Examining the relative activity of several dicistrovirus intergenic internal ribosome entry site elements in uninfected insect and mammalian cell lines

James R. Carter, Tresa S. Fraser and Malcolm J. Fraser, Jr

Comparisons of the relative activities of 11 intergenic region (IGR) internal ribosome entry site (IRES) elements of insect dicistrovirus with 5’ IRES elements of the hepatitis C and encephalomyocarditis viruses were performed in insect and mammalian cells. Dual luciferase assays were performed to determine the most effective dicistrovirus IGR IRES in the lepidopteran cell lines S9 (Spodoptera frugiperda) and BmN (Bombyx mori), and the dipteran cell lines S2 (Drosophila melanogaster) and ATC-10 (Aedes aegypti). Evaluation of dual luciferase expression from DNA plasmids and in vitro-transcribed RNA revealed apparent splicing with certain IRES elements. Though IRES activity depended upon the cell line examined, the black queen cell and Drosophila C dicistrovirus intergenic IRES elements were most effective for coupled gene expression in the diverse insect cell lines examined.

The family Dicistroviridae, consisting of a single genus Cripavirus, is a newly formed family of non-enveloped, positive-sense RNA viruses that infect invertebrates (Mayo, 2002). Initially, dicistroviruses were categorized as picorna-like viruses due to the morphological similarity of these viruses to members of the family Picornaviridae (e.g. poliovirus) (Mayo, 2002). In terms of overall genome organization, there are several similarities between picorna- and dicistroviruses (Jan, 2006); a VPg element is present at the 5’ end of the respective genomes, a 5’ internal ribosome entry site (IRES) within the 5’ untranslated regions (UTR) (Shibuya & Nakashima, 2006) and a poly(A) tail at the 3’ end of the genome of each member of these virus families.

IRES elements are cis-acting elements that function to recruit the necessary translation machinery to the desired initiator codon. These cap-independent initiators of translation tend to vary in their degree of secondary structure, GC content and requirement for the cellular proteins necessary to recruit ribosomes, thus promoting the debate over the necessity of classifying IRES elements into several classes (Baird et al., 2006). IRES elements preferentially operate during times of cellular stress, such as viral infection and DNA damage, or when cap-dependent translation initiation is compromised. These elements can be found in a wide variety of viral genomes, ranging from the retrovirus human immunodeficiency virus type-2 (Herbreteau et al., 2005) to DNA viruses such as Kaposi’s sarcoma-associated herpesvirus (Bieleski & Talbot, 2001; Grundhoff & Ganem, 2001; Hellen & Sarnow, 2001), and in a wide range of cell types (Baird et al., 2006), underlining the importance of IRES elements in nature.

Reported here is a comprehensive study of dicistrovirus intergenic region (IGR) IRES elements using a Renilla/firefly (RL/FL) dual luciferase assay (Honda et al., 2000; Lerat et al., 2000). The relative activities of IGR IRES elements from 11 of the 14 currently classified dicistrovirus family members (Table 1) were compared in uninfected mammalian and insect cell lines for potential future use in expression vectors.

A bicistronic RL/FL dual luciferase reporter plasmid (Parsons et al., 2000; Stables et al., 1999) was engineered to analyse the activity of each dicistrovirus IGR IRES (Fig. 1a). Expression levels obtained for the 5’ RL cistron are an indication of cap-dependent translation while the 3’ FL cistron is indicative of the IRES-dependent translation (Parsons et al., 2000; Stables et al., 1999). Utilization of this system allows for an accurate and easily quantifiable assay with expression of RL acting as an internal normalizer, enabling the relative comparison of IRES-directed FL activity.

Cell-specific promoters were employed to study dicistrovirus IGR IRES elements in several insect and mammalian cell lines. The Bombyx mori A3 cytoplasmic actin gene promoter, AC3 (Mange et al., 1997), was utilized for optimal expression in the lepidopteran B. mori BmN (Grace, 1967) and Spodoptera frugiperda pupal ovarian S9 (Vaughn et al., 1977) cell lines. These cell lines have been
Assessment of dicistrovirus intergenic IRES elements

**Table 1.** Dicistrovirus intergenic IRES elements and the control IRES elements analysed

<table>
<thead>
<tr>
<th>Strain or control IRES</th>
<th>GenBank accession no.*</th>
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<tbody>
<tr>
<td><strong>Strain IRES elements</strong></td>
<td></td>
</tr>
<tr>
<td>Aphid lethal paralysis virus (ALPV)</td>
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<td>Black queen cell virus (BQCV)</td>
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<td>Solenopsis invicta virus-1 (SINV)</td>
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<td>Taura syndrome virus (TSV)</td>
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<tr>
<td><strong>Control IRES elements</strong></td>
<td></td>
</tr>
<tr>
<td>Encephalomyocarditis virus (EMCV)</td>
<td>†</td>
</tr>
<tr>
<td>Hepatitis C virus (HCV)</td>
<td>‡</td>
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<tr>
<td>Stretch of 257 bp of pBSII SK+ (pXL), negative control</td>
<td>$</td>
</tr>
<tr>
<td>X-linked initiator of apoptosis (XIAP), positive control for splicing</td>
<td>BX119811</td>
</tr>
</tbody>
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*GenBank accession numbers are given, where applicable, for those IRES elements synthesized by BioBasic.
†Purchased from Clontech Laboratories.
‡Kindly provided by Annie Cahour.
§Purchased from Stratagene.

used previously to study baculovirus expression vector gene products (Argaud et al., 1998; Cohen et al., 1989; Grasela et al., 2000). The distal portion of the *Drosophila melanogaster* actin 5C promoter (Zhao & Eggleston, 1999) was chosen for use in the dipteran *Aedes aegypti* mosquito, ATC-10 (Singh & Pavri, 1967), and *D. melanogaster* embryo S2 cell lines (Schneider, 1972), which have also been used previously to study viral gene products (McIntosh et al., 2005; Theoekattikul et al., 2005). The *Xenopus* elongation factor 1α promoter, EF1α, previously used for mammalian cell gene expression (Johnson & Krieg, 1995), was utilized for expression of the dicistronic dual luciferase plasmids in COS-7 (Yamamura et al., 1985) and primary human embryonic kidney 293GP (Pear et al., 1993) cells.

All cell lines were maintained in their respective complete media: Leibovitz’s L-15 (Blaney et al., 2005) for ATC-10, ExCell 401 (JRH Biosciences) for BmN and SF9, Dulbecco’s modified Eagle’s medium (Gibco-BRL) for COS-7 and Eagle’s minimal essential medium (Gibco-BRL) for 293GP cells. All media were supplemented with 10% fetal bovine serum (Atlanta Biologicals) and an antibiotic/antimycotic solution consisting of 10 000 U penicillin ml⁻¹, 10 mg streptomycin ml⁻¹ and 25 μg amphotericin B ml⁻¹ (Gibco-BRL). For all transfections, cells were seeded at a density of 5 x 10⁵ per cm² dish into six-well plates. Cells were grown in complete media and allowed to adhere for 3–4 h, except for ATC-10 cells which were allowed 24 h for full adhesion.

The T7 promoter was inserted into the plasmid backbone for *in vitro* production of capped RNA transcripts (Fig. 1a; see Supplementary Table S1, available in JGV Online, for primer sequences). RNA was synthesized using the Megascript high yield transcription kit (Ambion). Briefly, DNA plasmids to be used as templates were linearized with Acc65I and capped RNA was obtained by adding GTP cap analogue (Promega) with a molar excess of GTP (at a 4:1 ratio). Synthesis was carried out at 37 °C for 4 h and RNA was purified via phenol/chloroform extraction and lithium chloride precipitation as described by the manufacturer (Ambion).

The dicistrovirus IGR IRES elements chosen for study (Supplementary Table S2, available in JGV Online), plus the first 150 nt encoding the N-terminal portion of their respective capsid protein cistrons, were fused in-frame with the FL gene (Fig. 1a). The 5' end of the second ORF was included since it has been previously reported that either a number of dicistrovirus IGR IRES elements require interactions with the 5' end of the second ORF (Sasaki & Nakashima, 1999; Shibuya et al., 2003) to maintain the proper secondary structure of these IRES elements through RNA–RNA interactions (Shibuya et al., 2003), or the intergenic region of the dicistrovirus genome also functions to help maintain the secondary structure of the capsid coding region (Shibuya et al., 2003). An arbitrary stretch of 257 nt from the pXL BS SK+ plasmid (pXL) served as a negative control of IRES function (background expression) (Table 1). The encephalomyocarditis virus (EMCV) and hepatitis C virus (HCV) 5' IRES elements served as positive controls for IRES function, and the X-linked inhibitor of apoptosis (XIAP) IRES served as a positive control for potential splicing within the IRES element (Van Eden et al., 2004).

To test the ability of dicistrovirus IGR IRES elements to initiate translation in uninfected insect cells, the relative activities of several dicistrovirus IGR IRES elements (Table 1) were examined in dipteran *A. aegypti* ATC-10 and *D. melanogaster* embryo S2 cells via an RL/FL dual luciferase assay (Parsons et al., 2000; Stables et al., 1999). For each IRES construct, *A. aegypti* ATC-10 (Fig. 1b) and *D. melanogaster* S2 (Fig. 1c) cells were transfected in triplicate in wells of a six-well plate, with either 1 μg bicistronic DNA or 2 μg *in vitro* transcribed, 5' - capped RNA per well, using Transfectin reagent according to the manufacturer’s protocol (Bio-Rad). At 48 h (S2 cells) or 72 h (ATC-10 cells) after DNA transfection or 8 h after RNA transfection, cells were lysed and each well was analysed in triplicate for RL and FL activities according to the dual-luciferase reporter assay system protocol (Promega) on the LMaxII384 luminometer (Molecular Devices). Values for RL and FL activity were corrected by subtracting the mean value of the cells transfected with transfection reagent alone from each of the values of the experimental wells. IRES activity was measured by taking the relative ratio of FL to RL for each well read in triplicate and in reference to the FL to RL ratio of the pXL.
control insert, which was set to 1 as previously described (Wilson et al., 2000). Three wells per IRES element were transfected and each was analysed for luciferase activity. This experiment was performed three times to give a total of nine replicates for each IRES element tested.

In A. aegypti ATC-10 cells, the XIAP putative IRES showed a high FL to RL ratio when analysed by DNA-dual luciferase activities resulting from DNA transfections, as shown previously in other cell systems (Van Eden et al., 2004; Holcik & Korneluk, 2000; Fig. 1b). However, the XIAP relative FL activity was greatly reduced when cells were transfected with in vitro-transcribed RNA. This difference has been described previously as being reflective of the splicing of FL/RL RNA that is expressed from the DNA vectors (Van Eden et al., 2004). Since mRNA splicing occurs in the nucleus, the increase in RL activity versus FL activity that was observed with DNA transfections compared with transfection with capped RNA produced via transcription in vitro probably reflects an artificial reduction in RL as a result of splicing (Matera et al., 2007).

In dual luciferase experiments employing DNA transfection, the IGR IRES of the dicistrovirus aphid lethal paralysis virus (ALPV) was observed to be approximately five times more active than background, and approximately 2.5 times greater than the HCV and EMCV IRES elements (Fig. 1b). However, when compared with dual luciferase activity following RNA transfections, ALPV displayed a threefold decrease in IRES activity. Black queen cell virus (BQCV), cricket paralysis virus (CrPV) and Drosophila C virus (DCV) intergenic IRES activities were determined to be approximately four, two and three times greater than background levels, respectively, for DNA and RNA assays (Fig. 1b). The activities of the other dicistrovirus IGR IRES elements were comparable to the amount of activity displayed by the pXL control, indicating that these elements were non-functional in the examined mosquito cells. Similar results were observed in Drosophila S2 cells (Fig. 1c). Dicistrovirus IGR IRES elements from ALPV, BQCV and CrPV also displayed an increase in the normalized FL to RL ratio over the control levels, indicative of increased FL activity and thus a functional IRES element. There was no evidence that this increase in activity was due to splicosomal activity, since similar dual luciferase relative activities were observed in the RNA transfections. The DCV IGR IRES activity appeared to be
greatly diminished compared with the IGR IRES elements from ALPV, BQCV and CrPV, as well as from the mammalian viruses HCV and EMCV. The difference in DCV IGR IRES activity between S2 and ATC-10 cells may be attributed to differences in the translational effectiveness of this IRES between these cell lines, since the same promoter (Ac5) and vector (pXL BacII) combinations were used in both cell lines with each of the IRES constructs.

In assessing the strength of dicistrovirus IGR IRES elements in the lepidopteron S9 and BmN cells, we utilized identical dicistronic constructs, but replaced the Drosophila Ac5 promoter with the B. mori Ac3 promoter for optimal expression in these cell lines (Mange et al., 1997). Transfection and analysis of dual luciferase activities were performed as described above. Upon transfection of DNA constructs into S9 and BmN cells (Fig. 1d, e), the ALPV, BQCV, CrPV and DCV IGR IRES elements produced activities three- to fourfold above background, and approximately twofold greater than HCV and EMCV. Similar activities were obtained with the BQCV, CrPV and DCV IGR IRES elements introduced as in vitro-transcribed RNAs. The ALPV IGR IRES showed a slight decrease in the RNA-luciferase assay compared with the DNA-dual luciferase assays. However, this difference was not statistically significant.

Several dicistrovirus IGR IRES elements displayed variations in overall activities in BmN cells (Fig. 1d) compared with their activities in ATC-10 and S2 cells. IGR IRES elements from Kashmir bee virus (KBV), Solenopsis invicta virus-1 (SINV) and Taura syndrome virus (TSV) displayed a twofold increase in relative FL activity compared with the pXL-negative control; their activity was statistically equivalent to the negative control when studied in ATC-10 and S2 cells.

To determine the relative activities of dicistrovirus IRES elements in mammalian cells, we analysed dicistrovirus IRES activity in COS-7 and 293GP cells relative to the flavivirus HCV and picornavirus EMCV IRES elements (Fig. 2a, b). We expected that IRES elements derived from mammalian viruses would possess greater activity in COS-7 and 293GP cells, since the dicistrovirus IGR IRES elements have demonstrated the same phenomenon in insect cells. To verify this, COS-7 and 293GP cells were transfected either with DNA plasmids or in vitro-transcribed RNA using the Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer’s protocol. At 48 h after DNA transfection or 8 h after RNA transfection, cells were lysed and analysed as described for the insect cells studied. As expected, the resulting DNA-dual luciferase activities for the IRES elements derived from the mammalian viruses HCV and EMCV exhibited a far greater level of activity (five to six times greater) than the dicistrovirus IGR IRES elements that were examined.

The BQCV IGR IRES displayed a consistent level of increased activity compared with the other dicistrovirus IGR IRES elements, as well as IRES elements derived from the mammalian HCV and ECMV (Figs 1 and 2). However, in the 293GP and COS-7 mammalian cells, HCV and EMCV IRES elements display greater activities compared with the dicistrovirus IGR IRES elements tested (Fig. 2). Diverse intracellular activities have been demonstrated for different picornavirus IRES elements in different cell types (Borman et al., 1997; Roberts et al., 1998). Several IRES elements (from the 5’ UTR and IGR segments) studied from members of the family Dicistroviridae have also been shown to function at varying degrees in a number of cell types from plants to mammals (Masoumi et al., 2003; Wilson et al., 2000; Woolaway et al., 2001). The reason for this discrepancy in activity levels from one cell type to the next may be due to the possibility that some cell types possess a trans-activator of IRES function while others possess inhibitory binding proteins which bind to the same IRES, thus preventing its activity. One possibility that has yet to be discussed is the potential issue of dicistrovirus mRNA half-life and overall stability in cells. Some mRNAs have longer half-lives in cells than others (Koch & Friesen, 1979); this may be due to potential cis- and trans-acting elements which stabilize an individual mRNA in a cell-specific manner (Weiss & Liebhaber, 1994, 1995). This may also be true of dicistrovirus IRES elements, though this hypothesis has never been...
the X-linked inhibitor of Apoptosis (XIAP) internal ribosome entry site-directed translation.


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References


