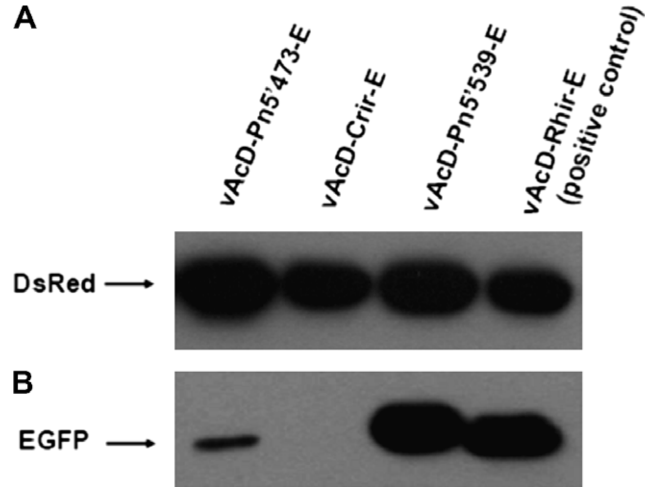

Fig. 3. Western blot analysis of (A) IRES efficiency and (B) molecular weight of EGFP proteins in recombinant virus-infected Sf21AE cells. (A) Sf21AE cells were infected as indicated (MOI = 1). Extracted proteins (13 μg per lane) were separated by 10% SDS–PAGE. The detected protein bands are DsRed (upper panel) and EGFP (lower panel).

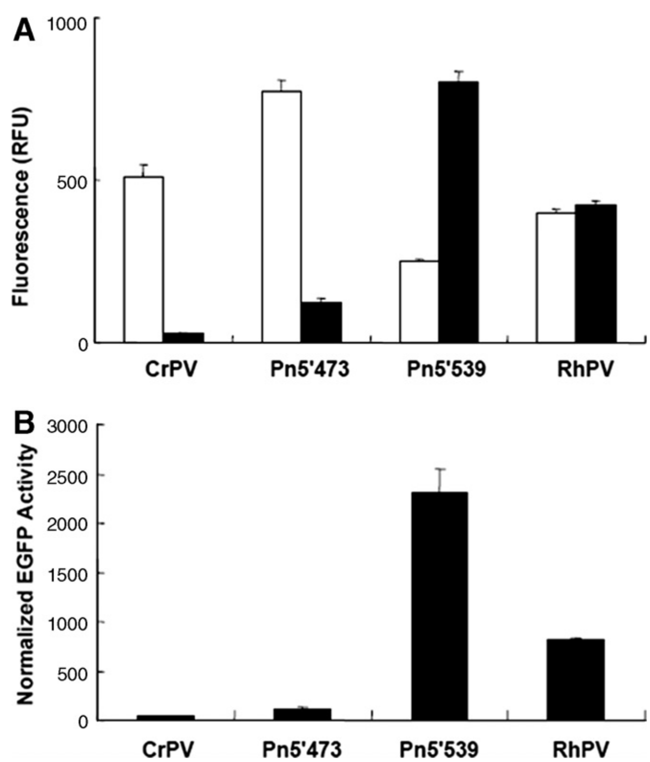

Fig. 4. Quantization of IRES efficiency in recombinant virus-infected Sf21AE cells. (A) Spectrofluorometric measurement of DsRed and EGFP protein expression levels in lysates (100 μl; 50 μg of protein) from Sf21AE cells infected with vAcD-Crir-E, vAcD-Pn5′473-E, vAcD-Pn5′539-E, or vAcD-Rhir-E. Emission was monitored at 583 nm for DsRed (unshaded bars) and at 507 nm for EGFP (solid bars). (B) Normalized green fluorescence intensity (relative to DsRed) in the same recombinant virus-infected Sf21AE cells. Data are the mean of three repeats; error bars show the standard deviation.

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

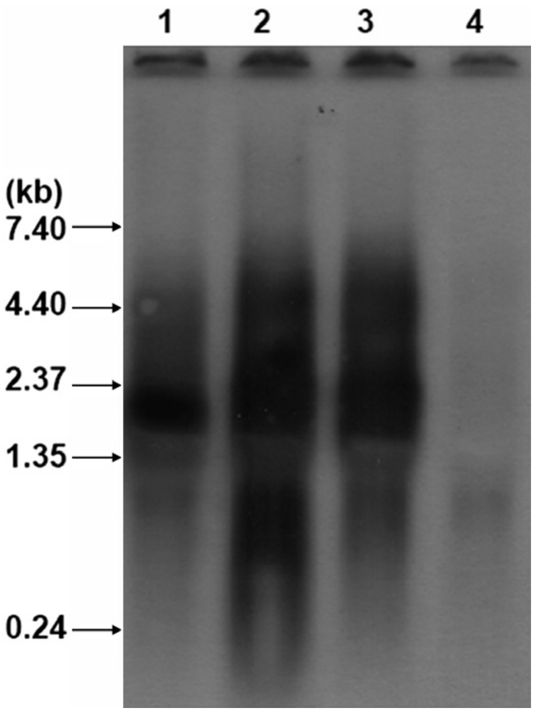

Fig. 5. Northern blotting of RNA transcripts extracted from vAcD-Crir-E (lane 1), vAcD-Rhir-E (lane 2) and vAcD-Pn5′539-E (lane 3) infected Sf21AE cells at 4 d post-infection, and uninfected cells (lane 4). A DIG-labeled probe specific for the EGFP sequence detected only a single species of RNA of the expected size.

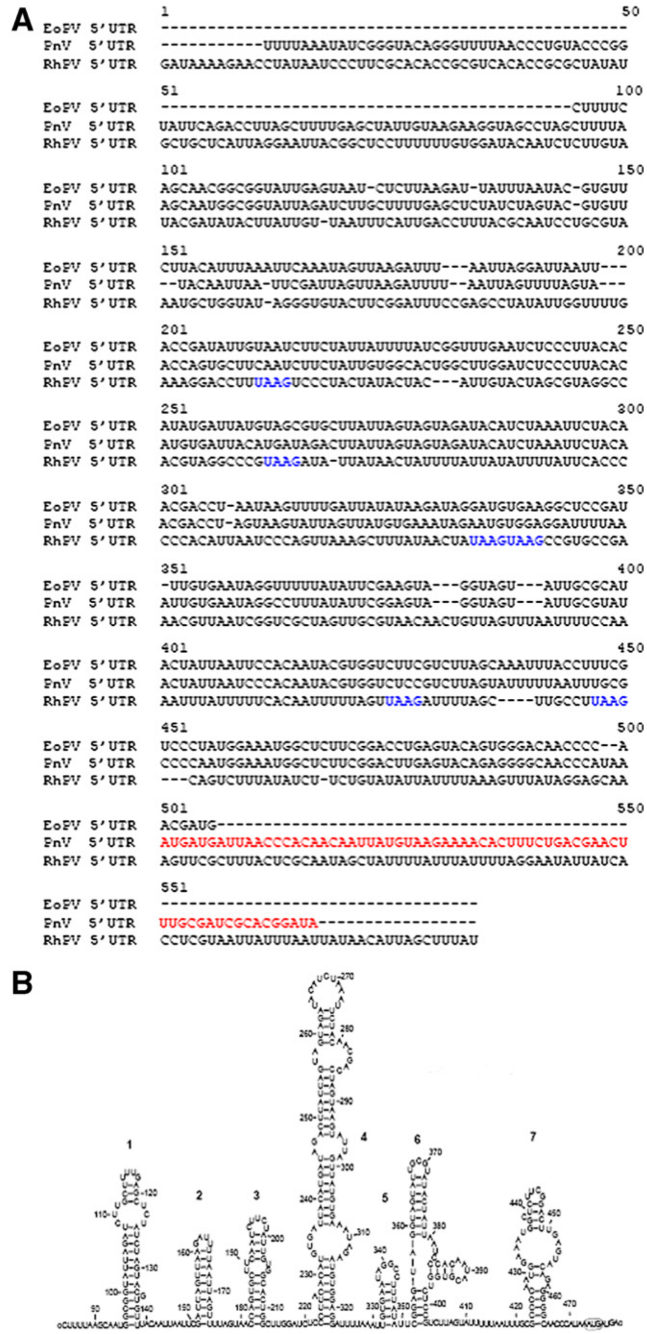

Fig. 6. Sequence comparison and secondary structure prediction of PnV 5′UTR. (A) Alignment of PnV 5′UTR sequence with the IRES of EoPV and RhPV. The first 66 nt of the PnV ORF are in red and the UAAG motifs in the RhPV 5′UTR are in blue. (B) Proposed secondary structure of the PnV 5′UTR. The seven stem-loops are indicated by Arabic numbers (bold).

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 conventional 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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