Hepatitis C virus-related internal ribosome entry sites are found in multiple genera of the family Picornaviridae

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The internal ribosome entry site (IRES) elements from porcine enterovirus 8 and simian virus 2, two members of a proposed new genus within the family Picornaviridae, were characterized. These IRES elements, in common with the porcine teschovirus 1 IRES, were found to be related functionally and structurally to the IRES element from Hepatitis C virus, a member of the family Flaviviridae. Partial secondary structure predictions were derived and functional assays demonstrated that these IRES elements continued to be active when eIF4G was cleaved and when the activity of eIF4A was blocked.

INTRODUCTION

Currently, there are nine genera within the family Picornaviridae, namely Enterovirus, Rhinovirus, Aphthovirus, Cardiovirus, Erbovirus, Hepatovirus, Parechovirus, Teschovirus and Kobuvirus. However, sequence analysis has shown that some members of this family differ significantly from these existing genera. Thus, it has been suggested (Krumbholtz et al., 2002; Oberste et al., 2003) that certain simian viruses, namely SV2, SV16, SV18, SV42, SV44, SV45 and SV49, together with porcine enterovirus 8 (PEV-8), should be classified into a new genus (the provisional name of Sapelovirus has been proposed; N. Knowles, personal communication). These viruses encode a leader protein and hence are quite distinct from the enteroviruses. It has been noted (Krumbholtz et al., 2002) that there is high level of sequence identity within a 90 nt region of the 5′ untranslated regions (UTRs) of PEV-8, SV2 and the porcine teschovirus 1 (PTV-1). Furthermore, Oberste et al. (2003) reported that the SV2 5′ UTR is 44% identical to the PTV-1 5′ UTR; however, the overall level of amino acid sequence identity among the various proteins of SV2 and the PTV-1 is only 14–39% and thus these viruses are clearly members of different genera.

Picornavirus RNAs are infectious and have to act both as mRNAs to produce the virus-encoded proteins and as templates for RNA replication. The 5′ UTRs of picornavirus RNAs are long (approx. 700–1300 nt) and are predicted to contain extensive secondary structure. These sequences include an internal ribosome entry site (IRES), which directs cap-independent initiation of protein synthesis on the viral RNA (see Belsham & Jackson, 2000, for a review). Multiple classes of picornavirus IRES element have been characterized. The enteroviruses [e.g. poliovirus (PV)] and rhinoviruses contain one type of structure, whilst the cardioviruses [e.g. encephalomyocarditis virus (EMCV)] and aphthoviruses [e.g. foot-and-mouth disease virus (FMDV)] contain a second class, which has a distinct secondary structure and different biological properties. For example, EMCV and FMDV IRES elements function efficiently in the rabbit reticulocyte lysate (RLR) in vitro translation system, whereas PV IRES does not. The hepatitis A virus (HAV) IRES is generally considered to represent a distinct minor class of picornavirus IRES. In contrast to the other picornavirus IRES elements, it requires the intact translation initiation eIF4F complex comprising eIF4E, eIF4A and eIF4G (Borman & Kean, 1997; Ali et al., 2001a). PV, EMCV and FMDV IRES elements have no requirement for the cap-binding protein eIF4E and can function when the eIF4G scaffold protein has been cleaved by either the FMDV leader protease or an enterovirus/rhinovirus 2A protease. These cleavage events take place at distinct but adjacent sites on eIF4G and separate the N-terminal fragment, which binds to eIF4E, away from the rest of the molecule, which interacts with eIF3 and eIF4A (two sites) (see Belsham & Jackson, 2000). The C-terminal fragment of eIF4G (often termed p100) is sufficient to support translation initiation on mRNAs containing the picornavirus IRES elements (except HAV) and even on capped mRNAs in vitro, albeit less efficiently (Ali et al., 2001b). Dominant-negative mutants of eIF4A (the prototype DEAD box RNA helicase) block the activity of these IRES elements (Pause et al., 1994; Svitkin et al., 2001).
Recently, we have characterized the IRES element from the PTV-1 Talfan strain, the prototype teschovirus (Kaku et al., 2002; Pisarev et al., 2004; Chard et al., 2006). Remarkably, it is quite different from the other characterized picornavirus IRES elements. The PTV-1 IRES is only approximately 280 nt long (compared with approx. 450 nt for FMDV, PV and EMCV) and functions when eIF4G is cleaved (Pisarev et al., 2004) but it is resistant to inhibitors of eIF4A function (Chard et al., 2006; Bordeleau et al., 2006). The assembly of 48S pre-initiation complexes on the PTV-1 IRES requires only purified 40S ribosomal subunits plus the ternary complex of eIF2, Met-tRNA and GTP (Pisarev et al., 2004); no eIF4 factors are necessary. The initiation factor eIF3 interacts directly with the PTV-1 IRES and enhances the formation of the 48S complex on the RNA. However, the PTV-1 IRES can form a binary complex with purified 40S ribosomal subunits alone (Pisarev et al., 2004). These characteristics are very similar to those described for the IRES elements from Hepatitis C virus (HCV), a hepacivirus, and Classical swine fever virus (CSFV), a pestivirus, which are both members of the family Flaviviridae (Pestova et al., 1998; Fletcher & Jackson, 2002; Sarnow, 2003). The PTV-1 IRES is indeed closely related to the HCV IRES (>50 % sequence identity) and is predicted to have a very similar secondary structure including a pseudoknot that is critical for IRES function (Sarnow, 2003; Pisarev et al., 2004; Chard et al., 2006).

Here, we have characterized the IRES elements from SV2 and PEV-8. These elements have distinctive properties and we have shown that they are both functionally and structurally related to HCV and PTV-1 IRES elements. Thus, multiple distinct viruses from different genera of the family Picornaviridae contain an HCV-like IRES.

METHODS

Secondary structure prediction. The HCV IRES is contained within nt 44–345 of the 5’ UTR (Honda et al., 1996) (GenBank accession no. AB016785), whilst the IRES element of PTV-1 is located within nt 125–405 of the PTV-1 Talfan sequence (Kaku et al., 2001, 2002; Pisarev et al., 2004) (GenBank no. AB038528). The sequences of PEV-8 (GenBank no. AF046813) and SV2 (GenBank no. AY064708) have been reported previously (Krumholz et al., 2002; Oberste et al., 2003). The sequences were aligned using CLUSTAL_W and manually edited using the GCG10 SeqLab program. Secondary structure elements (other than pseudoknots) within the PEV-8 and SV2 sequences were generated using Mfold (Zuker, 2003).

Mutagenesis. Plasmid construction, mutagenesis and analysis were performed using standard techniques (Sambrook & Russell, 2001).

The sequence of PEV-8 derived by Krumholz et al. (2002) lacks the extreme 5’ terminus of the viral RNA and the initiation codon has been identified at nt 444 in the known sequence. Forward and reverse PCR primers were designed to generate a nested set of cDNA fragments (between nt 20 and 439) including BamHI restriction sites at each terminus. The primer sequences are listed in Table 1. The fragments were generated by PCR using PEV-8 cDNA as template (kindly provided by Nick Knowles, IAH, Pirbright, UK) and ligated into p17Blue (Novagen). The cloned fragments were released by digestion with BamHI and ligated into similarly digested pGEM-CAT/LUC (van der Velden et al., 1995) to generate the plasmids indicated in Fig. 1(a). The orientation of the inserted was identified by restriction enzyme digestion and confirmed by DNA sequencing using the CAT forward primer (Table 1).

An amplicon corresponding to nt 13–1097 of SV2 cDNA (Oberste et al., 2003) was kindly provided by Steve Oberste (CDC, Atlanta, USA). The SV2 sequence is also presumed to be lacking some bases at the 5’ terminus (all picornavirus genome sequences commence with UU). The initiation codon has been predicted to be at nt 742 (Oberste et al., 2003), but our analyses indicated that this was incorrect (see below). The amplicon was used as a template in separate PCRs using the appropriate forward and reverse primers listed in Table 1. The fragments generated were ligated into p17Blue and the inserts were then released from these plasmids by digestion with BamHI and ligated into pGEM-CAT/LUC as above (see Fig. 1 and below).

To facilitate identification of the initiation codon, the dicistronic plasmids pG/SV2S/L, pGC/SV2L (see below) and pGC/PTV/L (Pisarev et al., 2004) were digested with XbaI and ClaI, treated with the Klenow fragment of DNA polymerase I with dNTPs to create blunt ends and religated. This process removed 1320 nt from within the firefly luciferase (ILUC) ORF; the reading frame was maintained but the expressed protein was reduced by 440 residues. The resulting plasmids were named pCAT/SV2S/ILUC, pCAT/SV2L/ILUC and pCAT/PTV/ILUC.

In vitro translation assays. Protein expression from the indicated plasmids, which each contained the T7 promoter, was achieved by using a coupled transcription and translation system (TnT; Promega) containing T7 RNA polymerase and RRL with [35S]methionine (Amersham Biosciences), essentially as described by the manufacturer. Products were analysed by 10 % SDS-PAGE and autoradiography.

Transient expression assays. Plasmids (2-5 μg), containing a T7 promoter, were assayed by transfection using Lipofectin (8 μg; Life

Table 1. Oligonucleotides used for characterization of the PEV-8 and SV2 IRES elements

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
</tr>
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<tbody>
<tr>
<td>PEVF1 forward</td>
<td>CCGGATCCCTAAAGTGTTGTGATCCCTCA</td>
</tr>
<tr>
<td>PEVA1 forward</td>
<td>GGATCCCATTAAGTGGAATGACAG</td>
</tr>
<tr>
<td>PEV2A forward</td>
<td>CCGGATCCACGTATGGAATGACAG</td>
</tr>
<tr>
<td>PEVA3 forward</td>
<td>GGATCCATGTAGTGGAATGACAG</td>
</tr>
<tr>
<td>PEVA4 forward</td>
<td>GGATCCATAGACATGGAATGACAG</td>
</tr>
<tr>
<td>PEBVF1 reverse</td>
<td>GGATCCACTAGCTAT</td>
</tr>
<tr>
<td>SV2FL forward</td>
<td>GGATCCCTGGCGCTGTACAG</td>
</tr>
<tr>
<td>SV2DA1 forward</td>
<td>GGATCCAGTGCAACACTTTGCG</td>
</tr>
<tr>
<td>SV2DA2 forward</td>
<td>GGATCCGTAGTGGAATGCTTAAGG</td>
</tr>
<tr>
<td>SV2DA3 forward</td>
<td>GGATCCGGATTAGCTTCAAGGGTGGCCG</td>
</tr>
<tr>
<td>SV2DA4 forward</td>
<td>GGATCCCGATATGTGATGTTAG</td>
</tr>
<tr>
<td>SV2A5 forward</td>
<td>GGATCCGGCGTTGTCGTCGCGGCGGCAGTAA</td>
</tr>
<tr>
<td>SV2A6 forward</td>
<td>GGATCCGAAGTAAAGCTTGGAGAATACAG</td>
</tr>
<tr>
<td>SV2S reverse</td>
<td>GGATCCGCCGTACCTGTATACCATAC</td>
</tr>
<tr>
<td>SV2L reverse</td>
<td>CTCATGGATCCCGCCATCTGAGAATGCTT</td>
</tr>
<tr>
<td>CAT forward</td>
<td>ACACCTCCCCCTGAACCTGACAT*</td>
</tr>
</tbody>
</table>

*Derived from the sequence of pSV2-CAT (Gorman et al., 1982).
Technologies) into baby hamster kidney (BHK) cells infected with the recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986), which expresses the T7 RNA polymerase, as described previously (Roberts et al., 1998). In some experiments, reporter plasmids (2 μg) were assayed alone or mixed, prior to transfection, with pGEM3Z/J1 (0–5 μg), which expresses the swine vesicular disease virus (SVDV) 2A protease (Sakoda et al., 2001). After 20 h, cell extracts were prepared and analysed for expression of CAT and LUC by immunoblotting and flUC activity was quantified using an flUC assay. Results shown in the graph are the mean values (±SD) determined from three separate transfections; flUC activity from the full-length PEV-8 5’ UTR was set at 100% in each case. Plasmid names are given in (a) and abbreviated in (b) and (c) to the name of the insert for clarity.

**RESULTS**

**Identification of the PEV-8 IRES**

Plasmids were constructed that expressed, from the T7 promoter, dicistronic mRNAs containing the ORFs for chloramphenicol acetyl transferase (CAT) and flUC separated by sequences derived from the 5’ UTR of PEV-8 (Fig. 1a). The initiation codon in the PEV-8 sequence has been identified as being at nt 444 (Krumbholz et al., 2002). Different lengths of PEV-8 sequence were positioned upstream of the flUC coding sequence with its own initiation codon appropriately positioned relative to the rest of the PEV-8 5’ UTR. Plasmids were initially assayed using an *in vitro* coupled transcription and translation (TNT) system containing RRL. Each of the plasmids tested produced similar amounts of the upstream CAT reporter protein as expected (Fig. 1b). Plasmids containing nt 22–433, 124–433 or 148–433 from the PEV-8 sequence all also expressed flUC efficiently, as did the positive control, pGC/PTV/L (Pisarev et al., 2004), containing the PTV-1 IRES (Fig. 1b). However, no flUC product was detected from the control vector lacking an IRES, nor when only nt 179–433 or nt 238–433 from the PEV-8 sequence was present. These results are consistent with the view that nt 148–433 (approx. 285 nt) of the PEV-8 cDNA includes an IRES element that functions in the RRL *in vitro* translation system.

To confirm and extend these results, the same plasmids were analysed in a transient expression assay within cells infected with the recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986) expressing the T7 RNA polymerase. Cell extracts were prepared and analysed by immunoblotting using anti-CAT and anti-flUC antibodies. As observed using the TNT reagents (Amersham). In addition, the extracts were assayed for flUC activity using a luciferase assay kit (Promega) and a luminometer. In some experiments, the transfected cells were treated with a small-molecule inhibitor of eIF4A (Bordeleau et al., 2006), named hippuristanol (0.5 μM), for 10 h prior to cell lysis.

**Fig. 1.** Functional analysis of the boundaries of the PEV-8 IRES. (a) The PEV-8 cDNA fragments indicated were generated by using PCR as described in Methods and inserted into the *Bam*HI-digested vector pGEM-CAT/LUC (van der Velden et al., 1995), which expresses a dicistronic mRNA containing the CAT and flUC coding sequences from the T7 promoter. (b) The indicated plasmids were used in TNT reactions containing [35S]methionine. Products were analysed by SDS-PAGE and autoradiography. (c) The plasmids indicated were used to transiently infect vTF7-3-infected BHK cells. After 20 h, cell extracts were prepared and analysed for expression of CAT and LUC by immunoblotting and flUC activity was quantified using an flUC assay. Results shown in the graph are the mean values (±SD) determined from three separate transfections; flUC activity from the full-length PEV-8 5’ UTR was set at 100% in each case. Plasmid names are given in (a) and abbreviated in (b) and (c) to the name of the insert for clarity.
system, the PEV-8 sequence from nt 148 to 433 was sufficient to direct the expression of Fluc efficiently within cells, but smaller fragments were non-functional (Fig. 1c). CAT expression was similar in each case as expected. Quantification of Fluc expression by enzyme assay indicated that the PEV-8 sequences within nt 148–433 were approximately 40% as efficient as the longer fragments of nt 124–433 or 22–433 (Fig. 1c). The expression of Fluc directed by these longer fragments of the PEV-8 5' UTR was similar to that observed with the PTV-1 IRES. No PEV-8 coding sequences were required for efficient production of Fluc in these systems.

Identification of the initiation codon in SV2

It was predicted by Oberste et al. (2003) that the SV2 initiation codon was located at nt 742 in the known sequence. This AUG codon is located just downstream of a polypyrimeridine tract. This arrangement is a conserved feature of all previously characterized picornavirus IRES elements except for the PTV-1 IRES (see Belsham & Jackson, 2000; Kaku et al., 2002). However, on the basis of sequence alignments among the SV2, PEV8 and PTV-1 sequences (see below; Krumholz et al., 2002), it appeared that the AUG codon positioned at nt 694 might be the authentic initiation codon. Use of this codon would simply extend the ORF within the SV2 sequence by 48 nt (resulting in a 16 aa extension to the leader protein). Dicistronic plasmids were constructed that contained either nt 15–746 (termed pGC/SV2L/Fluc) or nt 15–685 (termed pGC/SV2S/Fluc) of the SV2 sequence inserted between the CAT and Fluc ORFs (see Fig. 2a). When assayed either in vitro or in cells (not shown), it was apparent that both plasmids produced the Fluc protein, suggesting that both of the SV2 cDNA fragments expressed a functional IRES. However, Fluc expression in cells (as judged by Fluc assays) was approximately threefold higher from the longer SV2L construct than from the SV2S sequence. Furthermore, close examination of the original autoradiographs suggested that Fluc expressed in vitro from the SV2L sequence was slightly larger than the authentic Fluc product (~60 kDa) expressed from the SV2S, PEV-8 or PTV-1 elements. To facilitate further examination of this result, an in-frame deletion of 1320 nt was made within the Fluc coding sequence of these plasmids so that a shortened form of the Fluc protein (termed ΔFluc, ~15 kDa) was produced (see Fig. 2a). Analysis of the resultant plasmids pGC/PTV/ΔFluc and pGC/SV2S/ΔFluc indicated that an Fluc-related protein of 15 kDa was produced as expected (Fig. 2b). However, from the plasmid pGC/SV2L/ΔFluc a larger product of ~17 kDa was observed. These results strongly suggested that the upstream AUG codon at nt 694 rather than the AUG at nt 742 was being used as the initiation codon. Thus, the plasmid pGC/SV2L/ΔFluc produced a fusion protein, containing the N terminus of the SV2 leader protein linked to the ΔFluc protein. It is interesting to note that, as observed above, the presence of some of the SV2 coding sequence enhanced translation of the second cistron (Fig. 2b). A similar effect has been observed with the HCV IRES (Reynolds et al., 1995).

To define the 5' boundary of the SV2 5' UTR needed to direct internal initiation of protein synthesis, further plasmids were prepared that contained truncated portions of the SV2 5' UTR from within the sequence nt 15–746 (Fig. 3a). These plasmids were analysed by using the TNT system

![Fig. 2. Identification of the initiation codon in SV2 RNA. (a) The indicated fragments of SV2 cDNA were amplified by PCR and inserted into pGEM-CAT/LUC as shown in Fig. 1. A deletion of 1320 nt from within the Fluc coding sequence was generated by digestion with XbaI and Clal, end repair and religation (see boxed insert). Potential initiation codons at nt 694 and 742 are indicated. (b) The indicated plasmids were analysed using a transient expression system in vTF7-3-infected BHK cells. Products were detected by immunoblotting with an anti-Fluc antibody. Note the slower migration of the ΔFluc product generated from the SV2L insert. Plasmid names are shown in (a) and in abbreviated form (intercistronic region only) in (b) for clarity.](image-url)
(Fig. 3b) and within vTF7-3-infected BHK cells (Fig. 3c) as above. In each case, it was apparent that RNA transcripts including nt 253–746 (SV2Δ2) of the SV2 sequence expressed fLUC efficiently, whilst nt 285–746 were sufficient to produce approximately 66% of maximal activity in cells. Further truncation of the sequence to nt 326–746 resulted in a much reduced activity (Fig. 3b and c). Thus, nt 253–693 from the 5′ UTR plus a portion of the coding sequence (in this case approx. 60 nt) are required for optimal expression of the second cistron. Therefore, the functional element from within the SV2 5′ UTR (approx. 440 nt) is somewhat larger than the PEV-8, HCV and PTV-1 IRES elements.

**SV2 and PEV-8 5′ UTR sequences direct internal initiation when cap-dependent translation is inhibited**

A convenient method of confirming that a picornavirus 5′ UTR element does indeed contain an IRES is to show that expression of the second ORF within the dicistronic construct is maintained when cap-dependent protein synthesis is blocked. This analysis rules out the translation of a truncated RNA transcript produced from a cryptic promoter. This characteristic applies to all picornavirus IRES elements so far analysed except for the HAV IRES. Hence, dicistronic reporter plasmids containing the PEV-8 and SV2 5′ UTR sequences that efficiently directed the expression of fLUC were transfected into BHK cells alone or with the plasmid pGEM3Z/J1 (Sakoda et al., 2001), which expresses the 1D–2A region of the enterovirus SVDV. The 2A protease inhibited the cap-dependent expression of the upstream cistron (CAT), but had no significant effect on the PEV-8 and SV2 RNA-directed expression of fLUC (Fig. 4a and b). By using a control plasmid that contained the human coxsackievirus B4 IRES, it was observed that the activity of this IRES was enhanced under these conditions (data not shown), consistent with earlier results (Roberts et al., 1998; Sakoda et al., 2001). It was concluded that the PEV-8 and SV2 5′ UTRs each contain an IRES element that functions when eIF4G is cleaved and cap-independent initiation of protein synthesis is blocked.

**PEV-8 and SV2 IRES elements are unaffected by hippuristanol, a small-molecule inhibitor of eIF4A function**

Most picornavirus IRES elements are inhibited by dominant-negative mutants of eIF4A (Pause et al., 1994; Svitkin et al., 2001). In contrast, the HCV and PTV-1 Talfan IRES elements are insensitive to these proteins (Pestova et al., 1998; Svitkin et al., 2001; Chard et al., 2006) and to a small-molecule inhibitor of eIF4A function called hippuristanol (Bordeleau et al., 2006). Plasmids that express dicistronic reporter mRNAs containing the IRES elements from PEV-8, SV2 and EMCV were transfected in duplicate into vTF7-3-infected BHK cells as above. After 10 h, cell extracts were either treated with hippuristanol (0–5 mM) or left untreated. After 20 h, cell extracts were prepared and analysed for the expression of CAT and fLUC as above. The cap-dependent expression of CAT and fLUC was essentially abolished by hippuristanol treatment (Fig. 4a and b). By using a control plasmid that contained the human coxsackievirus B4 IRES, it was observed that the activity of this IRES was enhanced under these conditions (data not shown), consistent with earlier results (Roberts et al., 1998; Sakoda et al., 2001). It was concluded that the PEV-8 and SV2 5′ UTRs each contain an IRES element that functions when eIF4G is cleaved and cap-independent initiation of protein synthesis is blocked.
of the CAT reporter was significantly inhibited in the presence of hippuristanol and the EMCV IRES was also strongly inhibited (more than fivefold) under these conditions (Fig. 4c and d). These results are analogous to those observed with dominant-negative mutants of eIF4A in vitro (Pause et al., 1994; Svitkin et al., 2001). In contrast, the expression of fLUC directed by the SV2 and PEV-8 IRES elements was essentially unaffected (no more than a 20% decrease) by the presence of this inhibitor. Analogous results were obtained using TNT reactions (data not shown).

### Sequence comparisons among the IRES elements of HCV, PTV-1, SV2 and PEV-8

Significant sequence similarity has been reported previously between the 5' UTR of PTV-1 and the 5' UTRs of PEV-8 and SV2 (Krumholz et al., 2002; Oberste et al., 2003). Alignments were performed among the sequences defined as the IRES elements from PTV-1 (Pisarev et al., 2004), PEV-8 and SV2 (Fig. 5). The PTV-1 and PEV-8 IRES sequences were 62% identical in this region, whilst the

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**Fig. 4.** PEV-8 and SV2 IRES elements function when eIF4G is cleaved and eIF4A activity is blocked. (a, b) The indicated dicistronic plasmids were transfected alone or with pGEM3Z/J1 (which expresses the SVDV 2A protease; Sakoda et al., 2001) as indicated into vTF7-3-infected BHK cells. After 20 h, cell extracts were prepared and analysed for the expression of CAT and fLUC by immunoblotting. (c, d) Plasmids were transfected alone into vTF7-3-infected BHK cells as in (a) and (b). At 10 h prior to cell lysis, hippuristanol (hipp.; 0-5 μM) was added as indicated. Extracts were prepared at 20 h post-transfection and analysed for CAT and fLUC by immunoblotting and for fLUC enzyme activity. Results shown in the graph are the mean values (±SD) determined from three separate transfections; fLUC activity from each plasmid in the absence of hippuristanol was set at 100% in each case.

**Fig. 5.** Alignment of PTV-1, PEV-8 and SV2 IRES cDNA sequences. Nucleotide sequences corresponding to the IRES elements of PTV-1 and PEV-8 (a) and PTV-1 and SV2 (b) were aligned pairwise using CLUSTAL_W and edited manually. Predicted domains analogous to the HCV IRES domains are indicated. Sequences involved in the formation of the pseudoknots are marked above or below with an X. The initiation codons are boxed. Identical sequences are shaded. The polypyrimidine tract, located downstream of the initiation codon in the SV2 sequence, is also boxed (b).
HCV-related IRES elements in PEV-8 and SV2
PTV-1 and SV2 sequences were approximately 54% identical. The PEV-8 and SV2 sequences were approximately 65% identical to each other. As shown previously (Pisarev et al., 2004; Chard et al., 2006), the PTV-1 IRES showed a high level (approx. 53%) of sequence identity to the HCV IRES; similarly, the PEV-8 IRES was approximately 48% identical to the HCV IRES (not shown). It was apparent that these elements are closely related. Indeed, certain features that were identified previously as being highly conserved between the PTV-1 and HCV IRES elements were also highly conserved in the PEV-8 and SV2 sequences. For example, the HCV domain IIIe contains 12 nt; within the PTV-1 IRES, 11 of these are identical (Pisarev et al., 2004; Chard et al., 2006). In the PEV-8 sequence, this domain is identical to HCV, whereas in SV2 there are 9 nt that are identical to the HCV sequence and 10 nt that are shared with the PTV-1 IRES sequence. The four regions of sequence involved in base pairing to generate the pseudoknot provide considerable constraints on this alignment and divide the sequence into the two main domains. Furthermore, within domain III, the presence of the highly conserved domain IIIId and IIIe sequences provide additional landmarks. However, within domain II, the alignments should be regarded as provisional.

Conserved secondary structure elements predicted within the PEV-8 and SV2 IRES elements

Since there was a high level of sequence identity between these different IRES elements, it was possible to predict the presence of various structural elements that are found within the HCV IRES. Sequences required for the formation of a pseudoknot structure (termed domain IIIIf in HCV) were present within the PEV-8 and SV2 IRES elements and the structures closely corresponded to this critical feature of the HCV and PTV-1 IRES elements (Fig. 6) (Wang et al., 1995; Sarnow, 2003; Pisarev et al., 2004; Chard et al., 2006). Furthermore, a stem–loop structure analogous to the HCV and PTV-1 domain IIIId was also apparent. The terminal loop contained a conserved GGG motif in each case; these nucleotides are protected from modification when the HCV IRES is bound to 40S ribosomal subunits (Lukavsky et al., 2000; Kieft et al., 2001). The domains IIIId and IIIe are critical for the interaction of the HCV IRES element with the 40S ribosomes (Kieft et al., 2001).

FIG. 6. Partial secondary structure models for HCV, PEV-8 and SV2 IRES elements. Secondary structure models for domains IIIId, IIIf, IIIe and IV (where appropriate) were generated based on alignments with HCV IRES and its predicted secondary structure model (Sarnow, 2003); the base pairing predicted to form stem 1 (S1) and stem 2 (S2) of each IRES within the pseudoknot (domain IIIIf) is indicated. Note the complete identity between domain IIle of HCV and PEV-8 and the conserved GGG motif within domain IIIId. Initiation codons are indicated in bold.

DISCUSSION

In these studies, it was demonstrated that the 5' UTRs of SV2 and PEV-8 (members of a proposed new picornavirus genus) contain IRES elements that have structural and functional characteristics similar to those of PTV-1, the prototype teschovirus. The structural features included the presence of a predicted pseudoknot (domain IIIIf) and a highly conserved domain IIIe element (see Figs 5 and 6). In functional assays, these IRES elements maintained their activity when eIF4G was cleaved and also in the presence of hippuristanol, an inhibitor of eIF4A activity (see Fig. 4). In previous studies (Pisarev et al., 2004; Chard et al., 2006), we have shown that the PTV-1 IRES shares these functional and structural similarities with the IRES of HCV, a member of the family Flaviviridae. These structural features are also present in other picornavirus 5' UTRs [e.g. from avian
encephalomyelitis virus (L. Roberts & G. J. Belsham, unpublished observations) and Seneca valley virus (N. Knowles, personal communication). Thus, it is apparent that an HCV-like IRES element is present within multiple genera of the family Picornaviridae.

The conserved structural features helped the prediction of the whole PTV-1 IRES secondary structure on the basis of the HCV model (Sarnow, 2003; Chard et al., 2006). These structures both contain essentially two large, complex domains. The PEV-8 and SV2 IRES elements could also be predicted to form two major domains, which are separated by the stem 1 of the pseudoknot and are analogous to the domains II and III of the HCV IRES (not shown). However, there are differences among these various IRES elements. For example, it is interesting to note that the initiation codon of the SV2 IRES is predicted to be within a short hairpin structure (see Fig. 6), similar to domain IV of HCV (Sarnow, 2003), whereas there is no domain IV present in the PTV-1, PEV-8 or CSFV IRES elements (Fletcher et al., 2002; Chard et al., 2006). The presence of this feature seems to correlate with the enhancement of IRES activity when the HCV and SV2 sequences include a portion of the coding region (Reynolds et al., 1995; Figs 2 and 6). Studies have indicated that the low stability of this stem–loop structure in the HCV sequence is important: modifications that increase its stability inhibit translation (Honda et al., 1996). However, it has also been argued that the absence of any stable secondary structure around the initiation codon is the critical feature (Rijnbrand et al., 2001). We have found that modifications that reduce the stability of the SV2 stem also block translation (L. S. Chard & G. J. Belsham, unpublished data); hence, the role of this feature is not entirely clear.

The SV2 IRES is somewhat larger than the HCV and PTV-1 elements and additional sequences are included within the predicted SV2 domain II (see Fig. 5). Furthermore, whereas the PTV-1 IRES was predicted to lack a structure analogous to domain IIIa within the HCV IRES (Chard et al., 2006), the PEV-8 and SV2 IRES elements, in contrast, are predicted to contain a domain IIIa but lack a domain IIIc (see Fig. 5). However, the significance of these differences is currently unknown. Some of these sequences may be involved in RNA replication functions rather than translation initiation.

The use of the eIF4A inhibitor hippuristanol is a powerful and simple tool to distinguish between IRES elements that do or do not require eIF4A for activity. Thus, the EMCV IRES was highly sensitive to this inhibitor, whereas the PTV-1, PEV-8, SV2 and HCV IRES elements were unaffected (Fig. 4 and Bordeleau et al., 2006).

The results presented here (Fig. 2) indicated that the initiation codon on the SV2 RNA was at nt 694 rather than at nt 742 as predicted by Oberste et al. (2003). This result is fully consistent with the location of this AUG codon in relation to the IRES element and matches the location of the initiation codon in PTV-1, PEV-8 and HCV (see Figs 5 and 6). The consequence of this is that the leader protein of SV2 is 16 aa longer than previously suggested (Oberste et al., 2003). The AUG codon at nt 694 in the SV2 sequence is followed by a polyuridine tract (see Fig. 5b) and most picornavirus IRES elements include such a tract upstream of the initiation codon (see Belsham & Jackson, 2000). However, no such tract is present within the PTV-1 IRES or other HCV IRES-related elements. Thus, the presence of this feature within picornavirus RNA is not sufficient to determine the identity of the initiation codon. In contrast, the presence of the highly conserved HCV domain IIIe-like sequence and the pseudoknot appear sufficient to identify HCV-like IRES elements within picornavirus genomes.

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vaccinia virus that synthesizes bacteriophage T7 RNA polymerase.


