Polycistronic mRNAs and internal ribosome entry site elements (IRES) are widely used by white spot syndrome virus (WSSV) structural protein genes

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Introduction

While the eukaryotes can use either cap-dependent or cap-independent translation mechanisms, most cellular mRNAs utilize the cap-dependent mechanism for protein translation. In this mechanism, translation initiation factors recognize the 5′ cap structure (m7GpppN) first, and then the 43S pre-initiation complex is recruited (Kozak, 1978; Pestova et al., 1998). Next, the 43S pre-initiation complex scans along the mRNA to locate the first favorable initiation codon (usually ATG), with which it forms a 48S complex. The subsequent addition of a 60S ribosomal subunit leads to the formation of an 80S initiation complex, and this begins the translation.

Cap-independent translation was first discovered at an internal ribosome entry site (IRES) of poliovirus, where an RNA secondary structure in the 5′ untranslated region (5′ UTR) mediated internal translation without a 5′ cap structure (Pelletier and Sonenberg, 1988). Many IRES elements have since been identified in other positive-sense RNA viruses, such as hepatitis C virus (Tsukiyama-Kohara et al., 1992) and encephalomyocarditis virus (Jang et al., 1988), as well as in cellular mRNAs that are involved in the cell cycle or cell survival (http://www.rangueil.inserm.fr/iresdatabase). In the DNA viruses, the first IRES was discovered in Kaposi's sarcoma-associated Herpesvirus (KSHV), where a novel IRES element is located in the coding region of vCyclin (Bielecki and Talbot, 2001; Grundhoff and Ganem, 2001; Low et al., 2001). Since then, only a few IRESs have been identified in DNA viral genomes. These include the IRES in the thymidine kinase gene of herpes simplex virus (HSV; Griffiths and Coen, 2005), the IRES upstream of the vp31 coding region, and this region was able to promote the expression of a downstream firefly luciferase reporter. We show that vp31/vp39b/vp11 is representative of many other WSSV structural/non-structural gene clusters, and argue that these are also likely to produce polycistronic mRNAs and that use an IRES mechanism to regulate their translation.

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The genome of the white spot syndrome virus (WSSV) Taiwan isolate has many structural and non-structural genes that are arranged in clusters. Screening with Northern blots showed that at least four of these clusters produce polycistronic mRNA, and one of these (vp31/vp39b/vp11) was studied in detail. The vp31/vp39b/vp11 cluster produces two transcripts, including a large 3.4-kb polycistronic transcript of all three genes. No monocistronic vp39b mRNA was detected. TNT and in vitro translation assays showed that vp39b translation was independent of vp31 translation, and that ribosomal reinitiation was not a possible mechanism for vp39b. An unusually located IRES (internal ribosome entry site) element was identified in the vp31/vp39b coding region, and this region was able to promote the expression of a downstream firefly luciferase reporter. We show that vp31/vp39b/vp11 is representative of many other WSSV structural/non-structural gene clusters, and argue that these are also likely to produce polycistronic mRNAs and that use an IRES mechanism to regulate their translation.

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Furthermore, in some gene clusters such as \(\text{vp31/vp39b/vp11}\), \(\text{vp22/vp39a/wssv363/vp51c}\), and \(\text{vp60b/wssv478/wssv479/vp28}\), the polyadenylation signal is only found within the ORF or in the 3′ UTR of the last gene, while the preceding genes have no poly(A) signal within their ORF or 3′ UTR. This suggests that these clustered genes may share a long transcript with the same poly(A) tail (Tsai et al., 2004).

Previous studies have found that polycistronic mRNAs were transcribed from clusters of WSSV genes. For example: two transcripts, a polycistronic mRNA of about 5.5 kb and a monocistronic mRNA of about 1.3 kb, were detected using a \(\text{vp35}\)-specific riboprobe (Chen et al., 2002); two transcripts, a polycistronic mRNA of about 4.5 kb and a monocistronic mRNA of about 1.4 kb, were detected from the \(\text{wssv237/wssv240/wssv243}\) cluster using a \(\text{rr2}\)-specific riboprobe (Tsai et al., 2000); and two transcripts, a polycistronic mRNA of about 5.7 kb and a monocistronic mRNA of about 2.7 kb, were detected using a \(\text{protein kinase 1}\)-specific riboprobe (Liu et al., 2001). In addition, an IRES element in the 5′ UTR of \(\text{vp28}\) was discovered by Han and Zhang (2006). In the present study, we used Northern blot analyses to show that polycistronic mRNA was being transcribed from the \(\text{vp60b/wssv478/wssv479/vp28}\) cluster and from three other clusters, \(\text{vp31/vp39b/vp11}\), \(\text{wssv130/wssv133/vp36a}\), and \(\text{vp36b/vp51b/vp38a}\). To further investigate the gene expression mechanism of one of these gene clusters, \(\text{vp31/vp39b/vp11}\), we first used 5′/3′ RACE and found that two transcripts produced by this gene cluster were a 3.4-kb \(\text{vp31/39b/vp11}\) polycistronic transcript and a 1.6-kb monocistronic \(\text{vp11}\) transcript. Next we used coupled in vitro transcription-translation (TNT) assays, in vitro translation assays and dual luciferase activity assays to demonstrate that the expression of \(\text{vp39b}\) from the 3.4 kb transcript is regulated by an IRES element located in the coding region of \(\text{vp31/39b}\). Our results suggest that IRES elements are involved in the expression of WSSV structural genes, and further, that polycistronic mRNAs are widely used to regulate the transcriptional mechanism of other clusters of WSSV structural and non-structural genes.

Results

Polycistronic mRNAs are transcribed by WSSV gene clusters

Fig. 1 shows that polycistronic transcripts were produced by all four of the WSSV gene clusters investigated in this study. Using the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig1.png}
\caption{Polycistronic mRNAs were transcribed from WSSV gene clusters. (A) Schematic diagrams of the WSSV gene clusters \(\text{vp60b/wssv478/wssv479/vp28}\), \(\text{wssv130/wssv133/vp36a}\), \(\text{vp36b/vp51b/vp38a}\), and \(\text{vp31/vp39b/vp11}\). The gene-specific riboprobes used for Northern blot analysis are indicated by solid lines. (B) Northern blot analysis of total RNA isolated from the pleopods or gills of healthy (0 hpi) and WSSV-infected \(\text{P. monodon}\) (60 hpi) using \(\text{vp60b/vp28/vp36a/vp38a/vp31/vp39b/vp11}\)-specific riboprobes. For the first 3 clusters, about 20 μg of total RNA was isolated at 0 and 60 hpi, electrophoresed in the presence of formaldehyde, transferred onto Hybond N⁺ nylon membrane and probed with the DIG-labeled gene-specific probes. For the \(\text{vp31/vp39b/vp11}\) cluster, about 30 μg of total RNA was isolated at 0 and 60 hpi, electrophoresed in the presence of formaldehyde, transferred onto Hybond N⁺ nylon membrane and probed with the 35S-labeled gene-specific probes. The size standards are based on RNA markers (Amersham Pharmacia Biotech, USA or Promega). The arrows indicate the monocistronic, bicistronic, and polycistronic mRNA transcripts. The 18S rRNA is also indicated (arrowhead).}
\end{figure}
specific riboprobes (Fig. 1A). Northern blot analyses (Fig. 1B) showed that the vp60b/wssv478/wssv479/vp28 cluster transcribed: a ~3.4 kb polycistronic mRNA that encoded the vp60b and vp28 proteins (as verified by 5'/3' RACE analyses; data not shown); a ~1.3 kb bicistronic mRNA that included wssv479 and vp28; and a ~1.0 kb monocistronic mRNA containing only vp28. From the wssv130/wssv133/vp36a cluster, three mRNA transcripts were detected: a ~3.2 kb polycistronic mRNA containing all three ORFs; a ~1.8 kb bicistronic mRNA containing wssv133 and vp36a; and a ~1.1 kb monocistronic mRNA containing only the vp36a gene. The vp36b/vp51b/vp38a gene cluster transcribed a ~3.3 kb polycistronic mRNA containing all three genes and a ~1.1 kb monocistronic mRNA containing only vp38a.

When transcripts from the vp31/vp39b/vp11 cluster were detected with the vp31-, vp39b-, and vp11-specific riboprobes, all three probes detected a 3.4-kb band, while the vp11 probe also hybridized another band of 1.6 kb (Fig. 1B). These results suggest that vp39b is only being transcribed as a polycistronic mRNA, in which case the VP39B protein would have to be translated by a cap-independent mechanism. This made the vp31/vp39b/vp11 cluster a good subject for further investigation. As a first step, we next used 5'/3' RACE to determine the transcriptional units of vp31, vp39b and vp11.

The wssv396/wssv395/wssv394 gene cluster produces a vp31/vp39b/vp11 polycistronic transcript and a vp11 monocistronic transcript

The sequencing results showed that both vp31 and vp39b had the same two transcription initiation sites (TIS), and that these were located 32 and 35 nt upstream of the translational start codon of vp31 (GTG [the bold and underlined nucleotides are the initiation sites; Fig. 2B]). vp11 had only one TIS located 205 nucleotides upstream of its translational start codon (TAG; Fig. 2B). The results of 3' RACE showed that all three genes had the same poly(A) addition site, which was 17 nt downstream of the vp11 polyadenylation signal (Fig. 2B).

Based on these results, we concluded that this gene cluster transcribes two mRNAs with different 5' ends but with the same 3' ends. One transcript is a large, 3.4-kb mRNA encoding all three proteins and the other is a small, 1.6-kb mRNA encoding only VP11.

VP31, VP39B and VP11 are envelope proteins

Our previous proteomic study of purified WSSV virions (Tsai et al., 2004) suggested that the genes of VP31, VP39B and VP11 are viral structural proteins. To confirm this, the respective recombinant proteins were successfully expressed in E. coli, the corresponding antibodies were obtained, and Western blotting was used to detect the proteins in the envelope and nucleocapsid viral fractions. All three proteins were located in the envelope fraction (Fig. 3).

The Western blots also revealed the apparent molecular masses of the proteins. The apparent molecular masses of VP31 and VP39B (31 and 39 kDa, respectively) were in good agreement with their calculated molecular masses and also matched well with the previous proteomic results (Tsai et al., 2004). However, while the predicted molecular mass of VP11 is 48 kDa, the gene product of wssv394 was identified as an 11 kDa protein in the proteomic study (Tsai et al., 2004), and VP11 was detected here as a 38 kDa protein (Fig. 3). One possible explanation for these discrepancies is that wssv394 does in fact encode a 48 kDa protein, which, after synthesis, is then processed or degraded to the 38 and 11 kDa proteins. In support of this, several candidate proteolytic cleavage sites were predicted using Peptidecutter and other programs (data not shown, Table 1).
However, if this explanation is correct, then the 48 kDa protein must be very unstable, since it was never detected in the virions by Western blot. However, further study will be needed to test this hypothesis.

VP39B may be translated through an IRES-mediated mechanism

Theoretically, while VP31 and VP11 could both be translated from the corresponding 3.4- and 1.6-kb mRNAs using a cap-dependent mechanism, VP39B can only be translated from the 3.4 kb mRNA through a cap-independent mechanism. It is also possible that VP11 could be translated from the polycistronic 3.4 kb mRNA, but that possibility was not investigated here. To experimentally confirm that VP39B is being expressed via a cap-independent translational mechanism, a bicistronic expression construct pcDNA3-HA-VP31/VP39B (Fig. 4A) was assayed by coupled in vitro transcription-translation assay. As shown in Fig. 4B, lane 1, this bicistronic construct expressed both proteins, and the signal intensity of VP31 was stronger than that of VP39B. This result confirmed that the translation of VP39B was cap-independent, and since the assay was performed in rabbit reticulocyte lysate (RRL), it

Fig. 3. Western blot analyses of VP31 VP39B and VP11. VP31, VP39B and VP11 were all detected in the purified total WSSV virions and in the envelope fraction. The nucleocapsid protein VP51C (Tsai et al., 2004) was used as a fractionation quality control and was detected only in the total virions and the nucleocapsid fraction. Non-specific signals from the major envelope protein VP28 were also detected by the VP31, VP39B and VP11 antibodies.

Fig. 4. Coupled in vitro transcription-translation of frame-shifted and truncated vp31/vp39b constructs. (A) Two mutant forms of the pcDNA3-HA-vp31/vp39b bicistronic plasmid were constructed. In the frame shift mutant, vp31-F/vp39b, a base from the second codon of vp31 was deleted to generate a 20-codon nonsense open reading frame. In the truncation mutant, vp31-T/vp39b, codon 164 of vp31 was mutated to a stop. (B) Translation products from the indicated constructs. Size standards were determined by protein prestained marker (Bio-Rad).
also showed that the translation required no other viral or shrimp host proteins.

To further explore the cap-independent translational mechanism of vp39b, we next tested whether the expression of vp39b depends on the expression of vp31. To this end, the vp31-F/vp39b frame shift mutation construct (Fig. 4A) was subjected to the TNT assay. Our results showed that this frame-shifted construct still expressed VP39B (Fig. 4B, lane 2), from which we concluded that the expression of vp39b did not require the expression of vp31.

Our next assay was to determine which of the two kinds of cap-independent translational mechanism was being used in the expression of VP39B. One mechanism is translational reinitiation, which usually requires an optimal intergenic region of no more than 80 bp and is usually associated with a short upstream ORF (up to 30 codons) (Kozak, 1987). Although the vp31 gene has 262 codons, there are other known instances of large upstream viral genes that use translational reinitiation to regulate the expression of a downstream ORF (e.g., translational reinitiation is used to transcribe the gene immediately downstream of the feline calicivirus ORF2 gene, which encodes a ∼75 kDa precursor of the VP1 protein; Poyry et al., 2007). Therefore, to test if vp39b translation occurs through reinitiation, codon 164 of vp31 was mutated to become a stop codon in the vp31-T/vp39b construct (Fig. 4A). This mutation not only expressed a truncated VP31 protein but also increased the intergenic distance between vp31 and vp39b from 3 bp to 297 bp. In the TNT assay this construct successfully expressed the VP39b protein (Fig. 4B, lane 3).

In both the vp31-F/vp39b and vp31-T/vp39b constructs, the intergenic distance between the mutated vp31 and vp39b was far greater than 80 bp. With such a large intergenic region, we infer that the translation of vp39b could not have occurred through translational reinitiation. From the data presented here, we therefore conclude that the expression of vp39b must be effected by IRES-mediated internal translation.

The vp31/vp39b coding region is able to regulate firefly luciferase translation in vitro

To further substantiate whether there is a functional IRES element within the vp31/vp39b sequence, a partial fragment (nt 123 to 919) of the vp31/vp39b coding region (Fig. 5A) was cloned into the upstream region of the firefly luciferase gene in the T7/pRL-vector (Fig. 5). The vp31/vp39b coding region exhibits IRES activity in vitro. (A) Location of the putative IRES element in the vp31/vp39b coding region. (B) Schematic diagrams of the dicistronic constructs of T7/pRL-FL and T7/SL-pRL-FL (SL: stem–loop). The stable stem–loop structure with a free energy of −62 kcal/mol was introduced upstream of the Renilla ORF in the vector T7/SL-pRL-FL. (C) (D) After in vitro synthesis with T7 RNA polymerase, the indicated uncapped dicistronic transcripts were translated in Flexi RRL (Promega).
FL plasmid such that the ATG start codon of vp39b was fused in-frame with the downstream firefly luciferase. The resultant plasmid was designated T7/IRES123–919 (Fig. 5B). As a negative control, the same partial fragment of the vp31/vp39b coding region was inserted in reverse orientation to generate T7/IRES919–123. We also constructed a positive control plasmid T7/vp28–IRES that contained the recently identified IRES element from the WSSV vp28 gene (Han and Zhang, 2006). The dicistronic transcripts produced by these constructs were then translated in vitro. Fig. 5C shows that the IRES activity of IRES123–919 (lane 1) was weaker than that of the empty vector (lane 4), and it was only slightly higher than the IRES activity of the negative control (IRES919–123; lane 2).

However, when a thermodynamically stable stem–loop (SL) was inserted upstream of the RL ORF to block 5′ ribosomal scanning, we found that whereas the upstream RL translation was substantially reduced for all four dicistronic transcripts (cf Figs. 5C and D), the downstream FL translation of the T7/SL–IRES123–919 dicistronic transcript was now much greater than that of the other dicistronic transcripts (Fig. 5D). Because the stem–loop blocked ribosomal scanning and greatly reduced the possibility of translational reinitiation, we conclude that the increased expression of FL from the bicistronic transcript of T7/IRES123 must have been regulated by an IRES element. That is, in this assay, FL translation from the T7/SL–IRES123–919 dicistronic transcript occurred independently of RL translation and it was IRES-dependent.

The IRES element is functional in Sf9 cells

To test whether the IRES123–919 fragment was functional in Sf9 cells, it was inserted into the upstream region of the firefly luciferase gene in a dual-luciferase reporter plasmid driven by the WSSV ie1 promoter (Fig. 6). This reporter construct was transfected into Sf9 cells, and 48 h later, the transfected cells were harvested and assayed for Renilla luciferase and firefly luciferase activities. The ratio of firefly luciferase activity to Renilla luciferase activity was used to represent the IRES activity.

As shown in Fig. 6, the relative luciferase activity of the ie1/IRES123–919 construct was ∼1.7 times higher than that of the positive control (ie1/vp28–IRES) and ∼43 times higher than that of the empty plasmid (ie1/pRL-FL). The plasmid with the reversed vp31/vp39b fragment, ie1/IRES919–123, showed only a low level of activity. These results indicated that an IRES element was indeed located within the vp31/vp39b region and its IRES function was directionally dependent.

The transcript from the ie1/IRES123–919 bicistronic plasmid does not undergo splicing

It is possible that the firefly luciferase activity from the ie1/IRES123–919 bicistronic plasmid was due to splicing events that generated an abnormal monocistronic FL transcript. To exclude this possibility, total RNAs were isolated from ie1/IRES123–919-transfected (T) or untransfected (UT) Sf9 cells and subjected to RT-PCR analysis with two primer sets that amplified overlapping fragments of the entire transcript (Fig. 7A). The results (Fig. 7B) showed that only one RT-PCR product was amplified by each primer set (P1/P2 lane 2; P3/P4 lane 2), and in both cases, the product had the expected size as verified by the plasmid DNA templates (lane 4). Sequence analysis of these two bands also showed the presence of full-length bicistronic RNA and no smaller RNA species. We therefore conclude that when the ie1/IRES123–919 bicistronic plasmid is transfected into Sf9 cells, no RNA splicing events occur.

Discussion

The evidence presented here shows that the structural protein gene cluster vp31/vp39b/vp11 transcribes a 3.4 kb polycistronic mRNA and uses an IRES element to regulate the expression of vp39b. Northern blot and 5′/3′ RACE analyses of tissues from WSSV-infected shrimp (Figs. 1 and 2) detected only a single species of 3.4 kb tricistronic mRNA and no monocistronic vp39b mRNA. Since Fig. 3 shows that the VP39B protein was expressed in vivo, a cap-independent mechanism must therefore have been used to translate the VP39B protein from the tricistronic mRNA. After eliminating the possibility of ribosomal reinitiation (Figs. 4 and 5), taking all these data together, we conclude that the expression of VP39B protein is regulated in vivo by an internal ribosome entry site (IRES). The RRL and Sf9 experiments further showed that the IRES element was located in the IRES123–919 fragment (Figs. 5 and 6). There are several observations to be made on these experiments. First, we note that IRES activity was low both in the Sf9 cells (64.3 ± 12.9%) and in the RRL compared to the cap-dependent expression of FL (Figs. 5 and 6). This may be because these systems lack the specific IRES trans-acting factors (ITAFs). It is likely that Sf9 insect cells lack some of the ITAFs that are present in shrimp cells, while RRL has been shown to lack ITAFs that are necessary for IRES-dependent translation initiation (Byrd et al., 2005; Pudi et al., 2003). It is also possible that the fusion of the N-terminal region of vp39b to FL in the plasmids might have interfered with the translational efficiency of FL and/or affected its activity.
Secondly, we observed that when the stem–loop plasmids were used to rule out the possibility of translational reinitiation, the reduction of the upstream RL translation enhanced downstream translation of the FL (cf. Figs. 5C and D). To account for this, we hypothesize that in the absence of any competition with a cap-dependent mechanism, more free ribosomes would be available to load onto the IRES element to promote translational efficiency. Lastly, we note that translation of the positive control transcript (vp28-IRES) was much higher in the Sf9 cells than when it was translated from the stem–loop T7/SL-vp28-IRES plasmid in the RRL system (cf. Figs. 5D and 6). Although we do not know the reason for this, similar discrepancies have been reported for other IRES elements under different experimental conditions (Collier et al., 1998; Creancier et al., 2000; Hennecke et al., 2001; Kamoshita et al., 1997; Woolaway et al., 2001).

Although most IRES elements are located within the 5′ UTR or intergenic region of the mRNA, the IRES element that we identified here is located in the vp31/vp39 coding region. This is unusual, and to date, only a few IRESs have been found within the coding sequences of DNA or RNA viruses. These include an IRES located in the vCyclin ORF of KSHV (Bieselski and Talbot, 2001; Low et al., 2001), an IRES located in the VP2 ORF of SV40 (Yu and Alwine, 2006), and an IRES in the gag coding region of human immunodeficiency virus type 1 (Buck et al., 2001). Collectively, these IRES elements might represent a novel type of IRES, but their regulating mechanism remains unknown. Further study will be needed to discover how these elements can function as IRESs while still being part of the translatable coding region.

Our Northern blot analysis of the vp60b/wssv478/wssv479/vp28 gene cluster detected at least three transcripts (~3.4 kb, 1.3 kb, 1.0 kb) using the vp28-specific probe (Fig. 1B). Analysis of the 3.4 kb polycistronic RNA by 5′/3′ RACE revealed that vp60b and vp28 were both present and that they shared the same TIS and poly(A) tail. Marks et al. (2003) found that the TIS of vp28 was located only 33 nt upstream of the transcription start codon (TAAC), which almost matches our unpublished TIS data (TAACC) for the 1.0 kb monocistronic vp28 mRNA. By contrast, Zhang et al. (2002) found that the TIS of vp28 from a cDNA clone was located 482 nt upstream of the transcription start codon. This is consistent with the 1.3 kb wssv479/vp28 bicistronic mRNA detected in Fig. 1B. Using the same cDNA clone, Han and Zhang (2006) were subsequently the first to identify a 180 bp IRES element in the 5′ UTR of vp28, and infer that it was downstream of an in-frame minicistron of about 5 kDa. Taking all these data together with our Northern blot results, we conclude that there are at least three TISs for vp28, one in the 5′ UTR of the vp60b/wssv478/wssv479/vp28 polycistronic mRNA, one in the 5′ UTR of the wssv479/vp28 bicistronic mRNA, and one in the 5′ UTR of the monocistronic vp28 mRNA.

Analysis of the WSSV genome reveals many structural and non-structural genes that occur in clusters. In addition to the four clusters studied here, i.e. vp60b/−/wssv478/−/wssv479/−/vp28/+, wssv130/+ /wssv133/−/vp36a/+, wssv36a/+ /vp36b/+, vp31/−/vp39b/−/vp11/+, these include vp22/−/vp39a/−/wssv363/−/vp51c/+, vp41a/−/vp51a/+ /vp41b/−/wssv299/+, wssv237/−/wssv240/+ /wssv243/+ /wssv285/+ /wssv287/−/wssv288/+ , and wssv038/−/wssv035/−/wssv033/+, where the plus sign indicates a poly(A) signal near the stop codon of the gene. We have shown that the four gene clusters studied here all transcribe a polycistronic mRNA with a shared poly(A) tail (Fig. 1B), and we hypothesize that this is also probably true for most of the other gene clusters. This is especially likely for clusters such as vp22/vp39a/wssv363/vp51c, where the polyadenylation signal can only be identified within the ORF or the 3′ UTR of the last gene. Further, as shown by the present paper for vp39b, and by Han and Zhang (2006) for vp28, IRES elements are used to regulate the translation of these two polycistronic miRNAs, and it seems likely that an IRES mechanism is also being used to regulate translation of the polycistronic miRNAs transcribed from many of these other WSSV structural/non-structural gene clusters.

Although the significance of an IRES mechanism in WSSV gene expression is presently unknown, Gale et al. (2000) point out that a translational mechanism like IRES can be used to ensure that structural proteins can still be expressed even under unfavorable conditions, such...
as when virus-induced host shutoff decreases cap-dependent translation efficiency. For example, some RNA viruses (e.g., Picornavirus) use an IRES element in cap-independent translational mechanism to enable selective translation of viral mRNA during host shutoff (Belsham and Sonenberg, 1996; Gale et al., 2000). Other cap-independent mechanisms can also be used to achieve similar results. For example, Human papillomavirus transcripts, which are almost all bicistronic or polycistronic mRNAs, use translational control mechanisms such as leaky scanning or translational reinitiation to translate downstream ORFs (Zheng and Baker, 2006). In the case of WSSV, we hypothesize that IRES elements are being used to ensure the translation of important viral structural proteins (including vp28 and vp39b), which in turn would allow the virion to be successfully assembled. This hypothesis will need to be further explored in future studies.

Materials and methods

Virus purification of intact WSSV virions and fractionation of virus proteins

The virus used in this study, WSSV Taiwan isolate WSSV-TW (Lo et al., 1999), was originally isolated from a batch of WSSV-infected Penaeus monodon shrimp collected in Taiwan in 1994 (Wang et al., 1995). For purification and fractionation of intact WSSV virions from crayfish and for the fractionation of virus proteins into envelope and capsid proteins, we followed the methods described by Xie et al. (2006).

WSSV infection of P. monodon

Adult P. monodon (30 to 40 g) were collected from a culture pond in Tung Kang. The shrimps used for the challenge test were checked with a commercial WSSV diagnostic kit (IQ2000™, IntelliGene) and confirmed to be WSSV-free. Experimental shrimps were infected with WSSV by injection between the fourth and fifth abdominal segments using a method described previously (Tsai et al., 1999). Immediately before injection and at 60 h post injection (hpi), the pleopods, lymphoid organs, and gills of two randomly selected shrimps were excised, immediately frozen in liquid nitrogen and then stored in liquid nitrogen until used.

Transcriptional analysis of WSSV genes by Northern blot

About 20 μg of total RNA isolated from the pleopods or gills of WSSV-infected P. monodon was subjected to electrophoresis in 1% formaldehyde gel. RNA was transferred onto positively charged nylon membranes (Roche Applied Science) with 20x SSC overnight, and detected by digoxigenin (DIG)-labeled RNA probes (DIG System Users Guide from Roche Applied Science). The vp60b, vp28, vp36a and vp38a fragments used for the DIG-labeled probes were amplified from WSSV genomic DNA by PCR with primer sets vp60b-F/vp60b-R, vp28-F/vp28-R, vp36a-F/vp36a-R and vp38a-F/vp38a-R respectively (Table 1). The amplified fragments (10 to 25 ng) were ligated with T7 adaptor (5’-CATTAAAGCAGCTGCTTATCGAATATACGCTACTATGAGGAGA-3’) using T4 DNA ligase.

Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Usage</th>
</tr>
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<tbody>
<tr>
<td>vp31-EcoR1-F</td>
<td>5’-GAATTCGGATCCCTTACTGATGGCCAAAC-3’</td>
<td>Antibody preparation</td>
</tr>
<tr>
<td>vp31-NdeI-R</td>
<td>5’-AAGCTCTGATGCTATTGAAAAGCTGAAGC-3’</td>
<td>Antibody preparation</td>
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<td>vp39b-BamHI-F</td>
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<td>vp39b-EcoR1-R</td>
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<td>primers used in this study</td>
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ligase (Promega). The ligation products (1 μl) were subjected to PCR amplification by gene-specific forward primer (vp60b-F for vp60b, vp28-F for vp28, vp36a-F for vp36a and vp38a-F for vp38a) and T7 adaptor primer 1 (‘-CATTATACGACCTGAGCTATGAAATT-3’) to generate probes with the T7 promoter. DIG-labeled antisense RNA probes were then made by in vitro transcription at 37 °C for 2 h with 2 μl of 5× DIG-RNA labeling mix (Roche Applied Science), 40 U of T7 RNA polymerase (Promega), 4 μl of 5× transcription buffer and 100–200 ng of the corresponding gene-specific probe to a final volume of 20 μl. The resulting DIG-labeled RNA probes were treated with 2 μl of DNase I (RNase-free; Invitrogen) at RT for 15 min. This was followed by DNase I inactivation by the addition of 2 μl 25 mM EDTA and incubation at 65 °C for 10 min to stop the reaction. The DIG-labeled RNA probes were denatured by heating at 95 °C for 5 min and cooling quickly on ice for 1 min, and then mixed with preheated (68 °C) hybridization buffer (50% formamide [v/v] deionized, 5× SSC, 0.1% N-lauroylsarcosine [w/v], 0.02% SDS [w/v], 2% blocking solution [1/5 volume of 10× blocking solution]). Hybridization and detection were performed as described in the DIG System Users Guide from Roche Applied Science. After 16 h hybridization at 68 °C, the membranes were washed twice with 2× SSC/0.1% SDS at RT for 5 min, and then washed twice with 0.1× SSC/0.1% SDS at 68 °C for 15 min. The membranes were incubated with anti-digoxigenin-AP, Fab fragment (1:20,000 dilution) (Roche Applied Science) for 1 h at room temperature, and then exposed to X-ray film at RT for 30 min.

About 30 μg of total RNA isolated from the pleopods of WSSV-infected *P. monodon* was separated on a 1% formaldehyde gel, and transferred onto Hybond*+* nylon membranes (Amersham Pharmacia Biotech, USA) as described by Sambrook et al. (1989). The vp31, vp39b, and vp11 fragments used for riboprobe preparation were amplified from WSSV genomic DNA by PCR with the primer sets vp31-BamHI-F2/vp31-NotI-R1, vp39b-F2/vp39b-R2, and vp11-F1/vp11-R1, respectively (Table 1). Radioactively labeled vp31-, vp39b-, and vp11-specific probes were then generated as described previously (Chen et al., 2002). The membranes were prehybridized at 65 °C for 1 h with prehybridization buffer (0.25 M sodium phosphate buffer [0.5 M Na2HPO4, 0.5 M NaH2PO4], 1 mM EDTA, 1% BSA, 7% SDS), and then hybridized with the vp31-, vp39b- and vp11-specific [α-32P] rCTP-labeled riboprobes at 65 °C for 16 h. Membranes were washed for 5 min with wash buffer I (2× SSC and 0.5% SDS) at room temperature, 30 min with wash buffer II (2× SSC and 0.1% SDS) at 65 °C, and 30 min with wash buffer III (0.1× SSC and 0.1% SDS) at 65 °C, and then exposed to Kodak BioMax MR film with a intensifying screen for several days at −80 °C.

**5′ and 3′ RACE**

5′ and 3′ RACEs were conducted for all three genes to find their corresponding 5′ and 3′ ends. The frozen lymphoid organs from the WSSV-infected *P. monodon* at 60 hpi were homogenized with Trizol reagent (Invitrogen) to extract the total RNA, and 5′ RACE was performed using a commercial FirstChoice® RLM-RACE kit (Ambion, Inc., Austin, Tex.) according to the instruction manual. Briefly, DNase I-treated total RNA was reacted with calf intestine alkaline phosphatase, and then treated with tobacco acid pyrophosphatase to remove the 5′ cap structure, leaving a 5′-monophosphate. The full-length decapped RNA was then ligated to a 45 bp RNA adapter with T4 RNA ligase. Reverse transcription was performed by M-MLV reverse transcriptase with random decamers from the kit. The first round PCR was conducted with the 5′ RACE outer primer (supplied with the kit) and the first gene-specific primers: vp31-5′-RACE-sp1, vp39b-5′-RACE-sp1, and vp11-5′-RACE-sp1 for vp31, vp39b, and vp11 respectively (Table 1). The second round PCR was performed with 5′ RACE inner primer (supplied with the kit) and the gene specific-primers: vp31-5′-RACE-sp2, vp39b-5′-RACE-sp2, and vp11-5′-RACE-sp2 (Table 1). For 3′ RACE, the first strand cDNA was synthesized using an oligo(dT)-anchor primer (Roche Molecular Biochemicals), and then amplified with a gene-specific forward primer (wssv397-R1 for vp31, vp39b-R2 for vp39b, vp11-3′RACE for vp11, Table 1) and an anchor primer. The PCR products of the 5′ and 3′ RACE were cloned into the vector pGEM-T (Promega) and sequenced.

**Antibody preparation**

The full length coding regions of vp31 (nt 1–786) and vp39b (nt 1–852), and a partial coding region of vp11 (nt 148–570) were amplified by the primer sets vp31-EcoRI-F1/vp31-NotI-R1, vp39b-BamHI-F1/vp39b-EcoRI-R1 and vp11-N148-BamHI-F1/vp11-C538-HindIII-R1, respectively (Table 1). The corresponding PCR products were digested by the appropriate restriction enzymes and then cloned into pET28b (+). The recombinant proteins were expressed in BL21 Codon Plus *Escherichia coli* cells (Stratagene) and purified using Ni-NTA beads as previously described (Leu et al., 2005). The purified proteins were eluted directly from the beads with sodium dodecyl sulfate (SDS) sample buffer, and then subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The protein bands were sliced from the gel, minced, and mixed with Freund’s adjuvant for antibody production in rabbit.

**Western blot analysis**

The purified virions, envelope and nucleocapsid fractions were separated by SDS-PAGE, and then transferred onto a polyvinylidene difluoride (PVDF) membrane (MSI). After incubation in blocking buffer (5% skim milk, 50 mM Tris, 500 mM NaCl, pH 7.5, 0.5% Tween 20) at 4 °C overnight, the PVDF membranes were reacted with anti-VP31, anti-VP39B, anti-VP11 and anti-VP51C antibody (1:5000 dilution in blocking buffer) for 1 h at room temperature, and then washed twice with TBS-T (50 mM Tris, 500 mM NaCl, pH 7.5, 0.5% Tween 20). Membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody as the secondary antibody (1:5000 dilution in TBS-T) for 1 h, and then washed twice with TBS-T. A chemiluminescence system (Perkin Elmer, Inc.) was used for detection.

**Plasmid construction**

To produce the plasmid pcDNA3-HA-VP31/vp31, the vp31/vp39b coding region (1641 nt) was amplified using Pfu DNA polymerase (MBDio, Inc.) with primers (vp31-BamHI-F3/vp39b-EcoRI-R1; Table 1) containing a BamHI restriction site and a blunt-end. The PCR product was then cloned to the vector pcDNA3 (Invitrogen) upstream of a hemagglutinin (HA)-tag using BamHI and EcoRI restriction sites. A truncation plasmid, pcDNA3-HA-VP31/VP39b/VP39b, was also produced. In this plasmid the primer sets vp31-BamHI-F3/vp39b-REV489 and vp31-MU491(T→A)/vp39b-EcoRI-R1 (Table 1) were used to mutate the second base “T” of codon 164 of vp31 into an “A” so as to produce a stop codon. The third plasmid, pcDNA3-HA-VP31/VP39b, was a frame shift mutation, in which the primer set vp31-D4FS-BamHI-F4/ vp39b-EcoRI-R1 (Table 1) was used to eliminate the fourth base “T” from the second codon in vp31 to produce a 20-codon nonsense open reading frame.

The pRL-FL plasmid was constructed as described previously (Bielecki and Talbot, 2001), that is, the firefly luciferase from pGL3 plasmid (Promega) was inserted into pRL-null plasmid (Promega) to give the dual luciferase plasmid T7/pRL-FL. For transient DNA transfection in 5B cells, the WSSV ie1 promoter (−94/+52) (Liu et al., 2007) was used to replace the T7 promoter by amplification (ie1 promoter-SacI-F/ie1 promoter-Hael-R; Table 1) and cloning the promoter into the SacI-Hael sites of T7/pRL-FL to produce the construct ie1/pRL-FL. The putative vp31/vp39b IRES fragment and its antisense sequence (as a negative control) were PCR amplified (primer
sets vp31-IRF1-N123-Smal/vp39b-IRRI-C199-NcoI and vp39b-IRF1-C199-Smal-F/vp31-IRRI-N123-NcoI-R, respectively; Table 1) and cloned into pRL-FL to generate ie1/IRES23-919 and ie1/IRES919-123. As a positive control, the IRES element (180 bp) of vp28 was PCR amplified (primer set vp28IRES-F/vp28IRES-NcoI-R) and inserted into pRL-FL to generate the plasmid ie1/vp28-IRES. The empty vector ie1/pRL-FL was also used as a negative control for IRES activity. For in vitro translation, we used the plasmids T7/IRES23-919, T7/IRES919-123, T7/vp28-IRES, and T7/pRL-FL. These were identical to ie1/IRES23-919, ie1/IRES919-123, ie1/vp28-IRES, and ie1/pRL-FL except that they used the original T7 promoter. To block ribosome scanning in vitro, a stable 28-bp stem–loop (ΔG = −62 kcal/mol) made of a 60-nucleotide sequence (5′-GCTAGCGGTACGGCAGTGCCGACGCAATTCGCTGAGCGATCCGACTGCCGCTGAGCT-3′; Bieleski and Talbot, 2001) was cloned at the NheI site immediately upstream from the Renilla luciferase start codon to produce the plasmids T7/SL-IRES23-919, T7/SL-IRES919-123, T7/SL-vp28-IRES and T7/SL-pRL-FL.

**Coupled in vitro transcription-translation reactions**

In vitro transcription and translation assays were carried out using the TNT™ Quick Coupled Transcription/Translation System (Promega Inc.) according to the manufacturer’s instructions. Briefly, 1 μg of plasmid DNA was added to 50 μl of translation mixture containing 1 μl [35S] methionine (1000 Ci/mM, EASYTIDES™) and incubated for 90 min at 30 °C. The reaction products (10 μl) were then added to 10 μl of 2× SDS sample buffer for resolution by SDS-PAGE. After electrophoresis, the gel was dried, and exposed to X-ray film at room temperature for 16 h.

**In vitro transcription**

RNA transcripts were prepared using a RiboMAX™ Large Scale RNA Production System-T7 kit (Promega) as described by the technical manual. The various T7/pRL-FL based constructs were linearized with NolI, and T7 RNA polymerase (Promega) was used to transcribe uncapped transcripts from the linearized plasmid DNAs (1 μg) at 37 °C for 2 h. The DNA template was then removed by digestion using RNase-free DNase I (Invitrogen) as described above. RNA sizes were determined by separating the RNAs on 1% agarose-formaldehyde gel.

**In vitro translation**

In vitro translation of the uncapped transcripts was performed by Flexi Rabbit Reticulocyte Lysate (RRl; Promega) according to the manufacturer’s instructions. RNA (1.5 μg) was added to 25 μl reaction mixtures containing 16.5 μl of RRL, 1 μl of [35S] methionine, 0.5 μl of amino acid mixture minus methionine, 0.5 μl of RNasin® ribonuclease inhibitor (40 μg/μl; Promega), 0.5 μl of 100 mM DTT, and 0.4 μl of 2.5 M potassium chloride (final potassium ion concentration was 40 mM) and incubated at 30 °C for 90 min. An aliquot of the reaction products (5 μl) was then added to 5 μl of 2× SDS sample buffer and analyzed by SDS-PAGE and autoradiography as described above.

**IRES activity assay for transfected S99 cells**

For the IRES activity assays, S99 cells were seeded in 24-well trays (1 × 10³ cells/well) and grown in SF-900 II SFM serum-free medium (Invitrogen) overnight at 27 °C. Plasmid DNAs (0.5 μg of plasmid DNA per well) were transfected into S99 cells using the Cellfectin reagent (Invitrogen) according to the manufacturer’s recommendations. Cells were harvested at 48 h after transfection and analyzed for dual luciferase activities using the Dual-Luciferase® Reporter Assay System (Promega). Briefly, transfected cells were washed twice with 1× PBS, lysed with 100 μl of passive lysis buffer, and then incubated for 15 min at RT on an orbital shaker with gentle shaking. Cell lysate (20 μl) was used to measure luciferase activities with a Labsystems benchtop luminometer. The ratio of firefly luciferase activity to Renilla luciferase activity was used as an index of IRES activity. Transfection assays were performed in triplicate with three independent experiments, each using a different batch of purified plasmid DNA. Data are presented as mean ± SD (standard derivation) from the three triplicate experiments.

**Reverse transcription-PCR analysis**

Total RNA (1 μg) isolated by Trizol reagent (Invitrogen) from untransfected and transfected S99 cells was pretreated with DNase I and then reverse transcribed by SuperScript II Reverse Transcriptase (Invitrogen). PCR was performed with the primer sets pRL-FL-N219-F/pRL-FL-C1732-R and pRL-FL-N1071-F/pRL-FL-C2654-R (Table 1) using the following profile: 94 °C for 3 min; 30 cycles at 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 2 min; a final extension at 72 °C for 20 min. The PCR products were then cloned into the vector pGEM-T (Promega) and sequenced.

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**References**
