Factor-Independent Assembly of Elongation-Competent Ribosomes by an Internal Ribosome Entry Site Located in an RNA Virus That Infects Penaeid Shrimp

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The Taura syndrome virus (TSV), a member of the *Dicistroviridae* family of viruses, is a single-stranded positive-sense RNA virus which contains two nonoverlapping reading frames separated by a 230-nucleotide intergenic region. This intergenic region contains an internal ribosome entry site (IRES) which directs the synthesis of the TSV capsid proteins. Unlike other dicistroviruses, the TSV IRES contains an AUG codon that is in frame with the capsid region, suggesting that the IRES initiates translation at this AUG codon by using initiator tRNA⁶⁰⁰⁰. We show here that the TSV IRES does not use this or any other AUG codon to initiate translation. Like the IRES in cricket paralysis virus (CrPV), the TSV IRES can assemble 80S ribosomes in the absence of initiation factors and can direct protein synthesis in a reconstituted system that contains only purified ribosomal subunits, eukaryotic elongation factors 1A and 2, and aminoacylated tRNAs. The functional conservation of the CrPV-like IRES elements in viruses that can infect different invertebrate hosts suggests that initiation at non-AUG codons by an initiation factor-independent mechanism may be more prevalent.

The Taura syndrome virus (TSV) is the causative agent of Taura syndrome in penaeid shrimp and is responsible for worldwide mortalities in shrimp agriculture. The virus was first isolated in 1997 and was classified, based upon biochemical and biophysical properties, as an invertebrate picornavirus. Picornaviruses contain a positive-stranded RNA genome which encodes a single open reading frame (reviewed in reference 20). However, complete sequencing of the viral genome suggested that TSV belongs to the genus of cricket paralysis-like viruses (12) whose single-stranded positive-sense RNA genomes encode two nonoverlapping reading frames separated by a 230-nucleotide internal ribosome entry site (IGR). This mechanism of initiation is quite different from the mechanism by which 40S subunits are recruited via the 5′ cap structures in mRNAs. In this mechanism, 40S subunits scan the mRNA until a start-site AUG codon is located with which the initiator tRNA⁶⁰⁰⁰ is in base pair interaction, occupying the ribosomal P-site and thereby setting the reading frame for the ribosome to start the elongation phase of translation (5). We are interested in the prevalence of initiator-tRNA⁶⁰⁰⁰-independent translation, because this mode of initiation should allow continuous translation of mRNAs when many translation initiation factors become limiting, for example, during cell stress, apoptosis, or viral infection.

Here, we report that TSV, which infects a noninsect invertebrate host, is capable of directing protein synthesis in an AUG-independent as well as initiation factor-independent manner, similar to CrPV and PSIV. Although the mechanisms of translation initiation are similar, the RNA sequences that mediate recruitment of ribosomes are different, demonstrating conservation of function of this divergent IRES element to viruses that can infect insect and noninsect invertebrates.

MATERIALS AND METHODS

Plasmid constructions. Dicistronic luciferase plasmids containing the CrPV IGR IRES have been described previously (26). Dicistronic luciferase plasmids containing the TSV IGR IRES (pRC1) were created by PCR, using TSV cDNA plasmid E38 (12) encompassing nucleotides 6566 to 10,205 of the TSV genome plus the poly(A) tail (accession number AF277675) as the template. The primers
used for amplification of the TSV IRES were TSVECO+5/-, 5’-TCAGCTATTGG GAATTCAATATAAGC-3’, and TSVNCO+, 5’-ATTAGTCCTCTCTCCATGGTTGTTGATC-3’, which amplified nucleotides 6719 to 7005 of the TSV genome and introduced EcoRI and NcoI restriction sites at nucleotides 6729 and 6991, respectively. The TSV IRES was then ligated into the intergenic region of a dicistronic luciferase reporter plasmid (26) which was previously digested with EcoRI and NcoI. The presence of the TSV IRES in the construct was confirmed by sequencing. Mutated TSV IRESs were generated using the QuikChange kit (Stratagene) and confirmed by sequencing.

To generate monocistronic TSV IRES-containing plasmid pRC3, plasmid pRCI was digested with EcoRI and NcoI. The TSV IRES fragment was gel purified and ligated into the monocistronic CrPV plasmid (26), which was digested with EcoRI and NcoI. Insertion of the TSV IRES in the construct was confirmed by sequencing. Dicistronic luciferase reporter plasmids were also digested with NcoI, which cleaves at the luciferase ATG start codon. RNAs were transcribed as described above.

RESULTS

The TSV IGR contains an IRES element. Phylogenetic analyses have predicted that the IGR IRES elements in dicistroviruses fold into a complex RNA structure that includes three pseudoknots (Fig. 1A) (7, 8). For CrPV and PSIV, much of this structure has been verified experimentally (7, 8). The structure of the TSV IGR element which has been shown to function as an IRES in wheat germ extract (4) is expected to be similar, but the predicted structure (4, 12) has not been tested experimentally.

As shown in Fig. 1B, however, pseudoknot I (PKI) in the TSV IGR IRES is predicted to contain an extra internal stem-loop region that is absent in the insect dicistroviruses (4, 7, 8).
In addition, the TSV IGR IRES contains an AUG triplet that is in frame with the capsid coding region (12). Thus, it has been suggested that, unlike the IRES elements in insect dicistroviruses, the TSV IGR IRES uses initiator-tRNA\textsubscript{met} to initiate capsid protein synthesis (12). To address this possibility, we monitored the translation of in vitro-transcribed dicistronic RNAs (Fig. 2A) containing either the CrPV IGR IRES or wild-type and mutated TSV IGR IRES elements in RRL. The first cistrons in each dicistronic mRNA were translated with similar efficiencies (Fig. 2B). While both the CrPV and TSV IGR sequence elements functioned as IRESs in the RRL, the TSV IGR IRES was only half as active as the CrPV IRES (Fig. 2B). Lower translational efficiency of TSV IRES-containing mRNAs was observed under a variety of salt concentrations (Fig. 2C). Dicistronic RNAs lacking an IRES sequence (Fig. 2B) or containing an UAG stop codon insertion after the alanine-encoding GCU triplet (Fig. 1B and 2B, lane 5) failed to produce significant amounts of firefly luciferase. TSV IGR IRES elements which contained a single point mutation that changed the in-frame AUG triplet to a UUG triplet (Fig. 1B) did not mediate translation of the second cistron (Fig. 2B, lane 4). This result was curious, because Hatakeyama et al. showed that a similar mutated IRES, which contained an AUG-to-UUG change and two additional mutations in PKI, displayed IRES activity in wheat germ extracts (4). This discrepancy is unclear at present, because the AUG-to-UUG mutation-containing TSV IRES was also inactive in the wheat germ extract (data not shown). In any case, our results suggest either that the AUG triplet is essential for initiator tRNA\textsubscript{met}-dependent translation or that the single point mutation generated an altered, nonfunctional IRES.

Single adenosine-to-uridine mutation in the IRES alters IRES structure. To examine the requirements of factors which are essential in TSV IRES-mediated translation, we first tested whether the TSV IRES can assemble binary 40S-IRES complexes from purified, salt-washed 40S subunits and in vitro-transcribed RNAs. Briefly, radiolabeled TSV IRES was incubated with different amounts of purified 40S subunits, and formation of binary complexes was monitored in composite agarose gels. Figure 3A shows that addition of 40S subunits to radiolabeled RNA resulted in the appearance of slower-migrating complexes, suggestive that the TSV IRES can form binary complexes with 40S subunits, and formation of binary complexes was monitored in composite agarose gels. Figure 3A shows that addition of 40S subunits to radiolabeled RNA resulted in the appearance of slower-migrating complexes, suggestive that the TSV IRES can form binary complexes with 40S subunits, as was observed with the insect CrPV and PSIV IRES elements, and with the human hepatitis C virus IRES (19). The TSV IRES bound 40S subunits with an apparent $K_d$ of 56 ± 6 nM (mean ± standard error), which is higher than the $K_d$ with which CrPV IRES binds 40S (i.e., $K_d$ of 24 ± 6 nM) (7). The higher $K_d$ value correlates with the observed lower translational efficiency of the TSV IRES (Fig. 2). The fact that only 50% of the TSV IRES engaged in recruitment of 40S subunits, in comparison with 75% of CrPV IRES molecules (7), suggests that proper RNA folding is a limiting step in TSV IRES-mediated translation initiation.

To determine the positioning of 40S subunits on the TSV IRES, we examined binary 40S-IRES complexes by toeprinting analyses. Briefly, a deoxyoligonucleotide primer was annealed to RNA sequences located in the coding region, and cDNA synthesis was initiated after addition of reverse transcriptase and deoxyribonucleotides. When such reactions are performed with IRES-40S complexes, cDNA synthesis terminates when reverse transcriptase encounters the attached 40S subunit. It has been shown that the nucleotide at which reverse transcriptase stops is located approximately 15 to 17 nucleotides downstream of the start-site codon in the P-site of the ribosome (19). Figure 4 shows that reverse transcriptase stopped at nucleotides UUG\textsubscript{6947}-6949 in TSV IRES RNA, in the presence (lane 2) but not in the absence (lane 1) of 40S subunits, arguing that either AUG\textsubscript{6947}-6949 or CCU\textsubscript{6951}-6953 (Fig. 1) occupied the ribosomal P-site. A similar toeprint was noted in RNAs that

FIG. 2. Tests for IRES activity in the TSV IGR. (A) Diagram of dicistronic reporter construct used to test for IRES activity. (B) Translation of dicistronic mRNAs in RRL. Radiolabeled products were separated in sodium dodecyl sulfate-polyacrylamide gels and visualized by autoradiography. Products synthesized from dicistronic mRNAs with no insert (lane 1), the CrPV IGR (lane 2), the TSV IGR (lane 3), TSV IGR mutant A\textsubscript{6947}U (lane 4), or TSV IGR mutant AAC\textsubscript{6956-6958}UAG (lane 5) in the IGR are shown. (C) Effects of salt concentration on IRES activity. Dicistronic constructs containing either no insert, the TSV IGR, or the CrPV IGR were incubated in increasing amounts of salt, and firefly luciferase activity was monitored.
contained a UAG stop codon insertion at position 6956 (lane 6). Thus, the lack of translation of UAG6956-containing RNAs (Fig. 2B) was not due to failure to recruit 40S subunits. In contrast, RNAs that contained the single A6947-to-U change, resulting in an AUG-to-UUG triplet change, failed to display a UUG6963 toeprint (lane 4), suggesting that the translational inactivity of these RNAs resulted from the failure to bind 40S subunits. However, careful inspection of the nonspecific stops generated by reverse transcriptase in the presence or absence of 40S subunits (lanes 3 and 4) revealed that the overall structure of RNAs carrying the A6947-to-U change differed from the wild-type TSV IRES (lanes 1 and 2). To examine whether the translational inactivity of RNAs carrying the AUG-to-UUG mutations was due to elimination of the AUG triplet or to overall changes in IRES structure, we measured the affinity of 40S subunits for the mutated IRES in gel shift assays (see above). Figure 3B shows that only 8% of UUG mutation-containing RNAs bound 40S subunits with an affinity that was slightly lower than that of the wild-type IRES (i.e., 38 ± 6 nM). Thus, translational inactivity of UUG mutation-containing IRES RNAs was likely due to misfolded mRNA structure.

The TSV IRES is insensitive to the antibiotic edeine. To examine further whether the in-frame AUG codon was used as the start-site codon, we performed in vitro translation experi-
ments in the presence of the compound edeine, an antibiotic that inhibits translation of prokaryotic and eukaryotic ribosomes. Specifically, edeine interferes with the base pair interaction of initiator-tRNA^met and the start-site AUG codon in the ribosomal P-site (2, 9, 16). As a result, translation initiation that requires initiator-tRNA^met–AUG interactions is inhibited by edeine, while initiator-tRNA^met-independent translation is unaffected at low edeine concentrations (25). Figure 5 shows that, like the CrPV IRES, the TSV IRES was relatively insensitive to translational inhibition at 0.25 and 0.5 μM edeine. At these concentrations, translation of the first, initiator-tRNA^met-dependent cistron was inhibited by more than 95% (Fig. 5). Similar experiments performed with the EMCV IRES showed that this IRES was also inhibited at 0.25 and 0.5 μM edeine (data not shown). These data suggest that, like the CrPV IRES, the TSV IRES does not need initiator-tRNA^met to start protein synthesis from an AUG codon in the ribosomal P-site, even though mutagenesis of an in-frame AUG codon inhibited translation.

The TSV IRES can assemble 80S ribosomes without initiation factors. Edeine-insensitive translation suggests that the TSV IRES can assemble 80S subunits from purified subunits without canonical initiation factors. Figure 6 shows that both the CrPV and TSV IRES elements mediated the assembly of 40S-IRES and 80S-IRES complexes from purified subunits without canonical initiation factors. The slowest-migrating RNA-protein complexes are indeed IRES-80S complexes, because when identical reaction mixtures were sedimented in sucrose gradients they migrated as 80S complexes which were sensitive to EDTA (data not shown). In contrast, the EMCV IRES did not assemble into ribosome complexes as expected (25).

FIG. 5. Effects of the translation inhibitor edeine on TSV IGR IRES activity. Dicistronic mRNAs containing either the CrPV IGR IRES or the TSV IGR IRES in the IGR were incubated in RRL with the indicated concentrations of edeine. Cap-dependent translational activity of the first cistrons and internal initiation of the second cistrons are indicated. Activities are displayed as the percentage of the activity observed in the absence of edeine. Results from triplicate experiments are shown.

FIG. 6. 80S ribosomal assembly on various IRES elements. Radio-labeled IRES-containing RNAs were incubated with either 40S, 40S and 60S, or 60S subunits as indicated. 40S-IRES and 80S-IRES complexes were observed as a supershifted bands, indicated by the arrows. Note that the purified 60S fraction was slightly contaminated with free 40S subunits. An autoradiograph of the composite gel is shown.

FIG. 7. Ribosomal translocations revealed by toeprinting analysis in a reconstituted system. All numbered lanes are the result of extension of a 32P-labeled primer annealed to a dicistronic RNA that contained the wild-type TSV IGR IRES in the IGR. Reaction mixtures included the components listed at the top of the figure. Positions of toeprints are indicated at the right by arrows. All reactions were performed in the presence of cycloheximide. A sequencing ladder is shown at the left. An autoradiograph of the gel is shown.
The TSV IRES directs ribosome translocation in a reconstituted system. To test the hypothesis that the CCU triplet occupied the ribosomal P-site in 80S-IRES complexes, we examined whether elongation factor eEF1A could deliver elongator-tRNA^{Ala} to the alanine-encoding GCU triplet, which would then be predicted to reside in the ribosomal A-site (Fig. 1). Briefly, 80S-IRES complexes were assembled in the presence of selected elongator-tRNA species, elongation factors eEF1A and eEF2, and the elongation inhibitor cycloheximide. As was observed in the experiments shown in Fig. 4, reconstituted 40S-IRES complexes revealed a toeprint at UUG_{6963-6965} (Fig. 7, lane 2). A similar toeprint was observed in 80S-IRES complexes (Fig. 7, lane 3), which is in agreement with the findings that 40S-mRNA and 80S-mRNA complexes reveal similar toeprints (7, 19). Incubation of 80S-IRES complexes with eEF1A and eEF2 and aminoacylated elongator tRNA^{Ala} resulted in a new toeprint at AAA_{6966-6968}, which is three nucleotides downstream of UUG_{6963-6965}, arguing that the ribosome translocated by one codon (Fig. 7, lane 4). In contrast, addition of only elongator methionine-tRNA^{Met} produced the original toeprint at UUG_{6963-6965}, arguing that ribosome movement was only induced after tRNA^{Ala} occupied the ribosomal A-site. Lastly, addition of bulk charged tRNAs generated toeprint U_{6969} (Fig. 7, lane 6), a shift of six nucleotides downstream of UUG_{6963-6965} (Fig. 7, lane 6). These data suggest that the ribosome underwent translocation across two codons, after which translation elongation was arrested by cycloheximide. Taken together, these analyses argue that the first decoded triplet mediated by the TSV IRES is the GCU-alanine codon, located in the A-site of the ribosome. These data are in agreement with previously reported results that the N-terminal amino acid of the TSV structural polyprotein, isolated from purified virions, is alanine (12).

The TSV IRES directs peptide synthesis without initiation factors. Finally, we tested whether the observed ribosome translocations resulted in peptide bond formation. To this end, the TSV-IRES-containing dicistronic mRNAs were incubated with purified ribosomal subunits, eEF1A, eEF2, and bulk tRNAs which had been aminoacylated in the presence of [35S]methionine and [35S]cysteine (6). Reaction products were displayed in sodium dodecyl sulfate-polyacrylamide gels and visualized after autoradiography. Figure 8 (lane 6) shows that, like the CrPV IRES (Fig. 8, lane 2), the TSV IRES could mediate the synthesis of radioactive peptides in the 3.5-kDa range in this reconstituted system. The radioactive products were peptides, because they disappeared after incubation with proteinases (Fig. 8, lane 8), but not after treatment with ribonucleases (Fig. 8, lane 7). In addition, peptide synthesis required elongating ribosomes, because cycloheximide greatly inhibited product accumulation (Fig. 8, lane 9). In contrast, the EMCV IRES, which requires canonical initiation factors to initiate translation (18), did not mediate peptide synthesis in the reconstituted system (Fig. 8, lane 1), even though this IRES was active in RRL (data not shown). These data indicate that the TSV IRES can bind 40S and 60S ribosomal subunits to assemble 80S ribosomes that immediately enter translation elongation, resulting in the production of polypeptides.

**DISCUSSION**

The findings presented here show a conservation of function between two IRES elements found in two different viruses that belong to the same family but that infect either insect or noninsect invertebrate hosts. Specifically, we have shown that the TSV IGR IRES, like the CrPV and PSIV IRES elements, can recruit ribosomes in the absence of initiation factors and initiator tRNA^{Met}. This was somewhat unexpected, because an in-frame AUG initiation codon is positioned two codons upstream of the GCU-alanine codon that had been determined to be the first amino acid of the TSV structural polyproteins (12). AUG-independent translation initiation of the TSV intergenic IRES was further substantiated by its activity in the presence of eideine, a drug which inhibits initiator tRNA^{Met} binding to the P-site of the ribosome (2). The factorless assembled 80S-IRES complexes were able to direct the synthesis of peptides from only aminoacylated tRNAs, eEF1A, and eEF2, suggesting that the TSV and CrPV IRES elements assemble elongation-competent ribosomes.

Although the CrPV, PSIV, and TSV IRES elements display functional similarities, the TSV IRES is predicted to contain local structures that are different from the two insect viral IRES elements. For example, it has been predicted that the TSV IRES contains an internal stem-loop structure in the PKI region which is not present in the PKI region of the CrPV and PSVI IRES elements (4). Importantly, the integrity of this stem-loop structure is necessary for IRES function (4). Perhaps the internal stem-loop influences the overall IRES conformation by engaging in RNA-RNA interactions or by providing binding sites for RNA binding proteins that affect IRES...
conformation or activity. Despite structural differences, cryo-electron microscopy analyses of 40S-IRES complexes have shown that the mechanistically distinct hepatitis C virus and the CrPV IRES elements induce very similar changes in the bound ribosomal subunits. Specifically, the induced changes result in clamping down the IRES in the mRNA binding cleft of the 40S subunit (23, 24). Although structural analyses on the TSV IRES have not yet been reported, the functional similarities with the insect IRES elements indicate that the TSV will likely induce comparable changes in IRES-ribosome complexes.

Relatively little is known about the biology of TSV infection of penaeid shrimp. Our findings suggest that this virus may counteract phosphorylation of eIF2 by kinases of the innate immune system by initiating translation without requirement for initiator-tRNA^met molecules. Alternatively, replication of the viral genome on cellular membranes may cause stress, resulting in the activation of eIF2 kinases such as PERK and overall inhibition of initiator-tRNA^met-dependent translation. Analysis of eIF2 phosphorylation during the viral infectious cycle may provide some experimental tests of these hypotheses.

The conservation of IRES elements in viruses that infect insects and shrimp suggests that viruses that infect other animals, possibly even mammals, may have evolved similar IRES elements. Finally, the results that the dicistrovirus IGR IRES elements can perform protein synthesis with ribosomes of plant, insect, yeast, and mammalian origin are unique, because IRES elements can perform protein synthesis with ribosomes of different cellular origins. We are grateful to Karla Kirkegaard for critical reading of the manuscript.

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