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Short Communication

Characterization of internal ribosomal entry sites of Triatoma virus

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Triatoma virus (TrV) belongs to a new family of RNA viruses known as *Dicistroviridae*. Nucleotide sequence comparisons between different dicistroviruses allowed two putative internal ribosomal entry sites (IRESs) in the TrV RNA to be defined: the 5′UTR IRES of 548 nt and the intergenic region (IGR) IRES of 172 nt. Using monocistronic and bicistronic RNAs, it was shown that the TrV genome contains two functional IRESs that mediate translation initiation in a cap-independent manner. In addition, it was found that the two TrV IRESs were able to direct efficient translation of reporter genes in microinjected *Xenopus* oocytes, suggesting minimum requirements for host factors. The IGR IRES begins with a non-canonical CUC; however, mutations of this triplet to AUG or CCU did not impair IRES function, indicating that the CUC is not essential for the initiation process. Furthermore, translation efficiency from two TrV IRESs was differentially modulated by IFN-α and viral infection.

Triatoma virus (TrV) is a pathogen of *Triatoma infestans*, the most important vector of human trypanosomiasis in Argentina (Chagas' disease). TrV is widely distributed in the *T. infestans* populations in Argentina. Insects usually die after showing leg paralysis and ecdysis failure (Muscio *et al.*, 1987). Due to the vertical transmission and high pathogenicity, TrV is considered a potential agent for biological control of *T. infestans* (Muscio *et al.*, 1997).

We have previously reported the complete nucleotide sequence analysis of TrV and showed that this virus belongs to the family *Dicistroviridae* (Czibener *et al.*, 2000), formerly known as insect picorna-like viruses (Mayo, 2002). The members of this family possess single-stranded, positive-sense RNA genomes with a distinctive bicistronic arrangement. The RNA genome contains two open reading frames (ORFs) each encoding a polyprotein separated by an intergenic region. The non-structural proteins are encoded in the 5′-proximal ORF and the structural proteins are encoded in the second ORF (Czibener *et al.*, 2000; Domier *et al.*, 2000; Johnson & Christian, 1998; Sasaki *et al.*, 1998; Wilson *et al.*, 2000). For several members of this family, it has been demonstrated that the two ORFs are preceded by RNA structures that function as internal ribosomal entry sites (IRESs) for translation of the viral proteins (Domier *et al.*, 2000; Kanamori & Nakashima, 2001; Sasaki *et al.*, 1998; Wilson *et al.*, 2000; Woolaway *et al.*, 2001). The 5′UTR and the intergenic region (IGR) IRES exhibit different sequences and presumably different mechanisms of translation initiation. An unusual feature of the IGR-IRES is that translation of the capsid proteins initiates with an amino acid other than methionine. Usually, the initiation site selection for translation involves base-pair formation between an AUG codon and the anticodon triplet of an initiator methionine tRNA. In contrast, for several members of the *Dicistroviridae*, different initiation codons were found: CUU for *Plautia stali intestine virus* (PSIV) and CCU in the case of *Cricket paralysis virus* (CrPV). It has been proposed that secondary and tertiary structures of the RNA within the IGR enable Met-independent initiation of translation (Domier *et al.*, 2000; Jan *et al.*, 2003; Jan & Sarnow, 2002; Pestova *et al.*, 2004; Sasaki & Nakashima, 2000; Spahn *et al.*, 2004; Wilson *et al.*, 2000).

Translation initiation mediated by the 5′UTR and the IGR of TrV has not been examined. In order to investigate the translation of the two ORFs of TrV, we generated different RNA molecules carrying the firefly luciferase gene flanked by the 5′UTR or the IGR and the 3′UTR of TrV. To this end, we obtained viral particles from infected *T. infestans* and purified them using sucrose gradients (10–30 %) as previously described (Muscio *et al.*, 1988). RNA extraction was performed using TRIzol and directly used for reverse transcription and PCR amplification of the 5′UTR, the IGR and the 3′UTR. According to our previous sequencing and alignment analysis, we defined the 3′ boundary of the 5′UTR IRES at nt 549 and the IGR-IRES spanning nt 5934–6111 (GenBank accession no. AF178440). From sequence alignments, we deduced that the initiator triplet of ORF2 is CUC (Czibener *et al.*, 2000). Both the 5′UTR and the IGR (including the first 40 nt of the respective viral-coding sequences) were fused in-frame with the luciferase-coding

region. Amplification of the viral sequences was performed using the primers indicated in Fig. 1(a). *In vitro* transcriptions were performed to generate the RNA 5′UTR-TrV-Luc and IGR-TrV-Luc (Fig. 1b). Translation was evaluated by microinjecting the RNAs into *Xenopus* oocytes. This system has proved to be a useful tool to analyse IRES-dependent translation, since, in contrast to *in vitro* translation systems, it does not initiate translation of uncapped RNAs (Fig. 1c) (Gamarnik & Andino, 1996; Gamarnik *et al.*, 2000).

To determine whether the 5′UTR and IGR of TrV were capable of initiating translation in a cap-independent manner, we microinjected oocytes with 20 ng 5′UTR-TrV-Luc, IGR-TrV-Luc, or control uncapped RNAs carrying the 5′ and 3′UTRs of β-globin. We used two controls, one carrying the 5′UTR of β-globin and the 3′UTR of TrV and the second one bearing both the 5′ and 3′UTRs of β-globin (Fig. 1b). The luciferase activity measured with the uncapped RNAs carrying the 5′UTR or the IGR of TrV were 300- and 500-fold higher, respectively, than that observed for the control RNAs (Fig. 1d), suggesting that the viral sequences mediate translation initiation in a cap-independent manner. In addition, to determine whether specific host factors present in *T. infestans* enhance translation mediated by the TrV sequences, we co-injected the RNAs together with cytoplasmic proteins obtained from *T. infestans* embryos (150 ng protein per oocyte). The levels of luciferase observed with the 5′UTR-TrV-Luc and

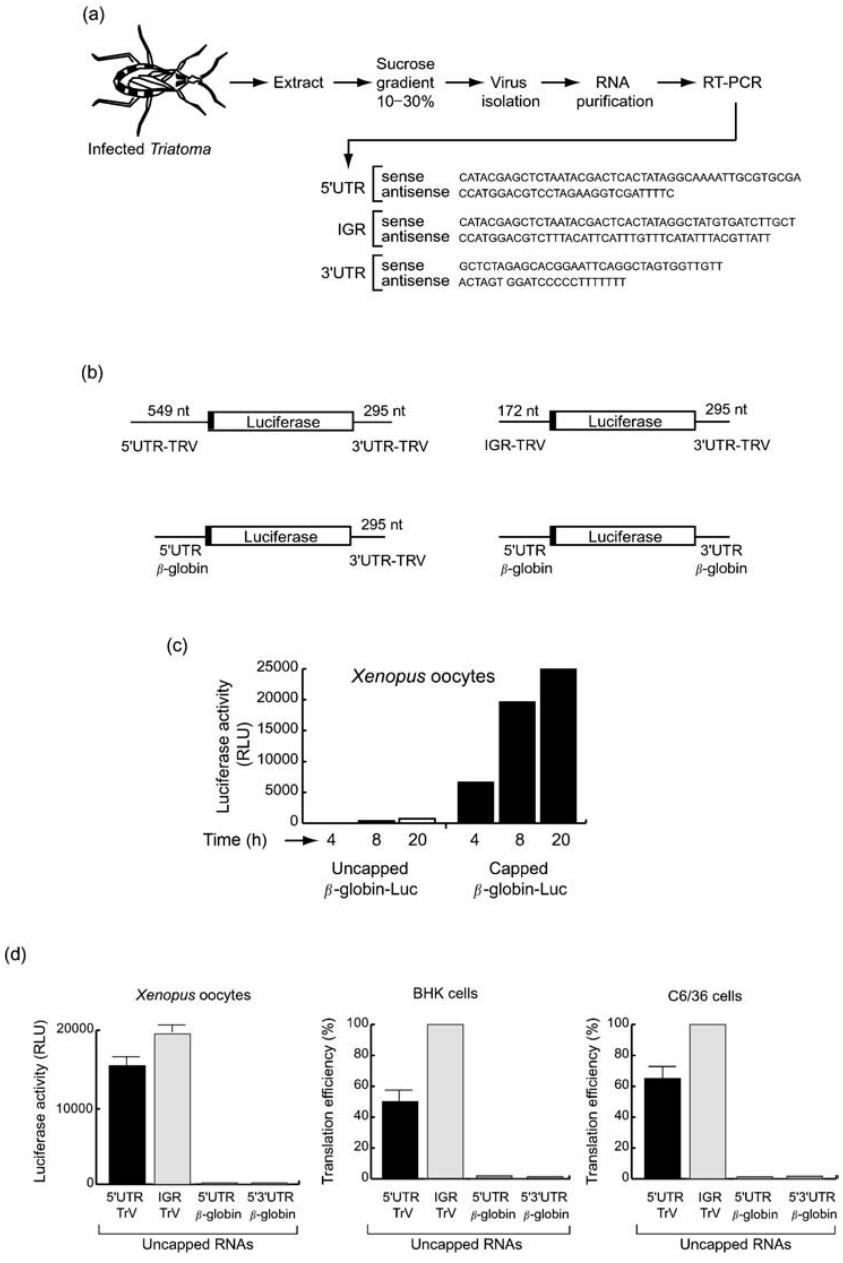

Fig. 1. Functional IRES activities in the TrV genome. (a) Schematic diagram of TrV isolation from infected insects. The sequences of oligonucleotides used to amplify the 5′UTR, IGR and 3′UTR of TrV are indicated. (b) Schematic representation of RNA molecules encoding firefly luciferase flanked by TrV or β-globin sequences. Different 5′- and 3′UTRs are indicated. (c) Translation of microinjected capped and uncapped RNAs in *Xenopus* oocytes. *In vitro*-synthesized capped and uncapped RNAs were microinjected into oocytes. The oocytes were incubated at 22 °C for 4, 8 and 20 h, as indicated. Translation efficiency was measured by luciferase activity expressed in relative light units (RLU). (d) Translation mediated by the TrV 5′UTR and IGR of uncapped RNAs in different cell types. The RNAs shown in (b) were microinjected into *Xenopus* oocytes or transfected into BHK and C6/36 cells as indicated on each graph. Translation levels of transfected RNAs were determined by firefly luciferase activity normalized by *Renilla* luciferase activity and expressed as a percentage of the levels of TrV IGR RNA.

IGR-TrV-Luc RNAs co-microinjected with Triatoma proteins or buffer control were similar (data not shown), suggesting that factors from *T. infestans* did not enhance translation mediated by TrV IRESs under our experimental conditions.

To extend these studies, we tested the ability of the 5′UTR and IGR of TrV to direct translation of the reporter in different cell types. RNA was transfected into baby hamster kidney (BHK) and insect (C6/36) cells using Lipofectamine 2000 (Invitrogen). The RNAs were *in vitro* transcribed and purified (RNeasy; Qiagen). In contrast to microinjection into oocytes, in which precise volumes of RNA can be delivered inside the cell, transfection of RNA into cells grown in culture required normalization. Thus, we co-transfected quantified RNAs with a second capped mRNA encoding *Renilla* luciferase. Translation efficiencies were expressed as the ratio of the activities measured for the firefly and *Renilla* luciferases in each case. Similar to the results observed in oocytes, translation of the RNA mediated by the 5′UTR or IGR of TrV was efficient, while the uncapped RNA control only showed background levels (Fig. 1d). In addition, in all the systems used, the TrV IGR was 30–50 % more efficient in directing translation than the viral 5′UTR.

The RNA molecules carrying the 5′UTR or IGR of TrV at the 5′ end also contained the complete 3′UTR sequence of TrV (295 nt) after the stop codon of luciferase. It has previously been shown that sequences and RNA structures present at the 3′UTR of viral and cellular mRNAs can modulate cap- and IRES-mediated translation initiation (reviewed by Mazumder *et al.*, 2003). To test whether the 3′UTR was important for efficient IRES activity, we replaced the 3′UTR of TrV with unrelated 3′UTRs [3′UTR of dengue virus (DV) or the 3′UTR of β-globin]. Translation of the RNAs carrying the 5′UTR or IGR of TrV was efficient for both TrV and the unrelated 3′UTRs (data not shown), suggesting that translation initiation mediated by the two putative IRESs of TrV is not modulated by specific 3′UTR elements.

To confirm the IRES during translation of the TrV genome, we constructed bicistronic mRNAs in which the 5′UTR and IGR of TrV were introduced preceding a second ORF. A schematic representation of the RNA constructs is shown in Fig. 2(a) (Bicis 5′UTR TrV-Luc and Bicis IGR TrV-Luc RNAs). The two bicistronic RNAs were microinjected into

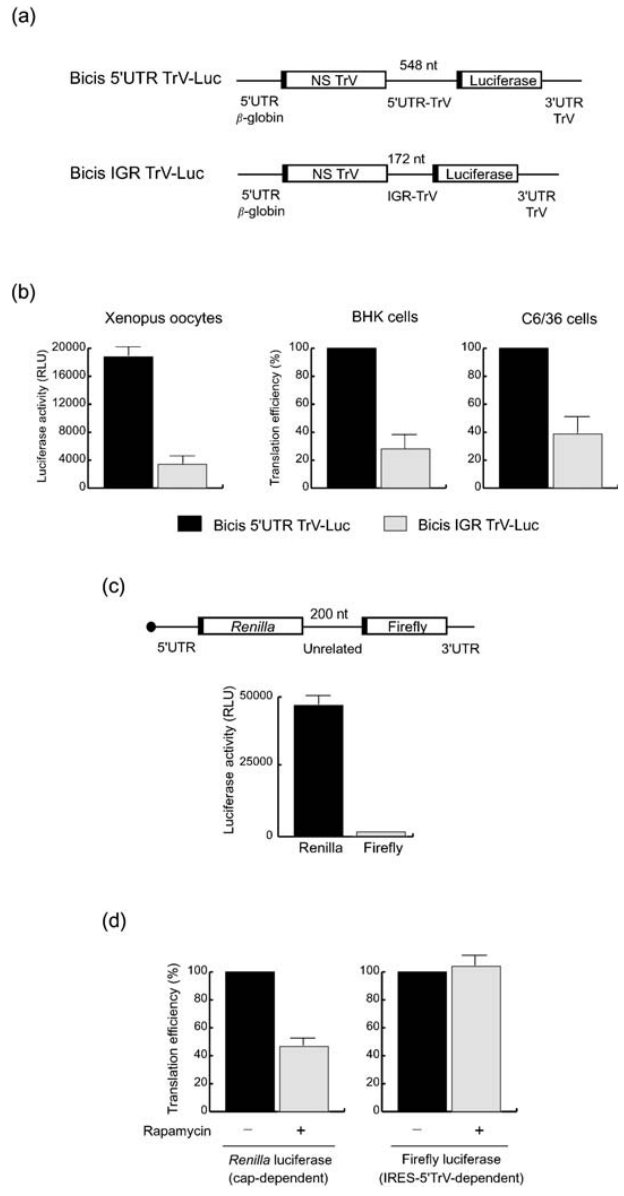

Fig. 2. Efficient translation initiation mediated by TrV 5′UTR or IGR in bicistronic constructs. (a) Schematic representation of bicistronic RNA constructs carrying the 5′UTR or IGR of TrV as intergenic regions preceding the firefly luciferase coding sequence. (b) Internal initiation of translation mediated by the 5′UTR and IGR of TrV. The Bicis 5′UTR TrV-Luc and Bicis IGR TrV-Luc RNAs were microinjected into *Xenopus* oocytes or transfected into BHK and C6/36 cells as indicated. Translation efficiency of microinjected RNAs was determined by firefly luciferase activity expressed in relative light units (RLU). Translation levels of transfected RNAs were determined by firefly luciferase activity normalized by *Renilla* luciferase activity and expressed as a percentage of the levels of 5′UTR TrV-Luc RNA. (c) Schematic representation of a bicistronic RNA construct encoding *Renilla* luciferase in the first ORF followed by an unrelated sequence of 200 nt in the IGR and the firefly luciferase-coding sequence. Translation efficiency of the two luciferases in BHK cells at 6 h post-transfection is expressed in RLU. (d) Translation efficiency of a capped RNA encoding *Renilla* luciferase and firefly luciferase in the second ORF under the control of the TrV 5′IRES, in the presence or absence of rapamycin. Luciferase activities in the presence of rapamycin are expressed as a percentage of the luciferase produced in untreated cells.

Xenopus oocytes or transfected into BHK and C6/36 cells as described above. Luciferase activity was observed with both RNAs in all cell types used, confirming that the 5′UTR and IGR of TrV can direct internal entry of ribosomes (Fig. 2b). To determine the background levels of translation of the second cistron due to leaky scanning, we constructed a bicistronic RNA control carrying an unrelated sequence of 200 nt in the intergenic region preceding a firefly luciferase-coding sequence. This bicistronic construct was capped and encoded *Renilla* luciferase in the first ORF. Analysis of the translation efficiency of both luciferases in transfected BHK cells showed efficient translation only from the first ORF (Fig. 2c).

In addition, we examined the translation efficiency mediated by the cap and the 5′ TrV IRES in the presence of rapamycin, which inhibits translation initiation dependent on IF4E (Beretta *et al.*, 1996). To this end, we incubated BHK cells with 20 ng rapamycin ml^{-1} in Opti-MEM medium (Invitrogen) or with control medium, and the RNA was transfected 1 h after treatment. Firefly and *Renilla* luciferase activities were measured at 6 h post-transfection. As shown in Fig. 2(d), translation of firefly luciferase mediated by the TrV 5′UTR was unaffected by rapamycin, while the levels of *Renilla* luciferase were reduced to about 50 %.

It has been reported that mutation of the initiator CCU in CrPV impairs IGR-IRES function, which is in agreement with the proposed formation of a pseudoknot structure during the initiation process (Wilson *et al.*, 2000). In contrast, the IGR-IRES of PSIV tolerates mutations in the initiator triplet CUU (Shibuya *et al.*, 2003). These observations indicate that, even though many similarities exist in the mechanism of initiation mediated by the IGR of different dicistroviruses, there are some features that are different among them. To examine the requirements of the TrV IGR-IRES, we mutated the initiator codon CUC to AUG or CCU in the bicistronic RNA constructs. Translation of the three RNAs with different initiator triplets was very efficient (data not shown), suggesting that the initiation site is flexible during translation mediated by the TrV IGR-IRES, resembling the initiation of PSIV.

We observed that translation efficiency of the RNAs carrying the TrV IGR directing initiation of a second cistron, which resembles the natural position in the viral genome, was consistently three to fivefold less that that observed with the 5′UTR IRES (Fig. 2b). These observations are intriguing, as it has been noted that the capsid proteins (ORF2) are produced in large excess over the non-structural proteins in cells infected with insect picorna-like viruses (Moore *et al.*, 1981), suggesting that the IRES activity present in the IGR should be more efficient than the IRES located at the viral 5′UTR. It is possible that changes in the cellular translation machinery during viral infection could result in a differential modulation of the two IRESs. Indeed, it has previously been reported that stress or direct phosphorylation of initiation factor IF2-α, conditions likely to occur during viral infection, enhances translation mediated by the CrPV IGR-IRES (Fernandez *et al.*, 2002). It has been postulated that translation initiation by the IGR-IRES independently of the IF-2–GTP–tRNAi complex could explain the advantage of translation of RNAs with this IRES over translation of other mRNAs in conditions with low active IF2-α (Fernandez *et al.*, 2002; Thompson *et al.*, 2001).

In order to examine whether the IRES activities present in the TrV genome were differentially modulated by an 'antiviral state' of the cell, we analysed the translation efficiency of the two IRESs in BHK cells pre-treated with IFN-α, which is known to phosphorylate IF2-α (reviewed by Katze *et al.*, 2002). For these experiments, we constructed a new bicistronic mRNA mimicking the genomic organization of TrV. The RNA contained: (i) the 5′UTR of TrV followed by the first ORF encoding firefly luciferase; (ii) the IGR-IRES of TrV followed by a second ORF encoding *Renilla* luciferase; and (iii) the 3′UTR of TrV (5′UTR-Fluc-IGR-Rluc RNA; Fig. 3a). BHK cells were treated with IFN-α (1000 IU per 35 mm culture plate) for 24 h before transfection with the 5′UTR-Fluc-IGR-Rluc RNA. The luciferase activities obtained in the untreated control cells were arbitrarily set to 100 % and the translation of the respective RNA in the treated cells was expressed relative to the controls. A large decrease in firefly luciferase activity in treated cells indicated that translation mediated by the 5′UTR of TrV was strongly inhibited under these conditions (Fig. 3b). In contrast, *Renilla* luciferase activity was higher in the treated cells, suggesting that translation by the IGR-IRES was not reduced by IFN-α (Fig. 3b). These results indicated that the relative translation efficiency of the two IRESs drastically changes, resulting in a sixfold increase in IGR-IRES translation upon IFN-α treatment.

To analyse further the possible differential modulation of the two TrV IRESs, we used insect cells. Because primary cultured cells obtained from *T. infestans* tissues were difficult to transfect with RNA, we used mosquito cells under conditions in which the host antiviral responses were activated. To this end, we examined the translation of 5′UTR-Fluc-IGR-Rluc RNA in C6/36 cells previously infected or not with DV. Cells were infected with DV type 2 strain 16681 (m.o.i. of 100) or mock infected and incubated at 28 °C. RNA transfection was performed at 48 h post-infection. By this time, the complete monolayer was infected, as determined by immunofluorescence using antibodies against DV antigens (data not shown). In the infected cells, firefly luciferase activity (5′UTR-IRES) decreased threefold compared with mock infections, while *Renilla* luciferase activity (IGR-IRES) increased by 30 % under the same conditions, suggesting that, in insect cells, the two IRESs are also differentially modulated under conditions in which general translation could be compromised.

Taken together our results confirm that TrV translation is mediated by two different IRESs. Translation activities of both IRESs were detected in different cell types, even in *Xenopus* oocytes, suggesting a minimum requirement of

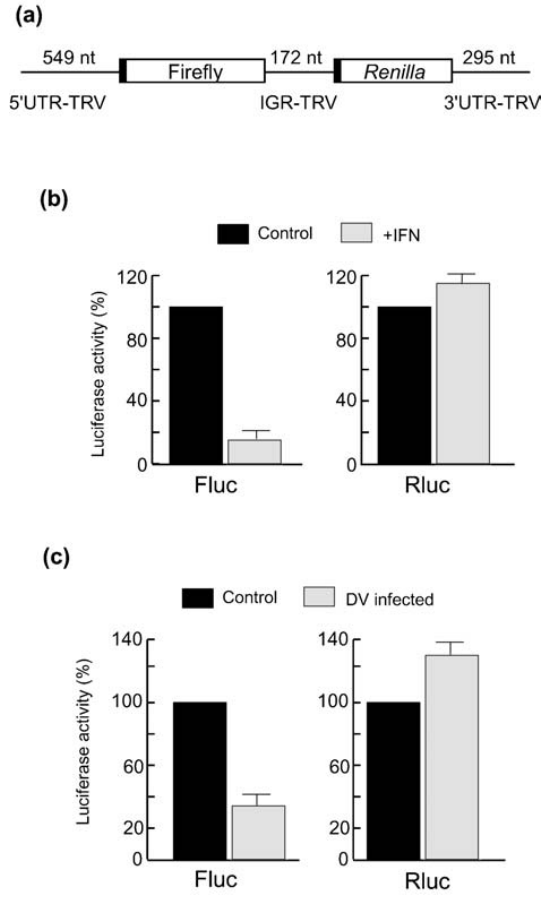

Fig. 3. Differential effect of IFN-α and viral infection on translation mediated by the two TrV IRESs. (a) Schematic representation of the dual luciferase RNA constructs. The ORFs encoding firefly and *Renilla* luciferases, respectively, are indicated by boxes. The TrV 5′- and 3′-UTRs and IGR are indicated. (b) Effect of IFN-α on translation mediated by the TrV IRESs. BHK cells were treated or not with IFN-α and transfected with the RNA represented in (a). The firefly (Fluc) and *Renilla* (Rluc) luciferase activities were used as an indication of the 5′UTR and IGR-IRES activities, respectively. Translation levels in treated cells were expressed relative to levels in untreated control cells, which were arbitrarily set at 100 %. (c) Effect of DV infection on translation mediated by the TrV IRESs. Mosquito C6/36 cells were infected or not with DV and transfected 48 h after infection with the bicistronic RNA represented in (a). The Fluc and Rluc luciferase activities were used as an indication of the 5′UTR and IGR-IRES activities, respectively. Translation levels in infected cells were expressed relative to the levels in the uninfected cells, which was arbitrarily set at 100 %.

host factors. Furthermore, the translation efficiency of the two IRESs was differently modulated under conditions that resemble virus infection, providing a mechanism to control the relative amounts of structural and non-structural viral proteins during replication.

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