**In vitro and in vivo identification of structural and sequence elements in the 5’ untranslated region of *Ectropis obliqua* picorna-like virus required for internal initiation**

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*Ectropis obliqua* picorna-like virus (EoPV) is a newly described insect virus that is classified as a putative member of the genus *Iflavirus*. The virus possesses a large, positive-sense RNA genome encoding a single polyprotein that shares physicochemical properties with those of members of the family *Picornaviridae*. The 5’ untranslated region (5’ UTR) plays an important role in picornavirus translation initiation, as it contains an internal ribosome entry site (IRES) that mediates cap-independent translation. To investigate translation in EoPV, an extensive range of mutations were engineered within the 5’ UTR and the effects of these changes were examined *in vitro* and *in vivo* by using a bicistronic construct. Results showed that deletions within the first 63 nt had little impact on IRES activity, whilst core IRES function was contained within stem–loops C and D, as their removal abrogated IRES activity significantly. In contrast to these findings, removal of stem–loop G containing two cryptic AUGs caused a remarkable increase in IRES activity, which was further investigated by site-directed mutagenesis at these two positions. It was also confirmed that initiation of protein synthesis occurs at AUG6 (position 391–394) and not at the AUG immediately downstream of the polypyrimedine tract. Mutation of the polypyrimedine tract (CCTTTC) had a slight effect on EoPV IRES activity. Furthermore, mutations of the RAAA motif led to a decrease in IRES activity of approximately 40% *in vitro*, but these results were not supported by *in vivo* experiments. In conclusion, this study reveals that the EoPV IRES element is unique, although it has features in common with the type II IRESs.

**INTRODUCTION**

*Ectropis obliqua* picorna-like virus (EoPV) is an insect RNA virus causing a lethal granulosis infection of the larvae of the tea looper (*Ectropis obliqua*) (Wang et al., 2004). The virus possesses a large, positive-sense RNA genome that encodes a single, long polyprotein. Structural and non-structural proteins, located at the N- and C-terminal regions, respectively, share physicochemical properties with those of members of the family *Picornaviridae*. EoPV infects invertebrate, but not mammalian, hosts. Several viruses with genome organization similar to that of EoPV, including *Infectious flacherie virus* (IFV; Isawa et al., 1998), *Sacbrood virus* of bees (Ghosh et al., 1999), *Perina nuda* picorna-like virus (Wu et al., 2002), deformed wing virus (Lanzi et al., 2006), Kakugo virus (Fujiyuki et al., 2004) and *Varroa destructor* virus 1 (Ongus et al., 2004), have also been found in various species of insects. Recently, these viruses have been grouped in the genus *Iflavirus* (Christian et al., 2002; Ongus et al., 2004), which has not yet been assigned to a virus family.

For the majority of eukaryotic cell mRNAs, initiation of protein synthesis involves the recognition of a 5’ cap structure (m’GpppN) by translation-initiation factors and subsequent recruitment of the 43S pre-initiation complex, which includes the 40S small ribosomal subunit (Hershey & Merrick, 2000; Dever, 2002). However, as no cap structure is present at the 5’ terminus of certain viral mRNAs (e.g. from picornaviruses), an alternative mechanism of translation has been described (Jang et al., 1988; Pelletier & Sonenberg, 1988). For picornavirus RNAs, it is now well established that the initiation of protein synthesis occurs by a cap-independent mechanism that is directed by an internal ribosome entry site (IRES) located within the 5’ untranslated region (5’ UTR) of the genome (Jackson et al., 1994; Jackson & Kaminski, 1995; Belsham & Sonenberg, 2000).

*In vitro* and *in vivo* translation experiments have been used to define IRES activity for representative members of the family *Picornaviridae*, such as aphthoviruses (Belsham & Brangwyn, 1990; Kühn et al., 1990), cardiortiviruses (Jang et al., 1988, 1989; Duke et al., 1992; Witherell et al., 1995),
enteroviruses (Percy et al., 1992; Haller et al., 1993; Niklasson et al., 1999), hepatoviruses (Brown et al., 1991, 1994; Glass et al., 1993) and rhinoviruses (Todd et al., 1997; Hunt & Jackson, 1999). Secondary and tertiary RNA structures within these viral IRES elements have been shown to play critical roles for translation initiation. At present, viral IRES elements can be classified into four types. Type I IRESs are found in enterovirus and rhinovirus genomes and are characterized by the particular folding of the IRES and by the site of translation initiation, which is located a considerable distance downstream of this structure (Agol, 1991; Pilipenko et al., 1992). Type II IRESs, found in cardiovirus and aphthovirus genomes, have a very different predicted secondary structure that is characterized by the presence of core stem–loops D to L (Pilipenko et al., 1989; Palmenberg & Sgro, 1997; Stewart & Semler, 1997). In this model, translation is initiated 12–15 nt downstream of a polypyrimidine tract located immediately 3′ to the core structural elements defining the IRES (Pilipenko et al., 1989; Belsham & Brangwyn, 1990; Belsham, 1992). Type III IRESs are found in hepatovirus genomes and appear to share characteristics of both type I and type II IRES elements (Brown et al., 1991). IRESs of another recently described type, HCV-like IRESs, are found in porcine teschovirus 1, porcine enterovirus 8 and simian virus 2 genomes and are similar functionally and structurally to the IRES elements from Hepatitis C virus (HCV) and Classical swine fever virus in the family Flaviviridae (Peitova et al., 1998; Fletcher & Jackson, 2002; Kaku et al., 2002; Sarnow, 2003; Pisarev et al., 2004; Chard et al., 2006a, b).

One common feature of picornavirus IRES elements is the presence at the 3′ border of a conserved, cis-acting element, the Yₙₜ–Xₙₜ–AUG motif (where Yₙₜ is a polypyrimidine tract and Xₙₜ is a random spacer sequence preceding an AUG triplet) (Jang et al., 1990; Pilipenko et al., 1992). In the case of type I and type III IRESs, translation is usually initiated at this AUG. In contrast, in the case of type II IRESs, the initiation codon is reached by ribosomal scanning downstream from this Yₙₜ–Xₙₜ–AUG motif. Interestingly, Foot-and-mouth disease virus (FMDV) represents an intermediate between these two extremes, with translation initiation occurring not only at the AUG at the 3′ end of the IRES, but also, at higher frequency, at the next AUG further downstream (Belsham, 1992). Another conserved feature that has been shown to be essential for IRES activity is an RAAA motif in the most distal loop of domain 3, which is shared by Encephalomyocarditis virus (EMCV) and FMDV (Hoffman & Palmenberg, 1995; López de Quinto & Martínez-Salas, 1997).

The aim of this study was to characterize the IRES for EoPV. The IFV genome can be translated efficiently in rabbit reticulocyte lysate (RRL) and wheatgerm systems (Hashimoto et al., 1984). However, efficient translation in insect cells has not been reported. This report describes secondary-structure modelling for each of the major predicted stem–loop structures in the EoPV IRES and investigates their requirement for efficient internal initiation of protein synthesis in vitro and in vivo. By using mutation analysis, we confirm the location of the initiation codon and the functional requirement of further conserved motifs. Of particular interest, we find that the polypyrimidine tract and RAAA motif are not crucial to the activity of the EoPV IRES. Furthermore, the mutation of AUG4 and/or AUG5 increases the activity of the EoPV IRES, in contrast to the IRESs of other, mammalian picornaviruses, whose activities are decreased by these changes. Together, these results indicate that the properties of the EoPV IRES are entirely different from those of all currently described picornavirus IRES elements.

METHODS

Secondary-structure determination. An RNA secondary structure for the 5′ UTR of EoPV was predicted by using the MFOLD program (Mathews et al., 1999), incorporating version 3.0 of the Turner rules (Zuker et al., 1999). The minimum-free-energy structure was determined for the first 390 nt. Resulting images were modified for the figures by using RnaViz 2.0 software (De Rijk et al., 2003).

Plasmid construction. Standard procedures were used for restriction-nuclease digestion and plasmid DNA construction and purification. The bicistronic constructs pRAEF and pRAE-EMCVF were kindly provided by Mark S. Carter (Carter & Sarnow, 2000). The construct pRAEF contains the Renilla luciferase (Rluc) reporter gene as the first cistron, the firefly luciferase (Fluc) reporter gene as the second cistron and a non-functional EMCV IRES (designated in the text as ΔΔ) in the intercistronic region under the control of the T7 promoter. The construct pRAE-EMCVF is inserted in a functional EMCV IRES between ΔΔ and Fluc in the construct pRAEF. Unique Xhol and EcoRI restriction sites are present between these two reporter genes. The integrity of all constructs generated was confirmed by restriction-enzyme digestion and nucleotide sequencing.

The 5′ UTR (nt 1–390) of EoPV was amplified by PCR from EoPV cDNA (Wang et al., 2004) by using forward primer Nos, containing an Xhol site at the 5′ end, and reverse primer Noa, containing an EcoRI site at the 3′ end (Table 1). The resulting PCR product was digested with Xhol and EcoRI and ligated into pRAEF prepared by similar restriction-enzyme digestion. Deletion constructs for the 5′ UTR of EoPV were generated by using PCR to displace ΔΔ using oligonucleotide primers No1 and Noa for pN1(35–390 nt), primers No2 and Noa for pN2(63–390 nt), primers No3 and Noa for pN3(112–390 nt), primers No4 and Noa for pN4(178–390 nt), primers Nos and No5 for pN5(1–299 nt), primers Nos and No6 for pN6(1–361 nt), primers Nos and No7 for pN7(63–361 nt), primers Nos and No5 for pN8(112–361 nt), primers Nos and No5 for pN9(63–299 nt), and primers Nos and OLNq− and OLNq+ and Noa for pNq(A90–234 nt) (deleted from nt 90–234). The amplified PCR products were digested with Xhol and EcoRI and inserted into similarly digested plasmid pRAEF between the Rluc and Fluc reporter genes. These constructs are summarized in Fig. 1.

In order to produce plasmids with part of the EoPV open reading frame (ORF) fused to the Fluc gene, the 5′ UTR (nt 35–390) and the first 73 codons of the ORF sequence were amplified by PCR from EoPV cDNA (Wang et al., 2004) using forward primer No1, containing an Xhol site at the 5′ end, and reverse primer NoF, containing an EcoRI site at the 3′ end (Table 1). The amplified PCR products were digested with Xhol and EcoRI and ligated into similarly digested plasmid pRAEF, giving the product pP(35–609 nt) shown in Fig. 2: the AUG pair mutations were constructed by site-directed mutagenesis of pP(35–609 nt).
Table 1. Oligonucleotides used to prepare deleted regions of the EoPV 5′ UTR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Location on genome*</th>
<th>Sequence (5′→3′)†</th>
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<tbody>
<tr>
<td>Nos</td>
<td>Sense/1</td>
<td>TAGCCCGGTGAGCTGTTTCACGCAGCG</td>
</tr>
<tr>
<td>Noa</td>
<td>Antisense/390</td>
<td>TCTACCGGAAATCCCTGTTGGGTTGTC</td>
</tr>
<tr>
<td>No1</td>
<td>Sense/35</td>
<td>TAGCCCGGTGAGATTCTTAAATACGTTG</td>
</tr>
<tr>
<td>No2</td>
<td>Sense/63</td>
<td>TAGCCCGGTGAGAAATTTCAATAGTTAAAG</td>
</tr>
<tr>
<td>No3</td>
<td>Sense/112</td>
<td>TAGCCCGGTGAGCTTCTATTTATTTATC</td>
</tr>
<tr>
<td>No4</td>
<td>Sense/178</td>
<td>TAGCCCGGTGAGATACATCTAAATTC</td>
</tr>
<tr>
<td>No5</td>
<td>Antisense/299</td>
<td>TGGCCAGGAATTCATATAATAGTATGCG</td>
</tr>
<tr>
<td>No6</td>
<td>Antisense/361</td>
<td>TCTACCGGAAATCCGAGGCAATTCCATA</td>
</tr>
<tr>
<td>NoF</td>
<td>Antisense/609</td>
<td>TCTACCGGAAATTCGTTGGTCGCCACAGGAC</td>
</tr>
</tbody>
</table>

*Numbering system is that used for the EoPV sequence (Wang et al., 2004) (GenBank accession no. AY365064). The sequences of the reverse primers (antisense) are complementary to the locations indicated. †Sequences within oligonucleotides that introduce restriction-enzyme sites are indicated in bold.

In vitro transcription and translation. Coupled transcription–translation (system programmed with plasmid DNA) of the bicistronic construct DNA, under the control of the T7 RNA polymerase promoter, was carried out by using the TNT T7 Quick Coupled transcription–translation system (Promega), containing [35S]methionine (Amersham Biosciences). Standard reactions were performed essentially as recommended by the manufacturer in a total reaction volume of 25 μl. Incubation was for 90 min at 30 °C. A 5 μl aliquot was separated on a 10% SDS-PAGE gel. These gels were subsequently fixed for 30 min at room temperature in 7% acetic acid/10% methanol, dried for 90 min at 80 °C and radioactivity was detected by a phosphorimager for preparation of figures. RLuc and Fluc activities were assayed by using the Dual Luciferase Reporter assay system (Promega) and a Turner Designs TD-20/20 luminometer according to the manufacturers’ protocols.

RESULTS

Features of the 5′ UTR of EoPV

The 5′ UTR nucleotide sequence of EoPV has many features in common with the analogous regions of mammalian picornavirus RNAs. The 5′ UTR of EoPV is predicted to be 390 nt in length, shorter than those of mammalian picornaviruses. It contains five cryptic AUGs and is highly A- and U-rich. By using the MFOLD program, we were able to model an RNA secondary structure for the EoPV 5′ UTR. The most energetically favourable structure contained seven major stem–loop structural domains, designated A to G, beginning at the 5′ terminus of the 5′ UTR (Fig. 1). The structures of stem–loop C, D, E, F and G were similar to those of stem–loops H, I, J, K and L in type II IRESs. Other critical features identified in other IRES elements were present, such as Ym-Xm-AUG and RAAA motifs, although a GNRA motif (a conserved loop motif in many mammalian picornavirus IRESs) was not predicted in the model. An AUG pair (AUGAUG) was evident at the 3′ terminus of the EoPV 5′ UTR. The features of the nucleotide sequence prompted us to examine the 5′ UTR of EoPV in more detail for the presence of an IRES element that could direct the cap-independent initiation of translation of the polyprotein.

Identification of the initiation codon on EoPV RNA

Sequence analysis reveals the presence of two in-frame AUG codons at positions 391 and 394 (AUG6 and AUG7 within the complete EoPV genome). In order to identify the location of the authentic initiation codon within the EoPV RNA sequence, a dicistronic reporter plasmid [pF(35–609 nt)] was constructed, containing the 5′ terminus of the EoPV genome (355 nt of the 5′ UTR plus the first

Transcript-expression assays. SL21 cells were prepared at 50–80% confluence in 24-well tissue-culture dishes and then infected with recombinant baculovirus AcT7N that expresses T7 RNA polymerase (a gift from Dr J. Vlak, University of Wageningen, The Netherlands; van Poelwijk et al., 1995). After 2 h at 28 °C, the medium was removed and the cell monolayer was washed with Grace’s insect cell culture medium (Gibco). DNA transfection was carried out by using 0.5 μg plasmid DNA mixed with Grace’s insect cell culture medium and Cellfectin (Invitrogen) as described in the supplier’s protocol. After 90 h incubation at 28 °C, the cells were harvested in 150 μl cell lysis buffer (Promega) and the lysates were clarified by centrifugation at 14 000 r.p.m. for 5 min at 4 °C. RLuc and Fluc activities were assayed by using the Dual Luciferase Reporter assay system (Promega) and a Turner Designs TD-20/20 luminometer according to the manufacturers’ protocols.
73 codons of the EoPV ORF) inserted between Rluc and Fluc reporter genes in plasmid pRDEF (Fig. 2a). In this plasmid, the reporter genes were both placed under the control of the T7 RNA polymerase promoter. Furthermore, the Fluc gene was in frame with the putative initiation AUG pair of codons, and the second fused cistron, consisting of the partial ORF of EoPV and Fluc, should generate a band of about 70 kDa. Additional plasmids containing mutations in the AUG pair were also constructed (Fig. 2a): the AUGs were mutated to AUA either individually or together. The plasmids were used to program the coupled in vitro transcription–translation system based on RRL with [35S]methionine, and the products were analysed by SDS-PAGE and autoradiography (Fig. 2b). Plasmid pRΔEF(ΔEMCV), containing a non-functional EMCV IRES, was used as a negative control, and plasmid pRΔE-EMCVF(EMCV), which was constructed by inserting the well-characterized EMCV IRES into pRDEF, was used as a positive control.

It could be seen (Fig. 2b) that both cistrons of the construct pF(35–609 nt) were translated efficiently, because bands of 70 and 36 kDa, close to the expected size, were produced. The unmodified EoPV 5’ UTR construct pF(35–609 nt) produced high levels of expression of the fusion protein containing the fragment of the EoPV ORF and Fluc. Expression of this 70 kDa protein was not detected by SDS-PAGE using the construct containing a mutation of AUG6 (Fig. 2b). However, expression levels using the plasmid containing a mutation of AUG7 were consistent with those

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Fig. 1. Proposed secondary structure of the EoPV IRES and schematic representation of the bicistronic construct pN0(1–390 nt) containing the EoPV 5’ UTR. Stem–loop domains (A to G) of the 5’ UTR are labelled, beginning at the 5' terminus of the 5’ UTR. The part of the 5’ UTR remaining in deletion mutants produced by using oligonucleotides pN1(35–390 nt), pN2(63–390 nt), pN3(112–390 nt), pN4(178–390 nt), pN5(1–299 nt), pN6(1–361 nt), pN7(63–361 nt), pN8(112–361 nt), pN9(63–299 nt) and pNq(Δ90–234 nt) is indicated.
The 5' UTR of EoPV contains an efficient IRES

To determine whether the 5' UTR of EoPV possesses an IRES domain, a bicistronic construct [pN0(1–390 nt)] was prepared containing the 5' UTR of EoPV. This construct was used to program the coupled in vitro transcription–translation system based on RRL (Fig. 3a). In parallel to these experiments, the coupled transcription–translation system (without [35S]methionine) was used to assay Rluc and Fluc activities by the Dual Luciferase Reporter assay system. Efficient translation of both cistrons was evident when an intact EoPV 5' UTR was present (Fig. 3b). The construct produced high levels of Fluc compared with the negative control, pRAEF. Thus, we concluded that the EoPV 5' UTR contained an efficient IRES element that was active in the RRL system.

Mapping the 5' and 3' boundaries of the EoPV IRES

To define the borders of the IRES, mutant constructs were initially produced by restriction-enzyme digestion and then tested in the coupled in vitro transcription–translation system, using the Dual Luciferase Reporter assay system to measure Rluc and Fluc activities. Representative results from these experiments are shown in Fig. 3(b).

The full-length construct pN0(1–390 nt) showed strong IRES activity compared with the negative control, pRAEF. Deletion of nt 1–35 [pN1(35–390 nt)] and nt 1–63 [pN2(63–390 nt)] had a slight stimulatory effect on IRES activity, indicating that this sequence was not required for internal initiation. However, truncation of the 5' UTR to position 112 [pN3(112–390 nt)] and 178 [pN4(178–390 nt)] resulted in reductions of twofold and fivefold, respectively, in IRES activity (Fig. 3b). These results demonstrated that nt 63–178 contained an element(s), probably full stem–loops C and D, important for full IRES function in the bicistronic system. In contrast to these findings, deletion of the 3' end of the EoPV 5' UTR [removal of nt 299–390 using construct pN5(1–299 nt)] resulted in a twofold increase in IRES activity. This indicates that the sequence between nt 299 and 390 (stem–loop G) contains an element that can negatively regulate IRES function. A further, smaller deletion at the 3' end, pN6(1–361 nt), generated a very similar result to pN1(35–390 nt) and pN2(63–390 nt). Therefore, the sequence of nt 299–390, which is predicted to contain partial E, F and G stem–loops, was not critical to IRES function. Addition deletions at the 5' end of pN6 [pN7(63–361 nt) and pN8(112–361 nt)], including the polypyrimidine tract and AUG, also had little effect upon IRES activity. Normalized fluorescence was increased by using the construct pN9(63–299 nt) in a similar manner to pN5(1–299 nt), providing further support that stem–loop G contained an element that inhibits IRES activity in vitro. Finally, the essential role of the predicted stem–loops C and D for IRES activity in this system was demonstrated by using pNq(AΔ90–234 nt), where internal initiation was abolished.

Identification of the role of cryptic codons within the EoPV 5' UTR

Five AUG triplets precede the initiation codon at position 391 in the EoPV genome. A site-directed mutational approach was performed on the parental plasmid pN1(35–390 nt) to determine the effect of each AUG codon. The AUG codons were mutated to AUA either
individually or together (Fig. 4a) and sequence analysis of the entire construct was used to confirm that only the desired change had been introduced. The resulting plasmids were used to program the coupled in vitro transcription–translation system (Promega) based on RRL, and the products were analyzed by the Dual Luciferase Reporter assay system (Promega). Plasmids containing mutations of AUG1 and/or AUG2 had little effect on EoPV IRES activity compared with the parental construct pN1(35–390 nt). In contrast, a plasmid containing a mutation of AUG3 led to a slight reduction in relative Fluc measured. Surprisingly, plasmids containing mutations of AUG4 and/or AUG5 generated an increase in IRES activity (Fig. 4b). These data indicate that AUG4 and AUG5 probably have a negative effect that slows the rate of EoPV IRES activity, possibly due to decreasing the opportunity to recognize the downstream initiation codon correctly. Mutational analysis was also performed by targeting a GUG codon at the 3′9 end of the EoPV 5′9 UTR, which is located downstream of the polypyrimidine tract (Fig. 4a). Results in the coupled in vitro transcription–translation system showed a small increase in IRES activity (Fig. 4b).

Table 2. Oligonucleotides used to introduce mutations into the EoPV 5′ UTR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Mutation</th>
<th>Sequence (5′→3′)*</th>
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<tbody>
<tr>
<td>OLA1†</td>
<td>Alter ATG1 to ATA CTTACACATAATAATGTAAGG</td>
<td></td>
</tr>
<tr>
<td>OLA1‡</td>
<td>Alter ATG1 to ATA CATAAATATGGAAGG</td>
<td></td>
</tr>
<tr>
<td>OLA2†</td>
<td>Alter ATG2 to ATA CACATATGATTATAAGCTTTAG</td>
<td></td>
</tr>
<tr>
<td>OLA2‡</td>
<td>Alter ATG2 to ATA CTAAATAAGCAGCTTTAATATGAGG</td>
<td></td>
</tr>
<tr>
<td>OLA1 + 2†</td>
<td>Alter ATG1 &amp; 2 to ATA CCAATGTTAGATGAGG</td>
<td></td>
</tr>
<tr>
<td>OLA1 + 2‡</td>
<td>Alter ATG1 &amp; 2 to ATA GTTATAGGTTAGGTTAGG</td>
<td></td>
</tr>
<tr>
<td>OLA3‡</td>
<td>Alter ATG3 to ATA TATATAAGAAGAG</td>
<td></td>
</tr>
<tr>
<td>OLA4†</td>
<td>Alter ATG4 to ATA TCTACCGGAATTGTTAGGTTAGG</td>
<td></td>
</tr>
<tr>
<td>OLA4‡</td>
<td>Alter ATG4 to ATA GTTATAGGTTAGGTTAGG</td>
<td></td>
</tr>
<tr>
<td>OLA5†</td>
<td>Alter ATG5 to ATA TCTACCGGAATTGTTAGGTTAGG</td>
<td></td>
</tr>
<tr>
<td>OLA5‡</td>
<td>Alter ATG5 to ATA GTTATAGGTTAGGTTAGG</td>
<td></td>
</tr>
<tr>
<td>OLA6 + 7†</td>
<td>Alter ATG6 &amp; 7 to ATA TCTACCGGAATTGTTAGGTTAGG</td>
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</tr>
<tr>
<td>OLA6 + 7‡</td>
<td>Alter ATG6 &amp; 7 to ATA GTTATAGGTTAGGTTAGG</td>
<td></td>
</tr>
<tr>
<td>OLG†</td>
<td>Alter 3′ end of EoPV 5′ UTR GTG to GTA TCTACCGGAATTGTTAGGTTAGG</td>
<td></td>
</tr>
<tr>
<td>OLRG‡</td>
<td>Alter TAAA to CUUU TAGTGTGAGGATAGGTCTACTAC</td>
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</tr>
<tr>
<td>OLRG†</td>
<td>Alter TAAA to CUUU TAGTGTGAGGATAGGTCTACTAC</td>
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</tr>
<tr>
<td>OLRC‡</td>
<td>Alter TAAA to GCCC TAGGTCGTTATGAGG</td>
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<td>OLRC†</td>
<td>Alter TAAA to GCCC TAGGTCGTTATGAGG</td>
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</tr>
<tr>
<td>OLpT‡</td>
<td>Alter CCTTTC to GGAAAG GAGATGTGCAATACAT</td>
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<tr>
<td>OLpT†</td>
<td>Alter CCTTTC to GGAAAG GAGATGTGCAATACAT</td>
<td></td>
</tr>
<tr>
<td>OLNg + ‡</td>
<td>Delete sequence from nt 90 to 234 GAAAACGAGCTTTGGAAAG</td>
<td></td>
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<tr>
<td>OLNg ‡</td>
<td>Delete sequence from nt 90 to 234 GAAAACGAGCTTTGGAAAG</td>
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</tbody>
</table>

*Introduced mutations are indicated in bold.
†Oligonucleotides used with Nos in the first-round PCR. Overlapping first-round PCR products were combined and subjected to second-round PCR with Noa and No1 (Table 1).
‡Oligonucleotides used with Noa in the first-round PCR. Overlapping first-round PCR products were combined and subjected to second-round PCR with Noa and No1 (Table 1).
§Oligonucleotides used with NoF in the first-round PCR. Overlapping first-round PCR products were combined and subjected to second-round PCR with No1 and NoF (Table 1).

The polypyrimidine tract is not crucial for EoPV IRES activity

In addition to 5′ UTR structural elements, a key element in the IRES of picornaviruses is the Yₙ–Xₚ–AUG motif, where Yₙ is a polypyrimidine tract, followed by an AUG located 5–25 nt downstream (Stanway et al., 2000). The sequence of...
EoPV contains a polypyrimidine tract (CCTTTC, nt 333–338) that is followed by an AUG triplet after a spacer region of 6 nt (Fig. 5a). However, precise mutation of this polypyrimidine tract resulted in only 20% reduction in IRES activity compared with pN1(35–390 nt) (Fig. 5b), thus suggesting that the presence of the polypyrimidine tract was not crucial to IRES function, yet it can contribute to maximal IRES activity.

Conserved motif RAAA is important for EoPV IRES activity

Mutations were introduced into the conserved RAAA motif located in stem–loop D, with the aim of analysing the contribution of this motif to IRES activity (Fig. 5a). Mutant RB altered the sequence of the apex of the stem–loop D from UAAA to CUUU, whilst mutant RC altered the sequence from UAAA to GCCC. Both mutations led to a reduction of approximately 40% in IRES activity compared with pN1(35–390 nt) (Fig. 5b). The result demonstrated the importance of the apical loop for the maintenance of full IRES activity in vitro.

The EoPV IRES functions in vivo

To examine the activity of the EoPV IRES activity in vivo, the constructs were assayed by transfection into Sf21 cells using
Cellfectin (Invitrogen) according to the methods above. Rluc and Fluc were assayed by using the Dual Luciferase Reporter assay system (Promega). As seen previously with the in vitro transcription–translation system (described above), the EoPV 5' UTR possessed IRES activity. Furthermore, the result of 5'- and 3'-end deletion mutants was similar to that seen in vitro. However, the effects of mutants RB and RC were not evident in this system compared with pN1(35–390 nt) within Sf21 cells (Fig. 6). These results indicated that the RAAA motif did not contribute to IRES activity in vivo.

**DISCUSSION**

EoPV is an invertebrate picorna-like virus, displaying similarities in genome organization and in the physico-chemical characteristics of the polyprotein to mammalian picornaviruses. Currently, EoPV is classified as a putative member of the genus *Iflavirus*, which has not yet been placed into a virus family. This work represents the first molecular analysis of EoPV and provides valuable insights that may contribute to the classification of this virus.

We examined the structure and function of the EoPV IRES, an element that, in picornaviruses, plays a central role in translation of the viral polyprotein and, in some cases, can also mediate virulence of the virus. In this study, we constructed a panel of 5' UTR mutants containing an extensive range of mutations, including point mutations, large mutations and large deletions. They were introduced into a bicistronic construct, which allowed changes in the expression of the second cistron to be correlated to IRES activity during in vitro and in vivo experiments. The results presented here demonstrate that the 5' UTR of EoPV contained an IRES element, the function of which can be measured in the coupled in vitro transcription–translation system based on RRL, and in vivo based on Sf21 cells.

Taken together, the in vitro and in vivo results allowed the IRES to be mapped and the contribution of specific elements within it to be assessed. Deletions within the first 63 nt of the 5' UTR were of little importance, whereas deletions of nt 112–178 had a significant effect on translation. This indicates that the 5' boundary of the IRES must therefore be located downstream of position 63. At the 5' end, removal of stem–loops E, F and G did not clearly reduce translation. These findings suggest that stem–loops C and D constitute the structural core of the IRES, as disruption of these stem–loops abrogated EoPV IRES activity. Stem–loops E and F of EoPV have predicted structures similar to those of stem–loops J and K of the type II IRESs. Among the type II IRES elements of cardioviruses and aphthoviruses in the family Picornaviridae, the Y-shaped domain carries the determinants for recruitment of eIF4G to the viral RNA. Although previous work has shown that stem–loops J and K appear to be most critical for translation initiation in cardioviruses and aphthoviruses (Duke et al., 1992; Hoffman & Palmenberg, 1995; Witherell et al., 1995), the structural stem–loops defined here for EoPV were unnecessary for the activity of the EoPV IRES. Separate removal of domain G (removal of nt 299–390) increases the IRES activity. This result resembles those for poliovirus and
Results from this study indicated that these sequences decreased the efficiency of IRES activity, presumably due to disruption of ribosomal scanning and subsequent correct initiation of protein synthesis. The results were consistent with those from pN5(1–299 nt) with stem–loop G deleted, because AUG4, AUG5 and GUG are all located within this predicted structure.

A short polypyrimidine tract is present at the 3' end of the EoPV 5' UTR, which is similar in sequence and location to a polypyrimidine tract near the 3' terminus of the FMDV 5' UTR. Our results also showed that mutation of the polypyrimidine tract was not critical to the activity of the EoPV IRES. It should be noted that the importance of the polypyrimidine tract for picornavirus IRES elements is not entirely clear (Belsham & Jackson, 2000). Initial studies on the polypyrimidine tract suggested that this is an essential element of the IRES for FMDV, EMCV and poliovirus (Jang & Wimmer, 1990; Kühn et al., 1990; Meerovitch et al., 1991; Nicholson et al., 1991; Pestova et al., 1991). In contrast, studies on the EMCV and Theiler's murine encephalomyelitis virus IRESs have demonstrated a remarkable insensitivity of the polypyrimidine tract to mutation (Kaminski et al., 1994; Filipenko et al., 1992). Results in this study indicate that the insensitivity of the polypyrimidine tract of the EoPV IRES to mutation was similar to that of type II IRESs.

The role of the well-conserved RAAA motif was also analysed in the EoPV IRES by in vitro translation of mutated RNAs. It was demonstrated that alteration from UAAA to CUUU or GCCC resulted in an approximate decrease of 40 % in EoPV IRES activity. Mutagenesis of this motif has also been performed on the FMDV IRES (López de Quinto & Martínez-Salas, 1997), also indicating a significant effect on IRES activity when the RAAA motif is disrupted. However, in vivo results in this study showed little effect on the EoPV IRES activity, distinguishing our findings from those with other type II IRESs.

The results presented here, derived from experiments using an extensive panel of mutants, provide strong evidence for the requirement for structural elements and conserved sequence motifs in the EoPV IRES. The data show that the EoPV IRES is similar in several functional and structural respects to those of cardioviruses and aphthoviruses, which contain type II IRESs. Particular similarities are the efficient function of the EoPV IRES in vitro and in vivo and the remarkable insensitivity of the polypyrimidine tract to mutation. However, there are some differences between the IRES of EoPV and other type II IRES elements. Notably, EoPV lacks a GNRA motif, the initiation codon of the polyprotein in the genome was at AUG6, which was not part of the Y_n'-X_m-AUG motif, and the RAAA motif was not important to IRES activity in vivo. These data therefore contribute significantly to our understanding of the structure and function of the 5' UTR in the insect picorna-like viruses.

hepatitis A virus RNAs (Nicklin et al., 1987; Brown et al., 1991) and suggests that stable helical structures of stem–loop G present an obstacle to efficient ribosomal scanning.

In addition to the analysis of these structural elements, site-directed mutagenesis of five cryptic AUGs and a GUG was also performed. These experiments showed that initiation of protein synthesis occurred at the AUG6 codon located at nt 391–393, which was consistent with the initiation site proposed previously (Wang et al., 2004). The AUG codon is the first codon of an AUG pair, which is preceded by a significant polypyrimidine tract, a conserved motif existing at the 3' end of the IRES elements of the picornaviruses (Meerovitch & Sonenberg, 1993). However, in the newly reported Equine rhinitis A virus, a member of the genus Aphthovirus, the second AUG of the first AUG pair is its dominant start site (Hinton et al., 2000). There are two additional AUG codons (AUG4 and AUG5) and a GUG codon (not in frame with the polyprotein and between the polypyrimidine tract and the authentic initiation site at nt 391) in the EoPV 5' UTR. Surprisingly, in contrast to mammalian picornavirus, the mutations of AUG4 and/or AUG5 resulted in a remarkable increase in IRES activity.

**Fig. 6.** Examination of EoPV IRES activity in vivo. The plasmids were transfected into AcT7N-infected Sf21 cells expressing T7 RNA polymerase. Rluc and Fluc activities were assayed by the Dual Luciferase Reporter assay system. Relative Fluc/Rluc ratios representing EoPV IRES activity are shown. Samples were normalized (to an Fluc/Rluc ratio of 1) by using control plasmid pRAEF.
ACKNOWLEDGEMENTS

We thank Dr Mark S. Carter for the provision of plasmids pRAEF and pRAE-EMCVF and Professor J. Vlak for the kind gift of the recombinant baculovirus AcT7N. We thank Professors Deyin Guo and Congyi Zheng for helpful discussions and for providing us with excellent laboratory facilities. We also thank Dr Donald P. King for critical reading of the manuscript. This work was supported by the National Natural Science Foundation of China (no. 30670084).

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