Short Communication

The *Rhopalosiphum padi* virus 5′ internal ribosome entry site is functional in *Spodoptera frugiperda* 21 cells and in their cell-free lysates: implications for the baculovirus expression system

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Received 27 January 2004
Accepted 19 February 2004

Initiation of protein synthesis on most cellular mRNAs involves the recognition of the 5′ cap structure (m′GpppN) by translation initiation factors and subsequent recruitment of the 43S pre-initiation complex which includes the 40S small ribosomal subunit (reviewed by Hershey & Merrick, 2000). However, translation initiation on certain viral mRNAs (e.g. from picornaviruses) occurs by a cap-independent mechanism. An internal ribosome entry site (IRES) element present within the 5′ untranslated region (UTR) directs initiation of protein synthesis at an internal position hundreds of nucleotides downstream from the 5′ terminus. The viral IRES elements are highly structured and position the ribosome at, or just upstream of, the initiation codon. The presence of IRES elements has also been reported within certain cellular mRNAs, such as those involved in apoptosis, cell cycle regulation or stress response (Carter et al., 2000; Hellen & Sarnow, 2001).

The best-characterized IRES elements are those from the mammalian picornaviruses (reviewed by Belsham & Jackson, 2000). The picornavirus IRES elements are grouped into two major classes based on their predicted secondary structure and their activity in vitro. One class contains IRES elements from the enteroviruses and rhinoviruses (e.g. poliovirus) 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 poliovirus and rhinovirus IRES elements are inefficient in this system unless the reaction is supplemented with HeLa cell extracts (Brown & Ehrenfeld, 1979; Dorner et al., 1984). Similarly, the activity of the hepatitis A virus IRES (which forms a third class of IRES) is stimulated by the addition of liver cell, but not HeLa cell, extracts (Glass & Summers, 1993). It is clear that cellular trans-acting factors play an important role in the mechanism of IRES action and may contribute to the cellular tropism of picornaviruses. Indeed, it has been demonstrated that different IRES elements function with different efficiencies in different cell types (Borman et al., 1997; Roberts et al., 1998).

We have previously demonstrated that the 5′ UTR of *Rhopalosiphum padi* virus (RhPV) mRNA contains an IRES element (Woolaway et al., 2001). This virus belongs...
to the family *Dicistroviridae*, which also includes cricket paralysis virus (CrPV), *Drosophila* C virus (DCV) and *Plautia stali* intestine virus (PSIV). The single-stranded, positive-sense RNA genome of these viruses contains two open reading frames (ORFs) that encode two polyproteins (Moon *et al.*, 1998). ORF1 encodes the non-structural proteins and ORF2 the structural proteins. All of these proteins possess sequence similarity with mammalian picornavirus proteins. It has been shown that the 5' UTRs and the intergenic regions (IGRs) of these virus genomes contain IRES elements (Sasaki & Nakashima, 1999; Domier *et al.*, 2000; Wilson *et al.*, 2000a; Woolaway *et al.*, 2001). The IGR IRES elements are unusual in that they direct translation initiation from non-AUG codons and they do not require any of the canonical initiation factors for assembly of initiation complexes on the mRNA (Wilson *et al.*, 2000b; Nishiyama *et al.*, 2003). In contrast, the 5' IRES of RhPV directs initiation from AUG codons. It has been shown to function in a *Drosophila* cell-based *in vitro* translation system and also functions efficiently in RRL and wheat germ lysates (Woolaway *et al.*, 2001). The ability of the RhPV 5' IRES to function in insect translation systems suggests potential utility of this IRES in insect cell expression systems. To explore this further, we have tested the function of this IRES in Sf21 cells and in a novel *in vitro* Sf21 cell-based lysate system (Kubick *et al.*, 2003). These cells are commonly used with baculovirus expression systems. We report here that the RhPV 5' IRES displays efficient activity in these cells and in the *in vitro* system.

To examine the activity of the RhPV 5' IRES within Sf21 cells, reporter plasmids of the form T7:CAT/IRES/LUC containing the RhPV IRES (previously referred to as RhPVΔ1; nt 1 to 579) or the mammalian picornavirus EMCV IRES were used, as described by Woolaway *et al.* (2001). Cap-dependent translation was monitored by measuring chloramphenicol acetyltransferase (CAT) expression and activity of the IRES element was assessed from the expression of luciferase (LUC). Mutated versions of the RhPV IRES plasmid containing 5' and 3' end deletions [named RhPVΔ2 (with nt 1 to 463), Δ3 (nt 1 to 374) and Δ4 (nt 100 to 588)] have also been described previously (Woolaway *et al.*, 2001). Sf21 cells were grown at 28°C in TC100 medium (Gibco-BRL) supplemented with fetal bovine serum (FBS; 10%), penicillin (5000 units penicillin G sodium ml⁻¹), streptomycin (5000 μg streptomycin sulphate ml⁻¹ in 0.85% saline) and 2 mM l-glutamine. The dicistronic reporter plasmids were assayed by transfection into Sf21 cells (60 mm dishes) using lipofectin (20 μl; 1 mg ml⁻¹ stock; Gibco-BRL). T7 RNA polymerase was expressed in the cells by prior infection with a recombinant baculovirus (AcT7N; gift from Dr J. Vlak, University of Wageningen, The Netherlands; van Poelwijk *et al.*, 1995). After 48 h incubation at 28°C, the cells were harvested in 400 μl cell lysis buffer (Promega) and the lysates clarified by centrifugation at 14 000 r.p.m. for 5 min at 4°C. CAT assays were performed using the CAT ELISA kit (Boehringer Mannheim) as described in the manufacturer’s instructions. Colour development was measured on an ELISA plate reader (Labsystems Multiscan Bichromatic) at 405 nm. As expected, each of the constructs induced a similar level of CAT expression (Fig. 1). LUC expression was measured using a LUC assay kit (Promega) and a Bio-orbit luminometer. The RhPV 5' IRES was able to direct LUC expression at a level 23-fold higher than the background LUC expression seen with pGEM-CAT/LUC (no IRES) and 400-fold higher than seen from the construct containing the EMCV IRES (Fig. 1). Presumably, the presence of the structured EMCV RNA sequences inhibited any translational read-through and

![Fig. 1. Analysis of the RhPV 5' IRES activity in Sf21 cells.](image-url)
hence LUC expression. As we have seen previously in \textit{in vitro} translation systems (Woolaway \textit{et al.}, 2001), the 5’ and 3’ end deletion mutants directed less-efficient expression of LUC in cells. However, the mutants still retained about 40 to 50% activity of the wild-type RhPV IRES within cells (Fig. 1). The antisense version of the RhPV IRES was unable to direct LUC expression as expected.

An Sf21 cell-based \textit{in vitro} translation system has recently been described using monocistronic constructs (Kubick \textit{et al.}, 2003). Since the RhPV IRES displayed activity in Sf21 cells, we wanted to explore the properties of the RhPV 5’ IRES within this system since a cell-free system can be more readily manipulated to define parameters important for RhPV IRES activity. We tested the ability of the RhPV IRES to direct translation of the downstream luciferase ORF in both untreated and nucleased Sf21 lysates. Capped transcripts from the linearized dicistronic plasmids were made \textit{in vitro} using T7 RNA polymerase (T7 Cap-Scribe; Roche). \textit{In vitro} translation reactions contained 5 μl 10 × buffer (30-6 mM HEPES/KOH pH 7-95; 1-5 mM magnesium acetate; 100 mM potassium acetate; 2-5 mM DTT; 0-25 mM spermidine); 1 μl 0-1 M creatine phosphate; 1-0 μl 0-1 M ATP; 0-5 μl 0-1 M GTP; 1-5 μl 1 mM total amino acid mix; 0-5 μl 10 mg creatine kinase ml⁻¹; and 1-25 μl 50 mM magnesium acetate. The mRNA was added at a final concentration of 100 ng μl⁻¹ in a total volume of 25 μl, containing Sf21 lysate (50 % by volume), and the reactions were incubated at 27 C for 90 min. Translation reactions in the nucleased lysate incorporating [³⁵S]methionine were analysed for CAT and LUC expression by SDS-PAGE and autoradiography. In addition, LUC activity was measured as above. Each of the dicistronic RNA transcripts containing the CAT gene expressed CAT at a similar level (Fig. 2A). However, only the dicistronic transcripts containing the RhPV IRES were able to direct efficient expression of luciferase, the EMCV IRES was essentially inactive in this system (Fig. 2A, B). Luciferase was also expressed from a monocistronic RNA control (pSP72LUC). Translational efficiencies were compared with those in untreated (un-nucleased) Sf21 lysate (Fig. 2C) to assess if the IRES could function in the presence of competing RNAs. All transcripts expressed CAT at a similar level as expected (data not shown). The RhPV IRES was able to direct expression of luciferase in both systems (Fig. 2A–C). In neither system was the EMCV IRES able to direct translation of luciferase better than the control plasmid lacking any IRES. The capped monocistronic luciferase control mRNA functioned well in the nucleased lysate (Fig. 2A, B) but, in the untreated lysate, very little luciferase expression was observed (Fig. 2C). Presumably, the RNA transcripts were unable to compete efficiently for the translational machinery in the presence of endogenous mRNAs (Fig. 2C). Indeed, the expression of luciferase was higher in the nucleased lysate than in the untreated lysate in each case, e.g. the RhPV IRES-directed luciferase expression was more than three-fold higher in the nucleased lysate. Consistent with these observations, translation of

![Fig. 2](http://vir.sgmjournals.org)  
**Fig. 2.** The RhPV 5’ IRES functions in Sf21 lysates. Capped mRNA transcripts of the form CAT/IRES/LUC were translated in nucleased (A and B) or untreated Sf21 lysates (C) as described in the text. Samples from the nucleased lysate reactions were analysed by SDS-PAGE and autoradiography (A) using a phosphorimager (Personal Molecular Imager FX; Bio-Rad) and by LUC assay (B). Samples from the untreated lysate reactions were analysed by LUC assay alone (C). The IRES-containing dicistronic transcripts are referred to by the name of the IRES insert. The results shown are from one complete experiment but are representative of three separate experiments.

CAT from the capped transcripts was also elevated in nucleased lysate as compared with that in untreated lysate (results not shown).
These results show that the RhPV 5’ IRES functions efficiently in Sf21 cells and in Sf21 lysates; the RhPV 5’ IRES also functions well in a Drosophila lysate (Woolaway et al., 2001). In contrast, the EMCV IRES is inactive in each of these systems (Finkielstein et al., 1999; Woolaway et al., 2001). It may be that insect cells lack an important trans-acting factor that is required for EMCV IRES function, or alternatively they may contain an inhibitor of EMCV IRES activity. Recently, Domier & McCoppin (2003) demonstrated the ability of the RhPV 5’ and IGR IRES elements to function in Sf9 cells. In their system the 5’ IRES seemed to function as efficiently as the IGR IRES to direct internal initiation. However, it should be noted that these authors reported just a three- to fourfold increase in expression directed by the 5’ IRES in Sf9 cells compared to a negative control containing a defective RhPV IGR IRES. In Sf21 cells, we observed a 23-fold increase in expression when the 5’ IRES was inserted into a dicistronic mRNA (compared to a dicistronic mRNA lacking an IRES). We have also observed a 180-fold increase in LUC expression from the RhPV IRES in mosquito cells using the same plasmids (Kohl et al., 2004). In a recent report, Masoumi et al. (2003) demonstrated that the 5’ and IGR IRES elements of CrPV display different levels of activity in a range of different insect cells. Surprisingly, neither of the IRES elements from the CrPV genome could function in Sf9 cells. This suggests that subtle differences may exist between the IRES elements from these related dicistroviruses.

Insertion of the RhPV 5’ IRES into an uncapped monocistronic mRNA increased the translational efficiency in the Sf21 lysate by fourfold (Kubick et al., 2003). In this study we have also demonstrated that the RhPV IRES directed LUC expression between three- and 10-fold over that seen from the CAT/no IRES/LUC control in vitro. However, within Sf21 cells, the increase in expression from the RhPV IRES was 23-fold. This is likely due to a lower background of internal initiation from the ‘no IRES’ dicistronic mRNA within cells compared with that seen in vitro. This is analogous to the mammalian systems in which a mutant EMCV IRES displayed around 0-25% of the activity of a wt EMCV IRES within cells, whereas in the in vitro system the activity was around 5% (van der Velden et al., 1995).

These data suggest that use of this IRES element within an insect cell-based expression vector can enhance translation, without the need for making capped transcripts in vitro. Similarly, insertion of the IRES between two ORFs may allow for expression within the baculovirus expression system of a protein of interest along with a reporter or selectable marker, or expression of two subunits of a protein within the same cells at the same time.

Acknowledgements
This work was supported by the BBSRC (L. O. R., G. J. B. and E. R.). K. E. W. gratefully acknowledges a BBSRC studentship award.

References


