Functional Analyses of RNA Structures Shared between the Internal Ribosome Entry Sites of Hepatitis C Virus and the Picornavirus Porcine Teschovirus 1 Talfan

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The internal ribosome entry site (IRES) of porcine teschovirus 1 (PTV-1), a member of the *Picornaviridae* family, is quite distinct from other well-characterized picornavirus IRES elements, but it displays functional similarities to the IRES from hepatitis C virus (HCV), a member of the *Flaviviridae* family. In particular, a dominant negative mutant form of eIF4A does not inhibit the activity of the PTV-1 IRES. Furthermore, there is a high level (ca. 50%) of identity between the PTV-1 and HCV IRES sequences. A secondary-structure model of the whole PTV-1 IRES has been derived which includes a pseudoknot. Validation of specific features within the model has been achieved by mutagenesis and functional assays. The differences and similarities between the PTV-1 and HCV IRES elements should assist in defining the critical features of this type of IRES.

Porcine teschovirus 1 (PTV-1) Talfan is the prototype member of the *Teschovirus* genus within the *Picornaviridae* family. PTV infection results in polioencephalomyelitis in swine, and there have been recent incidents of disease (resulting in paralysis or mortality) in both the United States and Japan (28, 42). Multiple serotypes of teschoviruses have been identified, and nearly complete genome sequences are available for a variety of strains (6, 14, 20, 43). The 5'-terminal region of the genome sequence is missing in each case, and it is possible that a poly(C) tract is present within the 5' untranslated region of the viral RNA in at least some strains (6, 43).

Picornavirus genomes are infectious and function as mRNAs. Initiation of protein synthesis on picornavirus RNA is dependent on an internal ribosome entry site (IRES) (see reference 2 for a review). Several different classes of picornavirus IRES element have been described. With the exception of the PTV-1 IRES (see below), they are all large, complex RNA structures of about 450 nucleotides (nt) which contain a polypyrimidine tract located about 20 nt upstream of an AUG codon at the 3' end of the element. (Note that in the cardio- and aphthovirus elements, this AUG is an initiation codon, but in the entero- and rhinoviruses, the AUG codon is not usually recognized and initiation occurs at the next AUG codon.) The poliovirus (PV) and human rhinovirus elements represent one class of IRES; these elements function poorly in the rabbit reticulocyte lysate (RRL) in vitro translation system unless it is supplemented with additional proteins (e.g., from HeLa cell extracts). In contrast, the cardio- and aphthovirus IRES elements (e.g., from encephalomyocarditis virus [EMCV] and foot-and-mouth disease virus [FMDV]) do function very efficiently in the standard RRL translation system and they have a secondary struc-

ture different from that of the entero- and rhinovirus IRES elements. The hepatitis A virus IRES is distinct again; most notably, it requires the intact translation initiation complex eIF4F (1, 3), whereas the other picornavirus IRES elements function when the eIF4G component of this complex has been cleaved or the cap-binding protein component eIF4E is sequestered (2, 27). However, dominant negative mutant forms of eIF4A, the third component of eIF4F, block the function of the PV, EMCV, and FMDV IRES elements (25, 39). In contrast, it should be noted that the IRES elements from hepatitis C virus (HCV) and classical swine fever virus (CSFV), which are members of the *Hepacivirus* and *Pestivirus* genera within the *Flaviviridae* family, are not affected by the dominant negative mutant forms of eIF4A (9, 26). GB virus B (GBV-B) is related to HCV and also contains a structurally related IRES element (12). There is some variation in the size of these elements. The HCV IRES is about 300 nt in length, whereas the CSFV and GBV-B IRES elements are rather larger, but each includes a critical pseudoknot structure (8, 12, 30, 41).

In earlier studies, we have shown that the PTV-1 Talfan genome contains an IRES that directs initiation of protein synthesis at nt 412 in the known sequence (15). This IRES is quite distinct from other picornavirus IRES elements. The PTV-1 IRES is only about 280 nt long, and it lacks a significant polypyrimidine tract upstream of the initiation codon (27). Remarkably, by using in vitro assays with purified components, it was shown that the PTV-1 IRES does not need any component of the eIF4F complex to form the 48S preinitiation complex in vitro. It only requires translation initiation factor eIF2 (within the ternary complex with GTP and met-tRNA) with 40S ribosomal subunits (27), although this process is enhanced in the presence of eIF3. Indeed, the PTV-1 IRES can form a binary complex with 40S subunits alone. These features are distinct from other picornaviruses but highly reminiscent of the HCV IRES (26, 36). The HCV and CSFV IRES elements appear to function most efficiently when linked to the usual virus coding sequences (9, 30); however, this is not always the

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TABLE 1. Oligonucleotides used for analysis of the PTV-1 IRES^a

Primer	Sequence (5'–3')
Talfan-3e-GATA.....	GGACTTGTGCTGCCTGATAGGGTCGCGGCTGGCCG
Talfan-3e-AACA.....	GGACTTGTGCTGCCTAACAGGGTCGCGGCTGGCCG
Talfan-3e-GACC.....	GGACTTGTGCTGCCTGACCGGGTCGCGGCTGGCCG
Talfan-3e-GAAA.....	GGACTTGTGCTGCCTGAAAGGGTCGCGGCTGGCCG
Talfan-3e-AAAA.....	GGACTTGTGCTGCCTAAAAGGGTCGCGGCTGGCCG
Talfan-3e-GATA.comp.....	CGGCCAGCCGCGACCCCTATCAGGCAGCACAAAGTCC
Talfan-3e-AACA.comp.....	CGGCCAGCCGCGACCCCTGTAGGCAGCACAAAGTCC
Talfan-3e-GACC.comp.....	CGGCCAGCCGCGACCCGGTCAGGCAGCACAAAGTCC
Talfan-3e-GAAA.comp.....	CGGCCAGCCGCGACCCCTTTCAGGCAGCACAAAGTCC
Talfan-3e-AAAA.comp.....	CGGCCAGCCGCGACCCCTTTAGGCAGCACAAAGTCC
PK S1 Mut Forward.....	TGGTGGCGACACCCCTACAGGAAGAGC
PK S1 Mut Reverse.....	GCTCTTCTGTAGGGTGTGCGCCACCA
PK S1 Comp Forward.....	CCGTCTGTAGGGTGTATAGTCAGT
PK S1 Comp Reverse.....	ACTGACTATACACCCTACAGACGG
PK S2 Mut Forward.....	ACAGGGTCGCGGCTCCCGTCTGTA
PK S2 Mut Reverse.....	TACAGACGGGGAGCCGACCCCTGT
PK S2 Comp Forward.....	TACTTTGTATAGGGAGTTGAAACTCAC
PK S2 Comp Reverse.....	GTGAGTTCAACTCCCTATACAAAGTA
PTV Spacer Forward.....	CATGGGCAGTCATAGGATCCC^b
PTV Spacer Reverse.....	ATGGGGATCCTATGACTGCC^b
CAT Forward.....	ACACCTCCCCCTGAACCTGAAACAT ^c
LUC1400 Reverse.....	CATACTGTTGAGCAATTAC ^d

^a Sequences in italicized bold font indicate restriction sites that were either included or present in the sequence. Nucleotides underlined are those that modify the WT sequence.

^b Oligonucleotide synthesized with a 5' phosphate group.

^c From the pSV2-CAT sequence (11).

^d From the pGEM-LUC sequence (Promega); EMBL accession number X65316.

case (32) and just a lack of extraneous secondary structure may be important. Analysis of the PTV-1 IRES indicated that there is no requirement for any coding sequence (15).

Comparison of PTV-1 and HCV IRES sequences revealed about 50% identity overall, including some very highly conserved regions (27). There has been extensive study of the structure of the HCV IRES. Although its size (ca. 300 nt) precludes analysis of the whole structure, several distinct elements from within the IRES have been studied by nuclear magnetic resonance analysis and X-ray crystallography (4, 16, 17, 18, 22, 23). There is a well-supported model of the secondary structure of the HCV IRES (see reference 36 for a review), which comprises two major features, termed domain II and domain III. The entirety of domain II of the HCV IRES is not absolutely essential for activity; deletion of part of this sequence reduces translation initiation about fivefold (31), and analogous results were obtained with the PTV-1 IRES (27). The structure of the HCV domain II has been determined by nuclear magnetic resonance analysis (18, 23); it appears to fold independently from domain III, and by cryoelectron microscopy it has been shown that domain II makes contact with the 40S ribosomal subunit in the P site (38).

Domain III of the HCV IRES is sufficient for interaction with 40S ribosomal subunits (16). This region comprises several smaller elements (termed domains IIIa to IIIf) including stem-loop structures and a pseudoknot (IIIc). HCV IRES domain IIIb is primarily responsible for the interaction with eIF3 (16, 37). By RNA protection studies, it has been shown that individual nucleotides involved in the interaction between the HCV IRES and the 40S ribosomal subunit are located in domains IIIc and IIIe (16, 22). These nucleotides are conserved within the PTV-1 IRES (27). Furthermore, a stretch of 14 nt, which includes domain IIIe of the HCV IRES, matches,

with just one nucleotide substitution, a region of the PTV-1 IRES. In each sequence, this region is located adjacent to the predicted pseudoknot, termed domain IIIc in HCV (27).

Key features of the PTV-1 IRES have been analyzed by mutagenesis and functional studies which have provided evidence to support a secondary-structure model of the whole IRES.

MATERIALS AND METHODS

Secondary-structure prediction. The HCV IRES is contained within nt 44 to 345 of the 5' untranslated region (12) (EMBL accession number AB016785), while the IRES element of PTV-1 is located within nt 125 to 405 of the PTV-1 Talfan sequence (14, 15, 27) (EMBL accession number AB038528). The sequences were aligned with ClustalW and manually edited with the GCG10 SeqLab program. Secondary-structure elements within the PTV-1 sequence (outside of the pseudoknot) were generated by Mfold (44).

Mutagenesis. Plasmid construction, mutagenesis, and analysis were performed by standard techniques (35).

Point mutations were introduced into the GACA loop (nt 360 to 363) of the PTV-1 IRES (corresponding to HCV IRES domain IIIc) to generate loop sequence AAAA, GAAA, GACC, AACA, or GATA by QuikChange mutagenesis (Stratagene) with the monocistronic pGEM-IB vector (15) as the template and the appropriate primers (Table 1). The plasmid contains nt 1 to 1544 of the PTV-1 sequence. To facilitate analysis of the activity of each mutant IRES, the mutant IRES elements were removed from these plasmids and inserted into a dicistronic reporter plasmid. To achieve this, 5'-phosphorylated Spacer primers (forward and reverse; Table 1) were annealed and ligated into the NcoI site present at the 3' end of the PTV-1 IRES in pGEM-IB and its derivatives. The Spacer oligonucleotides maintained the reading frame from the PTV-1 initiation codon to the firefly luciferase (fLUC) initiation codon and created a second BamHI site. The wild-type (WT) and mutant PTV-1 IRES sequences (as BamHI fragments) were then excised and inserted into the BamHI-linearized and phosphatase-treated pGEM-CAT/LUC vector (34). Final constructs were sequenced with the CAT Forward primer (Table 1) to confirm the orientation of the insert and the presence of the required mutation. Analysis of the WT PTV-1 IRES indicated that the Spacer oligonucleotides had no detrimental effect on the expression and activity of fLUC (data not shown).

Overlap PCR was used to make mutant forms of the predicted stem structures within the pseudoknot of the PTV-1 IRES with pGC/PTV/L (27) as a template with appropriate oligonucleotides (Table 1). The fragments generated were digested with BamHI and then inserted directly into the pGEM-CAT/LUC vector as described above. The mutations in stems S1 and S2 were designed to disrupt base-pairing interactions. Compensatory mutations were produced by the same approach with the S1 or S2 mutant pGC/PTV/L vector as the template for overlap PCRs. In this case, the internal primers contained the compensatory mutations (Table 1). Construction of the compensatory mutant forms in the dicistronic reporter vector was performed as described above. All of the pGC/PTV/L vectors containing the initial and compensatory mutations were sequenced to confirm the presence of the mutations.

Transient-expression assays. Plasmids (2.5 μ g) were assayed by transfection with Lipofectin (8 μ g; Life Technologies) into BHK cells infected with recombinant vaccinia virus vTF7-3 (10), which expresses the T7 RNA polymerase, as described previously (34). After 20 h, cell extracts were prepared and the products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gels) (21) and detected by immunoblotting with a rabbit anti-chloramphenicol acetyltransferase (CAT) antibody (Sigma) and goat anti-fLUC (Promega) with peroxidase-labeled anti-species antibodies (Amersham) and chemiluminescence reagents (Amersham). In addition, the extracts were assayed for fLUC activity with a luciferase assay kit (Promega) and a luminometer.

In vitro translation assays. In vitro RNA transcripts from pGC/PTV Δ 125/L (27) and pGC/EMC/L (formerly termed pGEM-CAT/EMC/LUC [34], which contains the EMCV IRES) were prepared with T7 RNA polymerase, and their translation was achieved in the RRL translation system (Promega) with [35 S]methionine (Amersham Biosciences) essentially as described by the manufacturer. Products were analyzed by SDS-PAGE (10%) and autoradiography. Incorporation into CAT and fLUC was determined with a phosphorimager (Molecular Imager FX; Bio-Rad).

Expression and purification of a dominant negative mutant form of eIF4A. The DQAD dominant negative mutant form of eIF4A (described previously [25]) was expressed in *Escherichia coli* BL21(DE3)/pLysS and purified to near homogeneity by phosphocellulose (unbound material) and Blue Sepharose chromatography. Peak fractions were pooled and dialyzed against 50 mM Tris HCl (pH 8.0)–50 mM NaCl–10 mM β -mercaptoethanol–0.1% NP-40 and stored frozen at -70°C .

RESULTS

PTV-1 IRES activity is unaffected by a dominant negative mutant form of eIF4A. A key difference between the properties of the HCV IRES and most picornavirus IRES elements is the role of the translation initiation complex eIF4F. The HCV IRES is unaffected by cleavage of eIF4G and is also insensitive to competition from dominant negative mutant forms of eIF4A (26, 39). The well-studied IRES elements from PV and EMCV are also insensitive to cleavage of eIF4G but, in contrast, are strongly inhibited by the dominant negative mutant forms of eIF4A (e.g., DQAD) (25, 39). To test the sensitivity of the PTV-1 IRES, the DQAD mutant form of eIF4A was expressed in *E. coli*, purified, and added into in vitro translation reaction mixtures containing RRL. The reactions were programmed with dicistronic RNA transcripts prepared from either pGC/PTV Δ 125/L or pGC/EMC/L. These mRNAs encode CAT by 5'-end-dependent translation initiation and fLUC by internal initiation (Fig. 1A). Addition of the DQAD mutant form of eIF4A inhibited the production of CAT and, more strikingly, the EMCV IRES-directed production of fLUC, consistent with earlier studies (25, 39). In contrast, the mutant form of eIF4A had no significant effect on the PTV-1 IRES-directed production of fLUC (Fig. 1AB). The insensitivity of the PTV IRES to the effect of the dominant negative mutant form of eIF4A was also observed in vitro by coupled transcription and translation assays, whereas the EMCV IRES activity was again strongly inhibited (data not shown). Thus, the PTV-1 IRES functions

FIG. 1. Insensitivity of the PTV-1 IRES to a dominant negative mutant form of translation initiation factor eIF4A. (A) In vitro translation reaction mixtures (10 μ l) containing RRL and [35 S]methionine were programmed with dicistronic RNA transcripts derived from plasmid pGC/PTV Δ 125/L or pGC/EMC/L with the structure CAT-IRES-LUC containing the PTV or EMCV IRES, respectively. Mutant (DQAD) recombinant eIF4A was added as indicated. Reaction products were analyzed by SDS-PAGE and autoradiography. The migration of the CAT and fLUC proteins is indicated. (B) The incorporation of [35 S]methionine into CAT (open bars) and fLUC (solid bars) in each reaction was quantified with a phosphorimager. The values in the absence of added protein were set at 100% in each case.

independently of eIF4F within RRL; this result is consistent with the lack of requirement for any component of eIF4F for the formation of 48S preinitiation complexes in vitro from purified components (27).

Secondary-structure prediction for the PTV-1 IRES. The high level (ca. 50%) of sequence identity between the PTV-1 IRES and the HCV IRES means that it was relatively easy to map certain features of the HCV IRES onto the PTV-1 sequence. For example, domain IIIe within the HCV IRES comprises 12 nt; of these, 11 nt are identical to a sequence within the PTV-1 IRES (27). With sequence alignments, phylogenetic comparisons (with other teschovirus sequences), and Mfold (44), a model of the secondary structure of the whole PTV-1 IRES has been derived (Fig. 2). In concert with the production of this model, a revised alignment of the PTV-1 IRES with the HCV IRES was derived (Fig. 3). The major change from the model presented previously (27) concerns the sequences which compose stem 1 of the pseudoknot; the new model provides uninterrupted base pairing between the two strands of this stem. This revision also lead to a realignment of the sequences within domain II, but domain III is little altered since a number of markers, e.g., the highly conserved domain IIIe sequence, provide tighter constraints. Like the HCV IRES, the PTV-1

FIG. 2. Predicted secondary structure of the PTV-1 IRES. The structure of the PTV-1 IRES was predicted by comparative sequence analysis with HCV, CSFV, and other teschoviruses and was based on the published secondary structures for HCV and CSFV. Domains are labeled according to corresponding domains in the HCV IRES. Except for the pseudoknot, particular elements were analyzed with Mfold (44), which predicted the most thermodynamically favorable structures for domains II, IIb, IIIc, and IIId.

IRES is composed of two major domains; for ease, it is convenient to refer to these elements with the same nomenclature as used for the HCV IRES. Thus, domain II is now predicted to comprise nt 121 to 221 of the known PTV-1 sequence (14). By deletion analysis, it was shown previously that nt 126 to 405 were sufficient for full IRES function within the context of a dicistronic mRNA whereas nt 151 to 405 were substantially less efficient (about 25% of the WT) at directing translation initiation (27).

Domain III is composed of multiple elements; unexpectedly, there appears to be no region analogous to HCV domain IIIa, although an AGUA motif, which is located in the loop of this

domain within the HCV and CSFV sequences, is present. The domain IIIb sequences are quite highly conserved between PTV-1 and HCV, although the apical loop sequence is smaller in the PTV-1 sequence (as is the case for CSFV [8]). In HCV, GBV-B, and CSFV, domain IIIc is predicted as a very short stem-loop structure (10 nt in total) (33) but this is not conserved in PTV-1. However, it is interesting that the sequence GAGAUUU, located upstream of domain IIIc in HCV, is also present in the PTV-1 sequence and is predicted to form part of a different stem-loop structure that we have termed IIIc. Surprisingly, this sequence is not conserved within the CSFV or GBV-B IRES (8, 12).

Domain IIId is essentially a stem-loop structure with a terminal loop containing a GGG motif which is conserved in HCV, CSFV, GBV-B, and the PTV-1 sequences. This motif is protected from modification when the HCV IRES binds to 40S ribosomal subunits (16, 22). In the HCV sequence, domain IIId has a hexanucleotide loop and the stem-structure is interrupted (19, 36). However, PTV-1 domain IIId is predicted to be a simple stem with an apical tetraloop (GGGA) which matches the GNRA consensus sequence. Such tetraloops are frequently observed in structured RNAs (5). In the GBV-B IRES, domain IIId is also predicted as just a simple stem-loop structure with a terminal GGGU loop (12).

As already mentioned, HCV domain IIIe only differs at one position from the PTV-1 sequence; in this case, the difference is within the 4-nt loop (Fig. 4). CSFV domain IIIe also differs from the HCV sequence by a single nucleotide, but this difference is located within the stem and preserves the secondary structure (reference 8 and Fig. 4A and B).

HCV domain IIIf is a pseudoknot (Fig. 4A) which is composed of two base-paired stem regions (S1 and S2) connected by two loops (L1 and L2). The sequences which make up this structure are quite distinct between the different elements, but the conservation of this structure is striking. Like the HCV IRES, stem 1 of the PTV-1 IRES is composed of uninterrupted base-paired nucleotides, whereas CSFV stem 1 is bipartite (Fig. 4). Stem 2 within the PTV-1 IRES could be up to 8 nt long, but whether each of these base pairs is able to form is not known.

The HCV and CSFV IRES elements can require about 40 nt from within the coding sequence for optimal activity (9, 30), but this is not the case for PTV-1 (15; L.S.C. and G.J.B., unpublished results). The HCV initiation codon is predicted to be located in the loop of another stem-loop structure termed domain IV (Fig. 4A); a similar arrangement is present in the GBV-B sequence (12). However, there is no evidence for a stem-loop structure containing the initiation codon at the 3' terminus of either the PTV-1 or CSFV IRES elements (8) (Fig. 4). In the studies described below, various features of the secondary-structure prediction for the PTV-1 IRES have been analyzed.

PTV-1 IRES GACA loop (domain IIIe) mutagenesis. As indicated above, there is a very high level of sequence identity between HCV domain IIIe and the analogous region of the PTV-1 IRES (27). This element is a simple stem-loop structure of 12 nt (compare Fig. 4A and C). The only difference between the HCV IIIe element and the PTV-1 sequence is within the loop, which is GAUA in HCV and GACA in PTV-1. In CSFV, this stem-loop structure also shares 11 out of 12 nt with those

FIG. 3. Alignment of the PTV-1 and HCV cDNA sequences corresponding to the IRES elements. Sequences were aligned with ClustalW and manually edited in the light of the predicted secondary-structure models. Identical nucleotides are shaded. The regions involved in the formation of the pseudoknot (domain IIIe) are marked by xxx. The limits of the predicted domains in both the PTV-1 and HCV structures are also shown. The initiation codons are indicated by an open box. The overall sequence identity from this alignment is 52.5%.

found in HCV (Fig. 4B) and the loop sequence is identical. Mutagenesis of the HCV IRES has shown that the U-to-C substitution (as in PTV-1) within the domain IIIe loop reduced IRES activity by 55% within cells (22). However, modification of the U to A had an effect similar to that of modification of the conserved GGG motif within domain IIId. It is interesting that with in vitro translation assays, Psaridi et al. (29) found that modification of each of the bases in the HCV domain IIIe GAUA loop had a major detrimental effect on translational activity but the GACA mutant form retained the highest level of activity. In contrast, in their cell-based assays all of the mutant forms displayed similarly deficient levels of activity.

To explore the properties of PTV-1 domain IIIe, five different mutant forms were produced with single or double substitutions within the loop; the sequences generated were GAUA (as in HCV and CSFV), GACC, AACA, GAAA, and AAAA (mutations are in boldface and underlined). The mutant sequences were assayed in cells within the context of the dicistronic vector pGC/PTV/L as described above. The WT and mutant plasmids were transfected separately into vTF7-3-infected BHK cells. After 20 h, cell extracts were prepared and analyzed by SDS-PAGE, followed by Western blotting (Fig. 5A). The expression of CAT was very similar from each plasmid, as expected. However, it was clear that each mutant form had an impaired ability to direct translation of the downstream fLUC open reading frame. In particular, IRES elements with the single-point mutation GACC and the double mutation AAAA were highly defective. Enzyme assays were also performed to quantify the fLUC activity directed by each PTV-1 mutant IRES. The results, from three separate experiments,

mirrored those observed in the Western blot analysis (Fig. 5B). IRES elements with the double mutation (AAA) and the GACC mutation displayed less than 10% of the WT (GACA) activity. The IRES with the GAAA mutation was also severely impaired in activity, with translation directed by this element reduced to 24% of WT levels. However, the elements with the AACA mutation (42% of WT efficiency) and the HCV-like GAUA mutation (50% of WT activity) both maintained a reasonable capacity to direct translation.

PTV-1 pseudoknot mutagenesis. The pseudoknot within the *Flavivirus* IRES elements has been shown to be critical for the activity of the IRES (8, 41). The nucleotides involved in the formation of the HCV pseudoknot are shown in Fig. 4A. For comparison, the CSFV pseudoknot is also shown (Fig. 4B). It should be noted that the proposed structure for stem 1 of the PTV-1 pseudoknot (Fig. 4C) is modified from that suggested previously (27). The revised PTV-1 stem 1 is fully base paired, as in the HCV IRES. To test the predicted model of the PTV-1 pseudoknot, mutagenesis experiments were undertaken which were designed to disrupt the predicted base pairs and then to restore the interactions by introducing compensatory mutations. It should be noted, however, that such compensatory changes do not result in the reformation of the true WT structure.

In stem 1 of the PTV IRES, nt 225 to 227 (GGG) are predicted to base pair with CUU (nt 386 to 388); hence, to disrupt this interaction, nt 225 to 227 were changed to CCC (termed S1 mut). These changes can therefore be expected to inhibit the activity of the IRES. Indeed, when assayed within the pGC/PTV/L vector, it was found that this stem 1 mutation

FIG. 4. Comparison of predicted secondary structures of domains IIIe and IIIf of the HCV, CSFV, and PTV-1 IRES elements. The highly conserved IIIe domains and predicted pseudoknots of HCV (A), CSFV (B), and PTV-1 (C) are shown. The two stems (S1 and S2) and loops (L1 and L2) that make up the pseudoknots are indicated. The initiation codon (AUG) is underlined in each case. Note that for HCV this codon is within a stem-loop structure (domain IV) but no such structure is predicted within the PTV or CSFV sequence. The sequences in bold within the PTV-1 IRES (C) have been modified and the effect on function analyzed (Fig. 5 and 6). The single-nucleotide difference between the domain IIIe loop of the PTV-1 and HCV IRES elements is indicated with an arrow.

(GGG-CCC) completely abrogated IRES activity, as indicated by the lack of expression of fLUC detected by immunoblotting (Fig. 6A). These results were confirmed by enzymatic LUC assays from three independent experiments (Fig. 6B). This shows that these nucleotides are critical for IRES function but does not indicate whether they are specifically required for the predicted interactions within the pseudoknot. To make compensatory changes in stem 1, nt 386 to 388 (CUU) were changed to GGG within the mutant background (to create S1 comp) to restore the predicted base pairing. Introduction of these compensatory mutations into the S1 mut background generated an element (S1 comp) with greatly increased IRES activity (about 95% of WT activity) (Fig. 6A and B), strongly suggesting that the base-pairing interactions within S1 were restored, which therefore had been predicted correctly.

To analyze stem 2 within the pseudoknot, nt 375 to 376 (GG) were mutated to CC to generate the S2 mut plasmid (Fig. 4C). These nucleotides are predicted to interact with nt 396 to 397 (UC); thus, these mutations would be expected to

prevent this interaction and destabilize the pseudoknot. Indeed, in transient-expression assays, the S2 mut element displayed greatly reduced IRES activity (ca. 5% of WT activity; Fig. 6). Modifications in stem 2 which aimed to restore nucleotide interactions and regenerate the pseudoknot (Fig. 4C) involved mutation of nt 396 to 397 (UC) to GG (in plasmid S2 comp). These compensatory mutations again greatly enhanced IRES activity (up to 62% of WT activity; Fig. 6). Thus, the predicted structure of stem 2 appears to be correct. However, the incomplete rescue of function may suggest that although the base-pairing interactions are restored, the pseudoknot structure itself or interactions with it are perturbed to some extent due to the modified sequences.

DISCUSSION

The PTV-1 IRES shows a high level of functional and structural similarity to the HCV IRES. An important functional distinction between the HCV IRES and the well-characterized

FIG. 5. The PTV-1 domain IIIe loop is critical for IRES activity in cells. (A) Dicistronic pGC/PTV/L plasmids (2.5 μ g) containing the indicated mutations (in bold) within the loop of domain IIIe were transfected into vTF7-3-infected BHK cells. After 20 h, cell extracts were prepared and analyzed for the expression of CAT and fLUC by SDS-PAGE (10%) and immunoblotting. (B) fLUC activity assays were performed on cell extracts from three separate transfections with each plasmid indicated. The results were standardized to expression directed by the WT PTV-1 IRES (GACA), which was set as 100% in each experiment. The mean ($\pm$ the standard deviation) was calculated for each mutant form.

picornavirus IRES elements is the effect of dominant negative mutant forms of eIF4A on their activity. The HCV IRES is unaffected by these mutant proteins, whereas the EMCV, PV, and FMDV IRES elements are strongly inhibited (25, 26, 39). We have now shown that the DQAD mutant form of eIF4A has no significant effect on the activity of the PTV IRES (Fig. 1), and hence it behaves like the HCV IRES. This result is fully consistent with the lack of requirement for any component of eIF4F for the assembly of 48S initiation complexes on the PTV-1 IRES (27).

Some features within the sequences of the PTV-1 and HCV IRES elements are extremely highly conserved. With these motifs as a starting point, it was possible to derive a secondary-structure prediction for the entire PTV-1 IRES which is very similar to that generated previously for the HCV IRES (13, 22, 36). A critical feature of this secondary structure is the presence of a pseudoknot (domain IIIf). There is relatively low sequence conservation within the pseudoknot between the PTV-1 IRES and the HCV IRES, but the evidence for conservation of the structure is compelling. Support for the base pairing within stems 1 and 2 was provided by the introduction of mutations which were aimed at disrupting these stems, and then second-site mutations were introduced to regenerate base pairing, albeit with altered sequences. It was apparent that the modifications of both the predicted S1 and S2 sequences behaved as predicted for the disruption and regeneration of the pseudoknot (Fig. 6), and hence these results strongly supported the predicted structure.

The most conserved region between the PTV-1 and HCV IRES elements includes domain IIIe. This structure consists of a stem with an apical loop consisting of GACA in PTV-1 and

GAUA in HCV. Modifications of this loop sequence suggested that each nucleotide is important for IRES activity (Fig. 5). However, it is interesting to look at how the different types of change affected this function. The two most active mutant IRES elements, which retained up to 52% of the WT activity, correspond to transition mutations (AACA and GAUA). The least active IRES elements each contained transversion mutations (GACC and GAAA) or contained two mutations (AAAA, includes one transversion). It should be noted that the GAAA loop, which conforms to the common, stable GNRA tetraloop sequence, was less functional than less stable structures containing transition mutations. Evidence from studies on the HCV IRES indicates that the G within this loop is protected from modification by the interaction with 40S ribosomal subunits (22). It may be that the whole of this loop is involved in this interaction, either alone or in conjunction with other sequences within the IRES. The latter possibility may be more likely since the GAUA loop sequence, which functions optimally within the HCV IRES, was significantly less active within the context of the PTV-1 IRES. Previous studies on the role of this loop within the HCV IRES have shown some disparities. In the studies by Lukavsky et al. (22), modification of GAUA to GACA (as in PTV-1) resulted in a reduction in activity of about 55% in cells; however, a similar drop in activity was also reported for a second mutation to form a GAAA loop or when highly conserved sequences within domain IIIId were modified. All of these assays were performed with a plasmid expressing a dicistronic reporter mRNA from within the nucleus. Studies on this same loop sequence by Psaridi et al. (29) showed that

FIG. 6. Functional analysis supports predicted PTV-1 IRES pseudoknot structure. (A) Dicistronic pGC/PTV/L plasmids (2.5 μ g) containing the indicated mutations (see text for details) within the predicted pseudoknot (domain IIIf) were used to transfect vTF7-3-infected BHK cells and analyzed for the expression of CAT and fLUC as for Fig. 5. (B) fLUC activity assays were also performed to quantify the fLUC expression directed by each PTV IRES element. The results were standardized to the expression directed by the WT PTV-1 IRES, which was set as 100% in each experiment. The mean ($\pm$ the standard deviation) was calculated for each mutant form.

all mutant forms had a major effect on IRES activity when tested in a similar transient-expression assay system, but when they were subjected to *in vitro* translation assays (with *in vitro* RNA transcripts), a wider spectrum of activities was detected and the GACA mutation (as in PTV-1) was the most active mutant sequence. In our studies, we have used a transient-expression system which produces the RNA transcripts within the cytoplasm, the normal cellular location for both picornavirus and flavivirus RNA transcription. It is noteworthy that evidence for a promoter element within the HCV IRES has been reported (7), although in other studies no evidence for the production of transcripts from within the HCV IRES was obtained (22, 40).

In the secondary-structure prediction for the PTV-1 IRES (Fig. 2), there is no apparent element that is equivalent to HCV domain IIIa although there is a conserved AGUA motif. The lack of a role for domain IIIa in translation is consistent with results obtained by cryoelectron microscopy which indicated that domain IIIa of the HCV IRES extends away from the surface of the 40S ribosome subunit rather than being involved in this interaction (38). If domain IIIa is primarily required for some other function, then it can be envisaged that this may be achieved by sequences outside of the IRES for PTV-1. There are at least 120 nt upstream of the IRES in the PTV-1 RNA, whereas in HCV there are only about 40 nt upstream of the IRES. The smaller size of the PTV-1 IRES compared to the HCV IRES may reflect the minimal presence of motifs which are not required for IRES function.

We have established that the PTV-1 IRES is highly related to the HCV IRES in sequence, initiation factor requirements (27), and overall secondary structure. Other members of the picornavirus family, including simian virus 2 and porcine enterovirus 8 (which are expected to form part of a new picornavirus genus; 20, 24), contain similar HCV-like IRES elements (L.S.C. and G.J.B., unpublished), and hence these elements represent an additional class of picornavirus IRES element. The various differences and similarities between them should assist in determining the mechanism of activity of this type of IRES.

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