The Picornavirus Avian Encephalomyelitis Virus possesses a Hepatitis C Virus-like internal ribosome entry site (IRES) element

Mehran Bakhshesh\textsuperscript{1,3}, Elisabetta Groppelli\textsuperscript{1,4}, Margaret M. Willcocks\textsuperscript{1}, Elizabeth Royall\textsuperscript{1}, Graham J. Belsham\textsuperscript{2} and Lisa O. Roberts\textsuperscript{1*}

\textsuperscript{1} Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK
\textsuperscript{2} The National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark.
\textsuperscript{3} Present address: Poultry Vaccines Department, Razi Vaccine & Serum Research Institute, Karaj, Iran, P.O.Box: 31975/148
\textsuperscript{4} Present address: Institute for Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

*Author for correspondence. Tel: 44 1483 686499; Fax: 44 1483 300374; e-mail: l.roberts@surrey.ac.uk

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SUMMARY

Avian encephalomyelitis virus (AEV) is a picornavirus that causes disease in poultry worldwide and flocks must be vaccinated for protection. AEV is currently classified within the hepatovirus genus since its proteins are most closely related to those of hepatitis A virus (HAV). We now provide evidence that the 494 nucleotide (nt) long 5’ untranslated region (UTR) of the AEV genome contains an internal ribosome entry site (IRES) element that functions efficiently in vitro and in mammalian cells. Unlike the HAV IRES, the AEV IRES is relatively short and functions in the presence of cleaved eIF4G, it is also resistant to an inhibitor of eIF4A. These properties are reminiscent of the recently discovered class of IRES elements within certain other picornaviruses, such as porcine teschovirus-1 (PTV-1). Like the PTV-1 IRES, the AEV IRES shows significant similarity to the hepatitis C virus (HCV) IRES in sequence, function and predicted secondary structure. Furthermore, mutational analysis of the predicted pseudoknot structure at the 3’ end of the AEV IRES lends support to the secondary structure we present. AEV is therefore another example of a picornavirus harbouring an HCV-like IRES element within its genome and thus its classification within the hepatovirus genus may need to be reassessed in the light of these findings.

INTRODUCTION

Translation initiation on the majority of cellular mRNAs is mediated by a cap-dependent mechanism. The cap structure (m7GpppN) found on all cytoplasmic mRNAs is recognized by the translation initiation factor complex eIF4F (reviewed in 29). This complex contains three proteins: eIF4E which is the cap-binding protein, eIF4A which has RNA helicase activity and eIF4G that acts as a protein scaffold.
between the mRNA and the 40S ribosomal subunit via its interaction with eIF3 (reviewed in 14). In contrast, initiation of protein synthesis on some viral mRNAs, for example from the picornaviruses, occurs by a cap-independent mechanism termed internal initiation. In this case, translation initiation is directed by an internal ribosome entry site (IRES) element located within the 5’ untranslated region (UTR) of the viral genome (reviewed in 2). These IRES elements are large, typically 450 nt in length, and contain extensive secondary structure; they have been shown to interact with a variety of cellular proteins (2). Most of these elements work without any requirement for eIF4E and hence can continue to function when cap-dependent protein synthesis is inhibited.

The picornavirus IRES elements are divided into several groups which display distinct secondary structures and biological properties. One group (class I) contains IRES elements from the entero- and rhinoviruses (e.g. poliovirus, PV) while the second contains the cardio- and aphthovirus IRES elements (e.g. encephalomyocarditis virus, EMCV). The cardio-/aphthovirus IRES elements function efficiently in the rabbit reticulocyte lysate (RRL) translation system. However, the PV and rhinovirus IRES elements are inefficient in this system unless the reaction is supplemented with additional proteins, e.g. from HeLa cell extracts (6, 10). The IRES element from hepatitis A virus (HAV) represents a third type of IRES. It is distinct from other picornavirus IRES elements in that it requires an intact eIF4F complex, including eIF4E, for function (1, 4). In contrast, the class I and II picornavirus IRES elements can function efficiently when eIF4G has been cleaved by the expression of an entero-/rhinovirus 2A or aphthovirus L protease (5, 35). This cleavage releases the N-terminus of eIF4G including its eIF4E binding site (reviewed in 20). The initiation
factor eIF4A has also been shown to be required by group I and II IRES elements since dominant negative mutants of this protein and inhibitors of eIF4A block their activity (9, 27, 39).

Recently, a new group of picornavirus IRES element has been identified. This group includes the IRES elements from porcine teschovirus-1 (PTV-1) Talfan strain (8, 17, 30), simian virus 2 (SV2) and porcine enterovirus-8 (PEV-8; 9). Strikingly, these IRES elements have many similarities to those from hepatitis C virus (HCV) and classical swine fever virus (CSFV), which both belong to the Flaviviridae. These recently characterised picornavirus IRES elements are predicted to share a very similar structure to the HCV-type elements, including a pseudoknot near the 3’ end of the IRES that is critical for function (12, 18, 41). These IRES elements are generally shorter than other picornavirus elements, e.g. about 280 nt in the case of the PTV-1 IRES (8). They can also function with cleaved eIF4G (9, 30) and, unlike the group I and II IRES elements, are resistant to both dominant negative mutants of eIF4A (8, 9) and to hippuristanol, a small molecule inhibitor of eIF4A (3). Like the HCV IRES element, the PTV-1 IRES element does not require any of the eIF4 initiation factors for assembly of 48S initiation complexes on the RNA (28, 30, 31).

Avian encephalomyelitis virus (AEV) is a picornavirus that infects young chickens, quails, pheasants and turkeys, causing ataxia and rapid tremors, especially in the neck. AEV is a worldwide problem and almost all flocks are susceptible unless they are vaccinated (7). Around 4 billion birds are vaccinated worldwide each year to protect them from infection (Dr I Tarpey, personal communication). The AEV genome is 7032 nucleotides (nt) in length (smaller than that of any other picornavirus).
encodes a polyprotein of 2134 amino acids that is processed to the individual viral proteins which are most closely related to the hepatitis A virus (HAV) proteins. AEV has therefore been assigned to the hepatovirus genus of the picornaviruses (25). The 5’ untranslated region (UTR) of the AEV RNA is 494 nucleotides (nt) in length, which is also shorter than most other picornaviruses (25). On the basis of sequence comparisons and secondary structure predictions it has recently been suggested that the AEV genome contains an HCV-like IRES element (15).

Here, we describe the biological properties of the AEV 5’ UTR. We demonstrate for the first time that this region contains an IRES element with clear functional differences from that of hepatitis A virus. We also show that the AEV IRES has significant functional and structural similarities to the other HCV-like picornavirus IRES elements.

**MATERIALS AND METHODS**

**Reporter plasmids.**

DNA preparations and manipulations were performed using standard methods as described in (37) or as stated in manufacturers’ instructions. The reporter plasmids pGEM-CAT/EMC/LUC containing the EMCV IRES cDNA and pGEM-CAT/LUC (lacking any IRES) have been described previously (35). These plasmids express from a T7 promoter dicistronic mRNAs encoding chloramphenicol acetyl transferase (CAT) and firefly luciferase (fLUC). Plasmids containing the HAV and HCV IRES elements between the cyclin and influenza virus NS sequences have also been described previously (references 4 and 33) and were a kind gift from Richard Jackson (University of Cambridge, UK).
To obtain a single cDNA fragment corresponding to the AEV 5’UTR, overlap PCR was performed. Two separate AEV cDNA clones (a gift from Ian Tarpey, Intervet, U.K. and Dave Cavanagh, Institute for Animal Health, Compton, U.K.) were used as templates to amplify by PCR fragments corresponding to nt 1 to 238 and 238 to 494 of the AEV 5’UTR using primers AEVF1 with AEVR266 and AEVF238 with AEVR494, respectively (Table 1). The two purified products were mixed and used in a further PCR using primers AEVF1 and AEVR494 to create a single fragment corresponding to the full-length AEV 5’ UTR (nt 1 to 494) flanked by BamHI sites. The PCR product was ligated into pGEMT easy (Promega) and from the resultant plasmid the AEV cDNA was released by BamHI digestion and then inserted, in both orientations, into similarly digested and phosphatased pGEM-CAT/LUC between the two open reading frames (ORFs). The plasmid containing the AEV 5’UTR cDNA in the sense (genomic) orientation was designated AEVs and that containing the fragment in the antisense orientation was called AEVas (Figure 1A). A further construct containing cDNA corresponding to the AEV 5’ UTR plus 30 nt of coding sequence (AEV+30) was created in a similar way using the reverse primer AEVR524 (Table 1) rather than AEVR494 in the PCRs.

Four other derivatives (see Figure 1) of the AEVs plasmid with truncated forms of the AEV 5’UTR cDNA were generated as follows:- the AEV3’Δ100 fragment (AEVm1) was created using primers AEVF1 and AEVR394, fragment AEV3’Δ200 (AEVm2) was produced using primers AEVF1 and AEVR294, AEV5’Δ100 (AEVm3) was made using primers AEVF100 and AEVR494 while AEV5’Δ200 (AEVm4) was synthesized using primers AEVF200 and AEVR494 (all primer sequences are given in Table 1). The various fragments were cloned, excised with BamHI and ligated into the pGEM-CAT/LUC dicistronic vector to produce the illustrated plasmids (Figure
The structures were confirmed by restriction enzyme analysis and sequencing of the inserts.

**In vitro translation reactions**

The dicistronic reporter plasmids (1µg) were assayed in the rabbit reticulocyte lysate (RRL) coupled transcription and translation (TNT) system (Promega) using[^S]-methionine as described by the manufacturer. Products were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Alternatively, uncapped mRNA transcripts were produced *in vitro* using the Ambion MegaScript kit with T7 RNA polymerase, following linearization of the plasmid DNAs with XhoI. Each mRNA was translated in RRL with[^S]-methionine and products were analysed by SDS-PAGE and autoradiography.

**Transient expression assays.** The dicistronic reporter plasmids (2µg) described above were transfected into 293 or HTK-143 cells alone, or with the plasmid pGEM3Z/J1 (0.2µg) which expresses the swine vesicular disease virus (SVDV) 2A protease as previously described (36). Briefly, the plasmids were transfected into cells (35mm dishes) previously infected with the recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase (13), using Lipofectin (8 µl; Invitrogen) and Optimem (192 µl; Gibco BRL). Cell lysates were prepared 20 h after transfection and were analysed by SDS-PAGE and immunoblotting to determine CAT and LUC expression and eIF4G cleavage. Detection was achieved with anti-CAT (Sigma), anti-fLUC (Promega) or anti-eIF4G (gift from Simon Morley, University of Sussex, UK) antibodies and peroxidase-labelled anti-rabbit (Amersham) or anti-goat (Dako Cytomation) antibodies respectively, using chemiluminescence reagents (Pierce).
fLUC expression was also quantified using a firefly luciferase assay kit (Promega) with a luminometer.

**RNA secondary structure prediction**

AEV 5’ UTR sequences (EMBL accession number: AJ225173) were aligned with those from HCV (EMBL accession number: AB016785) and PTV-1 (EMBL accession number: AB038528) using Clustal W and manually edited. Secondary structure elements (other than the pseudoknot) were generated in Mfold (42).

**Mutagenesis of the AEV cDNA**

Mutations were introduced into the predicted domain IIIe region in order to change the sequence in the loop region from GAUA to AAAA (nts 446-449). The plasmid pGEM-CAT/AEVs/LUC was used as the template for two PCR reactions, one with each primer set (AEVIIIeF with AEVR494 and AEVIIIeR with AEVF1 - see Table 1). After purification the products were mixed and a further PCR was performed using AEVF1 and AEVR494 primers. The *BamHI* digested product was ligated into pGEM-CAT/LUC as described above and the resultant plasmid named pGEM-CAT/AEVIIIemut/LUC. The plasmid was sequenced to verify the presence of the expected mutations.

Mutations within the stem sequences of the predicted pseudoknot were also created (termed S1mut and S2mut). For the S1mut mutant, the nts 273-275 (CUC) were changed to GGG and in the S2mut mutant, the nts 460-461 (CC) were changed to GG. The plasmid pGEM-CAT/AEVs/LUC was used as the template for the primary PCRs with the specific mutagenic primers and either CATForward or LUCReverse primers (see Table 1) as appropriate. Secondary PCRs used just the latter primers. The final
PCR products were purified, digested with *BamHI* and the ca. 500 bp fragment was ligated into *BamHI*-digested and dephosphorylated pGEM-CAT/LUC vector to generate pGEM-CAT/AEVmutS1/LUC and pGEM-CAT/AEVmutS2/LUC. Compensatory mutations were produced in the same way starting with the mutS1 or mutS2 plasmids as templates for the PCRs. The mutagenic primers specified the compensatory mutations (Table 1). The presence of all the expected mutations in the plasmids was confirmed by sequencing.

**Translation assays in the presence of hippuristanol**

The requirement of the AEV IRES element for eIF4A was investigated both *in vitro* and in cells using hippuristanol, a specific inhibitor of eIF4A (3). Dicistronic plasmid DNAs were expressed in the TNT RRL system with or without hippuristanol (10 µM; kind gift from Jerry Pelletier, McGill University, Canada). The products were analysed by 10% SDS-PAGE and autoradiography. The same plasmids were also assayed in HTK-143 (TK-) cells with or without the addition of 0.5 µM hippuristanol; cell lysates were prepared after 20 h and the inhibitor was added for the final 10 h.

**RESULTS**

**Identification of the IRES element within the 5’ UTR of AEV RNA.**

A dicistronic reporter plasmid was prepared in which cDNA corresponding to the AEV 5’ UTR (nt 1-494) was inserted between two reporter gene sequences, the first encoding CAT and the second encoding fLUC (AEVs; Figure 1A). A negative control containing the AEV sequence in the antisense orientation was also constructed (AEVs). Plasmid pGEM-CAT/EMC/LUC which contains the EMCV IRES was used
as a positive control and the plasmid pGEM-CAT/LUC, that lacks any IRES sequence, was used as a negative control. RNA transcripts were prepared from each of these constructs using T7 RNA polymerase and were analysed in translation assays in RRL. Translation of the first ORF was assessed by the level of CAT expression and a functional IRES element led to the expression of fLUC. Each of the mRNAs expressed CAT efficiently as expected (Figure 1B). RNAs containing the EMCV IRES and the AEV 5’ UTR sequence in the sense orientation also efficiently expressed fLUC (Figure 1B), but the AEV 5’UTR was less active in this system than the EMCV IRES. Only a background level of LUC expression was detected from the AEVas construct or the construct lacking any IRES element. Similar results were obtained in the RRL TNT system (data not shown). No IRES activity from the AEV sequence was detected in the wheat germ TNT system (data not shown). To confirm and extend the results from these in vitro assays, the same dicistronic plasmids were tested in a transient expression assay in cells. The dicistronic plasmids were transfected into 293 cells and after 20 h, cell extracts were prepared and analysed by SDS-PAGE and immunoblotting to detect CAT and LUC expression. As expected, all plasmids expressed CAT efficiently. The AEV and EMCV IRES elements directed efficient fLUC expression (Figure 1C). LUC assays performed in parallel were consistent with the immunoblotting results and the AEV 5’UTR generated about 50% of the fLUC expression observed with the EMCV IRES in 293 cells. These results indicated that the AEV 5’ UTR contains an IRES element that is functional both in the RRL system and within cells.

The AEV IRES element functions in the presence of an enterovirus 2A protease
The 2A protease from PV (plus other enteroviruses) and the FMDV L protease each inhibit cap-dependent translation by inducing the cleavage of eIF4G but these proteases have different effects on the various picornavirus IRES elements. Some IRES elements function very efficiently both in the presence or absence of the 2A and L proteases, for example the EMCV IRES. Other IRES elements, for example those from PV and other enteroviruses, are stimulated by these proteases within certain cell types, e.g. BHK cells (35). However, the IRES from HAV, the prototype hepatovirus, is strongly inhibited under these conditions since it requires the intact eIF4F complex (4). We first studied the effect of SVDV 2A on the AEV IRES activity in cells in order to discover any similarity with the HAV IRES. The dicistronic plasmids were transfected into HTK- cells either alone, or with the pGEM3Z/J1 plasmid which expresses the SVDV 2A protease. After 20 h, cell extracts were prepared and analysed by SDS-PAGE and immunoblotting to detect CAT and LUC expression. As expected, all plasmids expressed CAT efficiently when transfected into cells alone, but CAT expression was strongly inhibited in the presence of SVDV 2A protease (Figure 2A) as expected. The AEV and EMCV IRES elements directed efficient fLUC expression in HTK- cells (Figure 2A) in both the presence and absence of the 2A protease, although the AEV IRES did show some inhibition. Note that the AEV IRES displayed higher efficiency compared to the EMCV IRES in this cell type versus the 293 cells (80% versus 50%; Figure 1). Confirmation of eIF4G cleavage in cells expressing the SVDV 2A protease was achieved by Western blot analysis for eIF4G (Figure 2B); the C-terminal cleavage product was observed only in the presence of the protease. In addition, we compared the effect of addition of FMDV L protease on the activity of the AEV and HAV IRES elements in vitro. In the presence of FMDV L protease, both the EMCV and AEV IRES elements retained activity but the HAV IRES was severely
inhibited in the presence of the L protease (data not shown), in agreement with previous data (4). These data demonstrate that the AEV IRES directs internal initiation of translation which is cap-independent but it does appear that the intact eIF4F complex is required for optimal activity. This may be due to a direct requirement for binding of a component of eIF4F, or possibly to an indirect effect such as a requirement for an eIF4F-dependent factor. These results also indicate that the AEV IRES is different from the IRES element from the other hepatovirus, HAV, as it functions in the presence of cleaved eIF4G.

The 5′ terminal sequences of the AEV 5′UTR are not required for IRES activity.

To define the limits of the AEV IRES, four truncated fragments were made by removing sequences from either end of the AEV IRES cDNA. The residual sequences were inserted into the pGEM-CAT/LUC vector as described above. Each plasmid was analysed within transient expression assays within HTK- cells and in TNT assays as above. Deletion of 100 nt from the 5′ end of the 5′UTR (mutant AEVm3) had some effect on IRES activity in cells, reducing the activity to 50% of the wt (Figure 3). However, further deletion of 200 nt from the 5′ end (AEVm4) completely abolished IRES activity (Figure 3). Deletion of either 100 or 200 nt from the 3′ end of the AEV 5′UTR also completely inhibited IRES activity (AEVm1 and m2), indicating that the 3′ sequences are critical for IRES activity. Similar results were also obtained using TNT assays in vitro (data not shown).

The inclusion of 30 nt of viral coding sequence downstream of the AEV 5′UTR did not result in enhanced IRES activity within cells or in TNT reactions (data not shown) suggesting that these sequences do not play a significant role in AEV IRES function.
Similarity between the AEV IRES and IRES elements from HCV and porcine
teschovirus-1 (PTV-1)

As described above, the functional properties of the AEV IRES are clearly distinct
from those of the HAV IRES. To examine the relationship of the AEV IRES to other
picornavirus IRES elements, we performed sequence alignments of the AEV sequence
using ClustalW. We found that the AEV IRES shares a significant level of identity
with the recently characterised PTV-1 IRES (8, 17, 30). The PTV-1 IRES has been
shown to resemble the IRES element from HCV, a flavivirus, and the AEV IRES also
shares certain critical characteristics with them (Figure 4A and 4B). Notably, the
HCV domain IIIe is identical to a region of 12 nt within the AEV sequence. Overall,
the AEV IRES shares 48% sequence identity with the HCV IRES and 42% identity
with the PTV-1 IRES. The similarities are particularly apparent in the regions
surrounding and including the pseudoknot found in the HCV and PTV-1 IRES
structures (Figure 4C). We therefore believe that the pseudoknot structure found in
these IRES elements is also present in the AEV IRES.

Mutational analysis of the putative domain IIIe region within the AEV IRES

Studies on domain IIIe of the HCV IRES element have indicated that each of the
nucleotides within the highly conserved GAUA tetraloop is crucial for HCV IRES
activity (23, 32). This domain, together with domain IIId, plays an essential role in
binding the 40S ribosomal subunit (18, 26, 40). A closely related sequence is also
present within the PTV-1 IRES element although the loop sequence (GACA) has a
single nt difference from the HCV sequence; mutations in this loop of the PTV-1
IRES also greatly reduced IRES activity (9). To assess the importance of the GAUA
sequence within the AEV IRES element, corresponding to the HCV domain IIIe loop,
we mutated this motif to AAAA. Consistent with the results from similar mutations within the HCV and PTV-1 IRES elements, these changes resulted in a severely defective AEV IRES as assessed in cells (Figure 5A).

**Mutational analysis of the predicted pseudoknot region within the AEV IRES**

Evidence for pseudoknot structures within the IRES elements from HCV, CSFV and GBV-B has been obtained (41, 34, 12). Furthermore, the predicted structure of an analogous pseudoknot within the PTV-1 IRES element has been supported by mutagenesis studies (8). Disruption of base-pair interactions within this structure resulted in severely defective HCV (41) and PTV-1 (8) IRES elements. In order to test the structure of this region of the AEV IRES, mutagenesis was carried out to disrupt the predicted base-pairing, this was followed by the introduction of compensatory mutations to restore the interactions. Two sets of mutations were introduced into the predicted pseudoknot within the base paired stem regions (see Figure 4B). Mutations in stem 1 (S1) changed nt 273-275 (CUC) (which are predicted to base-pair with nt 471-473, GAG) to GGG. These mutations were predicted to disrupt these base-pair interactions. This mutant IRES was assayed within the CAT/IRES/LUC vector in cells. As expected (Figure 5B) the mutations in this region severely inhibited IRES activity. To confirm that it was the disruption of the base-pairing in S1 rather than the change in sequence alone that was responsible for the inhibition of IRES activity, compensatory mutations were introduced to restore the predicted base-pairing. The nt 471-473 (GAG) within the S1 mutant were changed to CCC, resulting in a total of 6 nt differences from the wt sequence; these additional modifications resulted in restoration of IRES activity to about 60-80% of wt IRES
activity. These results were confirmed in RRL (data not shown) and strongly suggest that the predicted secondary structure is correct.

Mutations in stem 2 (S2) changed nt 460/461 (CC) to GG, these changes were predicted to disrupt the interactions with nt 481/482 (GG) and hence destabilize the pseudoknot structure. The results from in vivo (Figure 5B) and in vitro (data not shown) experiments indicated that these mutations completely abrogated IRES activity as anticipated. Furthermore, compensatory mutations which changed nt 481/482 (GG) to CC, predicted to restore base-pair interactions, efficiently regenerated IRES activity (about 60% of wt AEV IRES activity in vivo, see Figure 5B). These results also supported the predicted pseudoknot structure shown in Figure 4. The fact that full restoration of IRES activity was not achieved may suggest that these nucleotides are also involved in other interactions (such as RNA-protein interactions) as well as forming the pseudoknot structure.

The AEV IRES is resistant to hippuristanol, an inhibitor of eIF4A

In previous studies it has been found that the HCV and PTV-1 IRES elements have no requirement for eIF4A for translation initiation (3, 28, 30). As the results presented above indicated that the AEV IRES element resembles these IRES elements, the requirement for eIF4A was studied in vitro and in vivo using hippuristanol, a specific inhibitor of eIF4A. Hippuristanol inhibits cap-dependent translation as well as the activity of type I and II picornavirus IRES elements. In contrast, the activity of the HCV and PTV-1 IRES elements is resistant to this inhibitor (3). Selected dicistronic reporter plasmids were assayed in cells in the presence and absence of hippuristanol. As expected, the eIF4A inhibitor severely reduced translation of the upstream cistron CAT (Figure 6A). As seen before (9), the EMCV IRES activity was also reduced to
about 10% of its activity in the presence of the eIF4A inhibitor. In contrast, the AEV IRES displayed marked resistance to this inhibitor (Figure 6A). Similar results were also observed *in vitro* (Figure 6B). However, we did note that the AEV IRES was partially inhibited in the presence of this inhibitor in both systems (reduced to about 50% activity in the presence of hippuristanol, which is similar to the reduction seen in the presence of the SVDV 2A protease) although a similar effect was also observed with the HCV IRES element (Figure 6B). In contrast, the HAV IRES was completely inhibited in the presence of hippuristanol, a previously unreported finding (Figure 6B). This result is in agreement with the suggestion that the HAV IRES requires the whole eIF4F complex for its function (4).

**DISCUSSION**

The studies presented here demonstrate that the 5’ UTR of the AEV genome contains an IRES element that functions efficiently in RRL and in mammalian cells. Until recently, picornavirus IRES elements were classified into one of three groups. However, a new group of picornavirus IRES elements has recently been described which includes IRES elements that closely resemble the HCV IRES elements - these include elements from PTV-1, PEV-8 and SV2 (8, 9, 17, 30). From database searches of sequences, other picornavirus genomes, including AEV, duck hepatitis virus-1 virus and Seneca valley virus, have recently been predicted to contain HCV-like IRES elements (see 9, 15). Here, we describe the functional analysis of the IRES from AEV and propose that this IRES element is included within this same group.

We have shown that the functional AEV IRES lies within nts 100-494 of the 5’UTR. In contrast to the PV, EMCV or HAV IRES elements the AEV IRES lacks a polypyrimidine tract near the 3’ end of the element. It can function when eIF4G is
cleaved and is also resistant to hippuristanol, an inhibitor of eIF4A activity. These features distinguish the AEV IRES from these picornavirus IRES elements but they are shared with the PTV-1, PEV-8 and SV2 IRES elements plus the HCV and CSFV IRES elements. Alignment of the nucleotide sequences of the HCV and AEV IRES elements confirmed that these elements share a striking similarity (about 48% overall identity). Indeed, within the HCV IIIe domain there is 100% sequence identity to a region of the AEV IRES (Figure 4). The secondary structure models for the HCV-like IRES elements include an important pseudoknot structure. The AEV sequence is also proposed to form this structure (Figure 4). We have obtained supporting evidence for the formation of this structure in the AEV IRES through mutational analysis of the sequences predicted to form the pseudoknot. Mutations within the S1 or S2 regions that were expected to disrupt the predicted pseudoknot structure inhibited IRES activity but compensatory mutations designed to restore the base-pairing in this structure efficiently rescued activity. Mutation of the GAUA motif within a portion of the AEV sequence that is identical to the domain IIIe of the HCV IRES also disrupted AEV IRES activity. Mutation of this loop region in the HCV and PTV-1 IRES elements also inhibited IRES activity (8, 23, 32). It is known that the IIIId and IIIe regions of the HCV IRES domain III interact with the 40S ribosomal subunit (18, 24) while the IIIb region has been shown to interact with eIF3 (16, 18, 38). A recent model of initiation complex formation on the HCV IRES suggests that regions IIIId and IIIe of the IRES bind to the 40S ribosomal subunit and this is followed by the interaction with eIF3 and the ternary complex (eIF2/met-tRNAi/GTP) to form a 48S pre-initiation complex (26). Recent data has shown that several proteins of the 40S ribosomal subunit, including p40, S3a, S5 and S16 are positioned close to hairpin IIIe of the HCV IRES element during the early stage of translation.
initiation (19). It has also recently been shown that domain II of the HCV and CSFV IRES elements plays a role in 80S ribosome assembly on the RNA and promotes eIF5-induced GTP hydrolysis and eIF2/GDP release, following 48S initiation complex formation (21).

The AEV IRES does not require any viral coding sequence for function and secondary structure predictions suggest that there is no equivalent region to the HCV IRES domain IV (15; Roberts, unpublished data). There are some differences between the picornavirus “HCV-like” IRES elements in this respect, as the SV2 IRES is predicted to contain a domain IV region, whereas the PTV-1 and PEV-8 IRES elements do not (9).

The discovery of a number of distinct picornaviruses harbouring an HCV-like IRES element in their 5’ UTR suggests that recombination between picornavirus and flavivirus genomes has occurred. Previous work has shown that the PV IRES can be replaced with the HCV IRES (22) to produce a viable chimeric virus, suggesting that they are functionally equivalent (although mechanistically very different). However, it is important to note that there are important structural differences between the picornavirus and HCV genomes (e.g. picornaviruses possess a 3’ poly(A) tail) that may be important in translation/replication. There are also differences in the sequence and predicted structures of the domain II regions of the picornavirus “HCV-like” and HCV IRES elements which may have a role in translation and/or replication. It remains to be seen if the diverse domain II structures found in the picornavirus “HCV-like” IRES elements have the same function or are involved in replication.

AEV has been tentatively classified as being a member of the hepatovirus genus within the Picornaviridae since it has the highest degree of protein sequence identity to HAV. However, its IRES element is clearly distinct from that of HAV in that
functions well in the presence of cleaved eIF4G, displays resistance to an inhibitor of eIF4A and shares a striking similarity to the HCV-like IRES elements. Due to these key differences between AEV and HAV, it is suggested that the placement of AEV within the hepatovirus genus should be reconsidered.

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Table 1: Oligonucleotides used for analysis of the AEV IRES

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tr>
<td>AEVF1</td>
<td>ATATGGATCCTTTGAAAGAGGCTC</td>
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<tr>
<td>AEVF238</td>
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<tr>
<td>LUCReverse</td>
<td>CATACTGTGAGGAATTAC</td>
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Sequences underlined indicate restriction sites included in the sequence. Nucleotides in bold italics are those that differ from the *wt* sequence.
FIGURE LEGENDS

**Figure 1:** The AEV 5'UTR displays IRES activity *in vitro* and *in vivo*. (A) The structures of plasmids used in this study are shown. Various fragments of the 5' UTR of the AEV genome were amplified by PCR using primers containing BamHI sites, digested, and inserted between the CAT and LUC ORFs (at the unique BamHI site) in plasmid pGEM-CAT/LUC as described in Materials and Methods. Nucleotide numbers corresponding to the fragments are shown. (B) *In vitro* translation reactions containing RRL and [35S]-methionine were programmed with RNA transcripts derived from the dicistronic plasmids containing the indicated virus sequences. Reaction products were analyzed by SDS-PAGE and autoradiography. The CAT and LUC proteins are indicated. (C) Transient expression assay in 293 cells. The dicistronic plasmids (2 µg) containing the indicated virus sequences were transfected into vTF7-3-infected 293 cells. After 20 h, cell lysates were prepared and analyzed for CAT and LUC expression by SDS-PAGE and immunoblotting. LUC assays were performed on cell extracts from three separate transfections and the results standardized to LUC expression directed by the EMCV IRES, which was set at 100%. LUC activities were normalised against CAT expression determined using a quantitative CAT ELISA (Roche). The mean values (+ SEM) are shown.

**Figure 2:** The AEV IRES functions in the presence of cleaved eIF4G. (A) Dicistronic plasmid DNA of the form CAT/IRES/LUC (2µg) containing the indicated IRES sequences were transfected into HTK- cells in the absence (-) or presence (+) of a plasmid encoding SVDV 2A protease (0.2µg). After 20 h, cell extracts were prepared and analyzed for CAT and LUC expression as for Figure 1. LUC assays
were performed on cell extracts from three separate transfections and the results standardized to LUC expression directed by the EMCV IRES, which was set at 100%. LUC activities were normalised against CAT expression as for Fig. 1. The mean values (+ SEM) are shown. (B) Samples were also analysed by immunoblotting to analyse the status of eIF4G. The C-terminal cleavage product of eIF4G is indicated (Ct).

**Figure 3: Delimitation of the AEV IRES sequences required for IRES activity.**

Dicistronic plasmids containing the AEV IRES and truncated versions of this sequence, AEVm1 (nt 1-294), AEVm2 (nt 1-394), AEVm3 (nt 100-494) and AEVm4 (nt 1-394) were transfected into vTF7-3-infected HTK- cells and cell extracts analyzed for CAT and LUC expression as in Fig. 1. LUC expression was also measured by LUC assay and the results are shown below the immunoblot in arbitrary units. Similar results were obtained in two independent experiments.

**Figure 4: Similarity between the HCV and AEV IRES elements.** (A) Alignment of the HCV and AEV IRES sequences. Sequences were aligned with ClustalW and manually edited. Individual domains associated with the HCV and AEV IRES elements are indicated above the sequence. Bold lines indicate regions involved in the formation of the pseudoknot structure within the HCV IRES and AEV IRES elements. The overall sequence identity from this alignment is 48.1% but note the 100% sequence identity within the domain IIIe region. (B) Proposed secondary structure of the entire AEV IRES. Domains are labelled according to corresponding domains of the HCV IRES (inset). The structure was predicted by comparative sequence analysis and using Mfold (42) to predict the most thermodynamically
favourable structures. (C) Comparison of predicted secondary structures of the domain IIIe and IIIf regions of the HCV, PTV-1 and AEV IRES elements. The two stems (S1 and S2) and loop regions (L1 and L2) that form the pseudoknot are shown. A domain IV structure is also present in the HCV IRES but not in the PTV-1 or AEV IRES elements. Within the AEV sequence, the nucleotides indicated in bold are those that were modified in the experiments shown in Fig. 5.

**Figure 5: Mutation of the domain IIIe loop or pseudoknot structure results in loss of AEV IRES activity.** (A) Dicistronic plasmids containing the wt AEV IRES or the IRES containing the loop IIIe mutation were transfected into vTF7-3-infected HTK- cells and analyzed for CAT and LUC expression as in Fig. 1. LUC activities (normalised against CAT expression) are shown and the results are the mean LUC values (+ SEM) from three experiments. (B) Dicistronic plasmids containing the indicated mutations within the predicted pseudoknot region were transfected into HTK- cells as described above and analyzed for CAT and LUC expression as in Fig. 3. The results are representative of two independent experiments.

**Figure 6: The AEV IRES is resistant to an inhibitor of eIF4A.** (A) Dicistronic plasmids containing the indicated IRES sequences were transfected into HTK- cells in the absence (-) or presence (+) of 0.5µM hippuristanol (Hipp.), an inhibitor of eIF4A. Cells were harvested after 20 h and the inhibitor added for the last 10 h of the incubation. Cell extracts were analyzed for CAT and LUC expression as in Fig. 1. LUC assays were performed on cell extracts from three separate transfections and the results standardized to LUC expression directed by the EMCV IRES, which was set at 100%. The mean values (+ SEM) are shown. (B) Dicistronic plasmids containing the
indicated IRES elements were also assayed in RRL TNT in the presence of 10 µM hippuristanol. The HAV and HCV IRES elements are within a cyclin/NS dicistronic construct. All proteins are indicated. Note the slower migration of the HCV IRES-directed NS product compared to the HAV IRES-directed product (arrows) due to the inclusion of 30 nt of the HCV coding sequence in this plasmid (reference 33).
Figure 1A
Figure 1
Figure 2A

Figure 2B
Figure 3
Figure 4A
Figure 4B
Figure 4C
Figure 5

(A) Bar graph showing LUC activity percentages for different samples: CAT/LUC, AEVs, AEVIIIemut, and No DNA. The graph indicates that AEVs have the highest LUC activity, followed by CAT/LUC, AEVIIIemut, and No DNA.

(B) Western blot analysis showing bands for CAT and LUC proteins in samples AEVs, AEVmutS1, AEVS1 comp, AEVmutS2, AEVS2 comp, and No DNA. The intensity of the bands correlates with the fLUC activity values provided below the blots.

fLUC activity:
- CAT: 1617, 18, 1374, 31, 938, 0.004
- LUC: 1617, 18, 1374, 31, 938, 0.004
Figure 6