JOURNAL OF VIROLOGY, Jan. 2005, p. 677–683 Vol. 79, No. 2
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.2.677–683.2005

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

Randal C. Cevallos and Peter Sarnow*

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California

Received 30 June 2004/Accepted 30 August 2004

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 tRNA^{met}. 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 agricultures (11). The virus was first isolated in 1997 and was classified, based upon biochemical and biophysical properties, as an invertebrate picornavirus (1). 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. Based upon this characteristic, the cricket paralysis-like viruses have been reclassified as a new family, *Dicistroviridae* (13). Thus far, several insect viruses have been found to belong to this family of viruses, including Himetobi P virus, acute bee paralysis virus, *Plautia stali* intestine virus (PSIV), cricket paralysis virus (CrPV), and the black queen-cell virus (3, 10, 14, 22, 26). TSV, however, is the first identified virus of this family that infects a noninsect invertebrate host.

Work performed with the PSIV and CrPV RNA genomes has indicated that the intergenic region (IGR), which separates the two nonoverlapping reading frames, mediates protein synthesis of the second, capsid-encoding cistron by internal ribosome entry (4, 6, 7, 15, 17, 21, 22, 25, 26). These IGR internal ribosome entry site (IRES) elements are unusual because they can assemble 80S ribosomes without the aid of known canonical initiation factors (7, 15). Both biochemical analyses (7) and structural analyses by cryo-electron microscopy (24) have shown that in the case of the CrPV IGR IRES element, the IRES RNA occupies the ribosomal P-site in the 80S-RNA complex. Specifically, three pseudoknot structures in the IRES are involved in positioning the IRES on the ribosome, triggering the ribosome into elongation mode (7). This notion was substantiated by the finding that assembled 80S-IRES complexes could indeed mediate the synthesis of peptides after the addition of only elongator tRNA molecules and elongation factors (6, 17). Thus, the IRES mimics the function of initiator-tRNA^{met} in the ribosomal P-site and can begin protein synthesis from the A-site of the ribosome. 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 tRNA^{met} is engaged 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-tRNA^{met}-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

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305. Phone: (650) 498-7076. Fax: (650) 498-7147. E-mail: psarnow@stanford.edu.

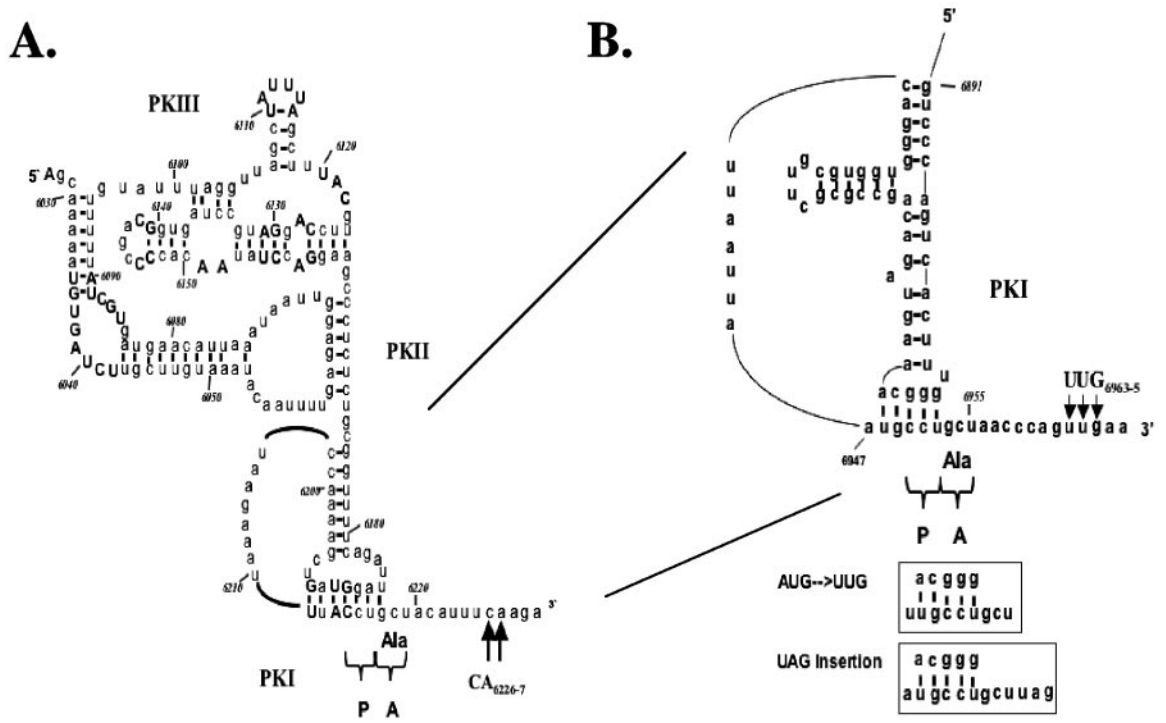

FIG. 1. Predicted structure of the IGR IRES in dicistroviruses. (A) Structure of the CrPV IGR IRES displaying the three predicted PK structures. (B) Proposed structure of the PKI region of the TSV IGR IRES, adapted from Hatakeyama et al. (4). IRES mutations used in this study are shown below the structure. Nucleotide positions of toeprints produced by bound 40S and 80S ribosomes are indicated by arrows. P and A refer to the triplet sequences that are predicted to occupy the ribosomal P- and A-sites, respectively.

used for amplification of the TSV IRES were TSVECO+, 5′-TCAGCTATTG GAATTCAACTAATAGC-3′, and TSVNCO-, 5′-ATTAGTCCTCCTCCCATG GTTGTTGTATC-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 pRC1 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 was confirmed by sequencing.

In vitro transcription and translation. To generate dicistronic RNAs, the respective luciferase reporter plasmids were linearized with BamHI and transcribed using the Ribomax large-scale RNA production system with T7 RNA polymerase (Promega). The dicistronic RNAs were translated in rabbit reticulocyte lysate (RRL; Promega) in the presence of 154 mM potassium acetate and 0.8 μCi of [^{35}S]methionine and [^{35}S]cysteine/μl. The dicistronic mRNAs displayed indistinguishable stabilities in the translation lysate (data not shown).

To generate monocistronic TSV IRES-containing RNAs, plasmid pRC3 was digested with NarI, which cleaves 33 nucleotides downstream of the firefly luciferase ATG start codon. CrPV IRES- and encephalomyocarditis (EMCV) IRES-containing plasmids were also digested with NcoI, which cleaves at the luciferase ATG start codon. RNAs were transcribed as described above.

Nondenaturing gel mobility shift assays and K_d measurements. Ribosomal subunits were purified from HeLa cells, and gel mobility shift assays were performed as previously described (6, 7). Briefly, 5′-end-labeled RNAs (0.5 nM final concentration) were incubated with noncompetitor RNA (7), 71 nM 40S subunits, 66 nM 60S subunits, or with both 40S and 60S subunits in buffer E (20 mM Tris-HCl [pH 7.5], 100 mM potassium chloride, 2.5 mM magnesium acetate, 2 mM dithiothreitol, 0.25 mM spermidine) for 30 min at 30°C. Noncompetitor RNA represented nucleotides 880 to 948 synthesized from plasmid pcDNA3 (Invitrogen). Gel shifts were performed in composite gels as described elsewhere (6). To measure the approximate K_d of binary TSV IRES-40S complexes, results were quantitated with a PhosphorImager (Molecular Dynamics) and plotted using GraphPad Prism.

Toeprinting analysis with purified 40S subunits. Dicistronic RNAs (0.25 μg/μl final concentration) were first annealed to primer prEJ94 (7) in 40 mM Tris-HCl (pH 7.5) and 0.2 mM EDTA by heating to 65°C, followed by slow cooling to 37°C. Annealed RNAs were incubated in buffer E with 35 nM 40S ribosomal subunits at 30°C for 10 min, and toeprinting analysis was performed as described previously (7).

Ribosome translocation analysis. Experiments were performed as described elsewhere (6), but using 25 ng of wheat germ eukaryotic elongation factor eEF1A/μl and 30 ng of wheat germ eEF2/μl. Where indicated, 0.8 and 0.44 μM elongator methionine-tRNA^{Met} and elongator alanine-tRNA^{Ala}, respectively, were used in place of bulk aminoacylated tRNAs. All reactions were performed in the presence of cycloheximide at a final concentration of 500 μg/ml.

Peptide synthesis analysis. Peptide syntheses were performed as described previously (6). Reaction mixtures contained a final concentration of 50 ng of dicistronic RNA/μl, 80 nM 40S subunits, 150 nM 60S subunits, 1 mM ATP, 1 mM GTP, 80 ng of eEF1A/μl, 100 ng of eEF2/μl, and 380 ng of [^{35}S]met- and [^{35}S]cys-containing aminoacylated bulk tRNAs/μl.

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).

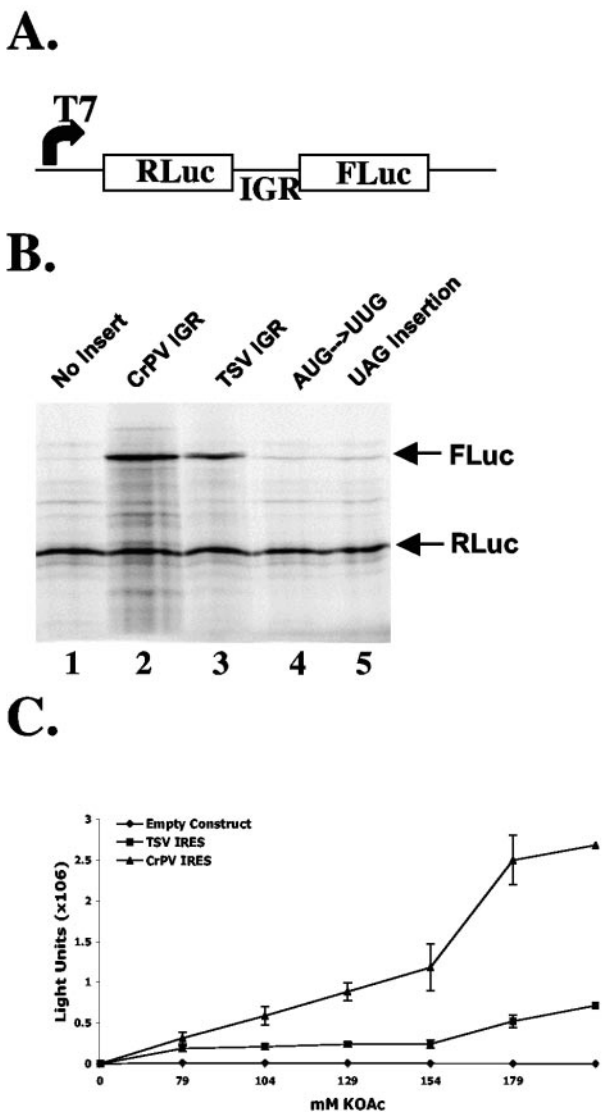

FIG. 2. Tests for IRES activity in the TSV IGR. (A) Diagram of dicistronic reporter construct used to test for IRES activity. (B) Translation of dicistronic mRNAs in RRL. Radiolabeled products were separated in sodium dodecyl sulfate-polyacrylamide gels and visualized by autoradiography. Products synthesized from dicistronic mRNAs with no insert (lane 1), the CrPV IGR (lane 2), the TSV IGR (lane 3), TSV IGR mutant $A_{6947}U$ (lane 4), or TSV IGR mutant $AAC_{6956-6958}UAG$ (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.

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-$tRNA^{met}$ 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-UGA 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 $tRNA^{met}$-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, 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 $UUG_{6963-6965}$ in TSV IRES RNA, in the presence (lane 2) but not in the absence (lane 1) of 40S subunits, arguing that either $AUG_{6947-6949}$ or $CCU_{6951-6952}$ (Fig. 1) occupied the ribosomal P-site. A similar toeprint was noted in RNAs that

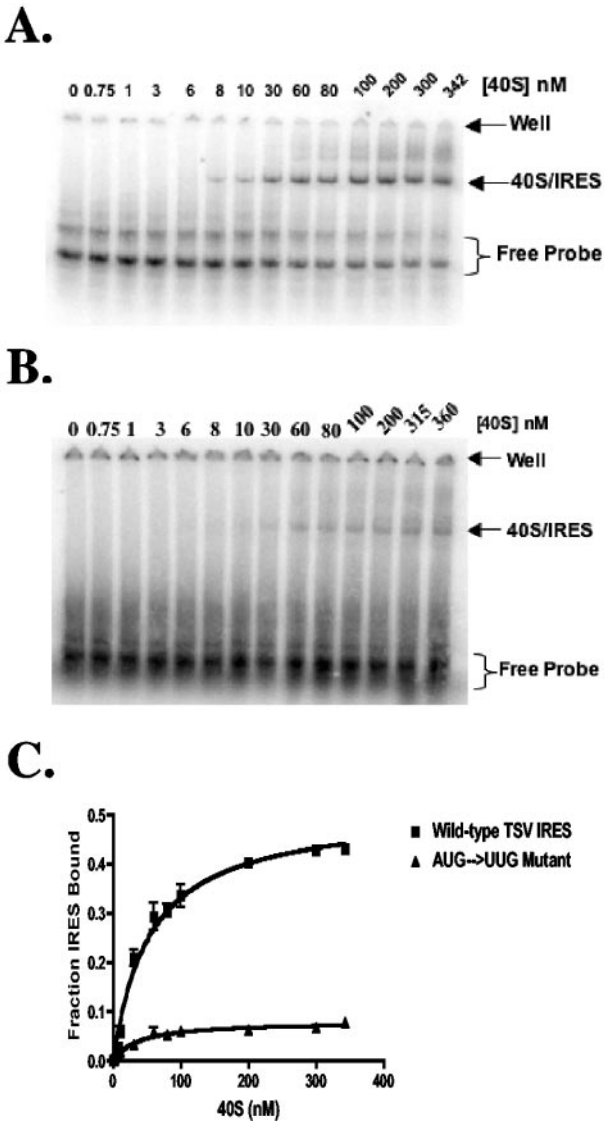

FIG. 3. Affinity measurements of 40S-TSV IRES complexes. (A) Gel mobility shift assays of wild-type IRES-40S complexes. (B) Gel mobility shift assays of mutant (AUG→UUG) IRES-40S complexes. Radiolabeled TSV IRESs were incubated with increasing amounts of purified 40S subunits and loaded onto composite agarose-acrylamide gels. The final concentrations of 40S subunits are indicated at the top of the gels. Formation of 40S-IRES complexes was monitored by the appearance of a slower-migrating band, indicated by the arrows at the right. Phosphorimaging analyses of the gels are shown. (C) Quantitation of wild-type and mutant TSV IRES-40S complexes. The fractions of bound IRESs at different 40S concentrations were determined in four independent experiments. Curve fitting was aided by GraphPad Prism.

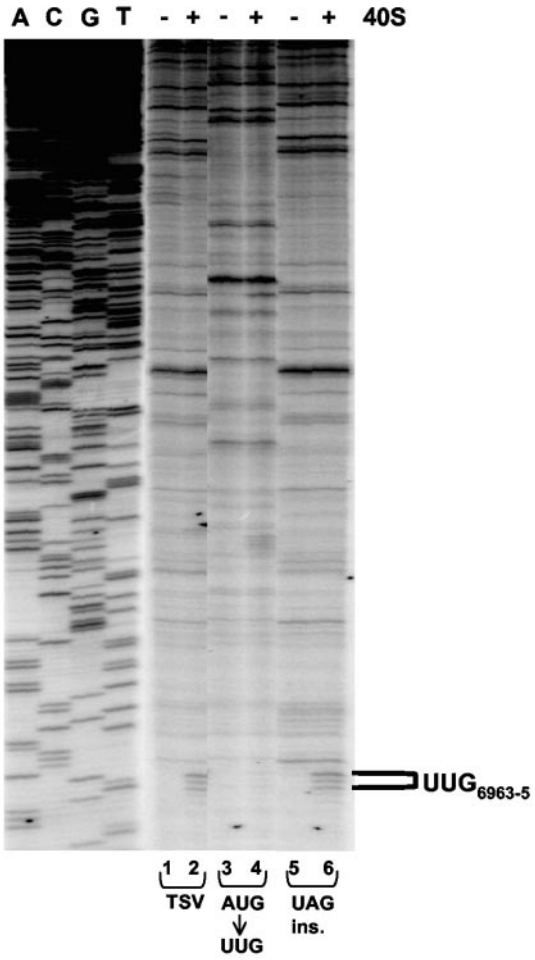

FIG. 4. Toeprinting analysis of 40S-IRES complexes. Dicistronic RNAs containing wild-type TSV IGR IRES (lanes 1 and 2), the TSV $A_{6947}U$ mutant IRES (lanes 3 and 4), or the TSV $AAC_{6956–6958}UAG$ mutant (lanes 5 and 6) in the IGR were analyzed by toeprinting analysis (see Materials and Methods) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of purified 40S subunits. An autoradiograph of the gel is shown. A specific toeprint is indicated on the right, and a sequencing ladder of the wild-type TSV IGR sequence is shown on the left.

contained a UAG stop codon insertion at position 6956 (lane 6). Thus, the lack of translation of UAG_{6956}-containing RNAs (Fig. 2B) was not due to failure to recruit 40S subunits. In contrast, RNAs that contained the single A_{6947}-to-U change, resulting in an AUG-to-UUG triplet change, failed to display a $UUG_{6963-6965}$ 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 A_{6947}-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-

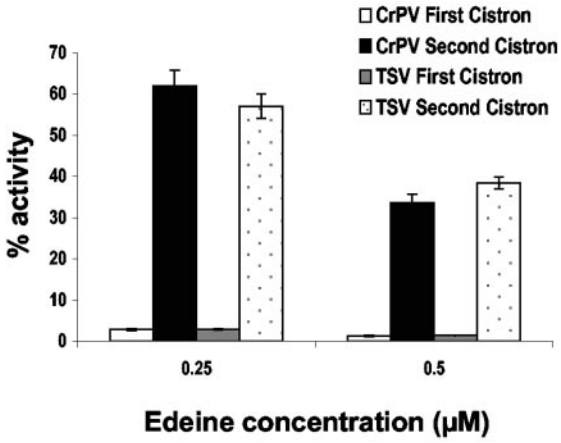

FIG. 5. Effects of the translation inhibitor edeine on TSV IGR IRES activity. Dicistronic mRNAs containing either the CrPV IGR IRES or the TSV IGR IRES in the IGR were incubated in RRL with the indicated concentrations of edeine. Cap-dependent translational activity of the first cistrons and internal 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.

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

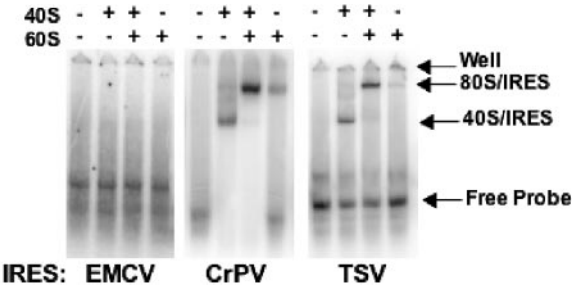

FIG. 6. 80S ribosomal assembly on various IRES elements. Radiolabeled IRES-containing RNAs were incubated with either 40S, 40S and 60S, or 60S subunits as indicated. 40S-IRES and 80S-IRES complexes were observed as a supershifted bands, indicated by the arrows. Note that the purified 60S fraction was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

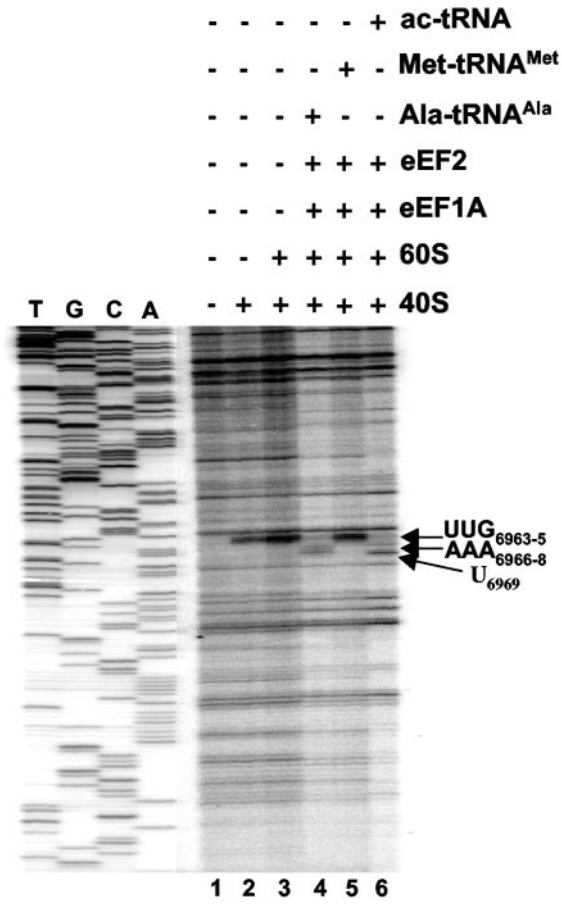

FIG. 7. Ribosomal translocations revealed by toeprinting analysis in a reconstituted system. All numbered lanes are the result of extension of a ^{32}P-labeled primer annealed to a dicistronic RNA that contained the wild-type TSV IGR IRES in the IGR. Reaction mixtures included the components listed at 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.

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 initiator-tRNA^{met} and eIF2, as was observed for the CrPV IRES (7). To test this prediction, purified 40S and 60S ribosomal subunits were incubated with radiolabeled EMCV, CrPV, and TSV IRES elements, and formation of ribosome-IRES complexes was examined in composite agarose gels. 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).

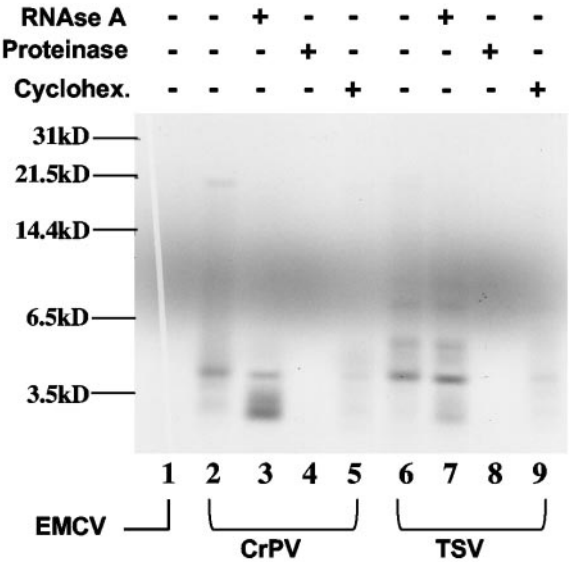

FIG. 8. Peptide synthesis mediated by the TSV IGR IRES in a reconstituted system. Dicistronic RNAs containing the EMCV IRES, the CrPV IGR IRES, or the TSV IGR IRES in the IGR, as indicated, were first incubated with purified 40S and 60S subunits, and then eEF1A, eEF2, and aminoacylated tRNAs were added. Reactions were then treated with 0.7 mg of RNase A/ml or 2 mg of proteinase/ml, as indicated. Control reactions were also performed in the presence of 500 μg of cycloheximide/ml, as indicated. Reaction products were separated on Tris-Tricine 16.5% polyacrylamide gels. An autoradiograph of the dried gel is shown. The migration of known molecular mass markers is shown on the left.

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 40-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 [^{35}S]methionine and [^{35}S]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 edeine, 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 usually display specificity for ribosomes of a particular group of hosts. This finding may indicate that IGR IRES-like mRNAs are translationally initiated using ribosomal features that are universal. Of course, the presence of such divergent IRES elements in cellular mRNAs would also indicate that the number of predicted open frames may be greater than anticipated so far.

ACKNOWLEDGMENTS

We thank Bill Merrick and Karen Browning for supplying eEF1A and eEF2. We also thank Donald Lightner for TSV cDNA plasmids. We are grateful to Karla Kirkegaard for critical reading of the manuscript.

This work was supported by grant GM55979 from the National Institutes of Health.

REFERENCES

1. **Bonami, J. R., K. W. Hasson, J. Mari, B. T. Poulos, and D. V. Lightner.** 1997. Taura syndrome of marine penaeid shrimp: characterization of the viral agent. J. Gen. Virol. **78:**313–319.
2. **Dinos, G., D. N. Wilson, Y. Teraoka, W. Szaflarski, P. Fucini, D. Kalpaxis, and K. H. Nierhaus.** 2004. Dissecting the ribosomal inhibition mechanisms of edeine and pactamycin: the universally conserved residues G693 and C795 regulate P-site RNA binding. Mol. Cell **13:**113–124.
3. **Govan, V. A., N. Leat, M. Allsopp, and S. Davison.** 2000. Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect-infecting RNA viruses. Virology **277:**457–463.
4. **Hatakeyama, Y., N. Shibuya, T. Nishiyama, and N. Nakashima.** 2004. Structural variant of the intergenic internal ribosome entry site elements in dicistroviruses and computational search for their counterparts. RNA **10:**779–786.
5. **Hershey, J., and W. C. Merrick.** 2000. Pathway and mechanism of initiation of protein synthesis, p. 33–88. *In* N. Sonenberg, J. Hershey, and M. B. Mathews (ed.), Translational control of gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
6. **Jan, E., T. G. Kinzy, and P. Sarnow.** 2003. Divergent tRNA-like element supports initiation, elongation, and termination of protein biosynthesis. Proc. Natl. Acad. Sci. USA **100:**15410–15415.
7. **Jan, E., and P. Sarnow.** 2002. Factorless ribosome assembly on the internal ribosome entry site of cricket paralysis virus. J. Mol. Biol. **324:**889–902.
8. **Kanamori, Y., and N. Nakashima.** 2001. A tertiary structure model of the internal ribosome entry site (IRES) for methionine-independent initiation of translation. RNA **7:**266–274.
9. **Kozak, M., and A. J. Shatkin.** 1978. Migration of 40S ribosomal subunits on messenger RNA in the presence of edeine. J. Biol. Chem. **253:**6568–6577.
10. **Leat, N., B. Ball, V. Govan, and S. Davison.** 2000. Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. J. Gen. Virol. **81:**2111–2119.
11. **Lightner, D. V.** 1996. Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. Rev. Sci. Tech. **15:**579–601.
12. **Mari, J., B. T. Poulos, D. V. Lightner, and J. R. Bonami.** 2002. Shrimp Taura syndrome virus: genomic characterization and similarity with members of the genus Cricket paralysis-like viruses. J. Gen. Virol. **83:**915–926.
13. **Mayo, M. A.** 2002. A summary of taxonomic changes recently approved by ICTV. Arch. Virol. **147:**1655–1663.
14. **Nakashima, N., J. Sasaki, and S. Toriyama.** 1999. Determining the nucleotide sequence and capsid-coding region of himetobi P virus: a member of a novel group of RNA viruses that infect insects. Arch. Virol. **144:**2051–2058.
15. **Nishiyama, T., H. Yamamoto, N. Shibuya, Y. Hatakeyama, A. Hachimori, T. Uchiumi, and N. Nakashima.** 2003. Structural elements in the internal ribosome entry site of Plautia stali intestine virus responsible for binding with ribosomes. Nucleic Acids Res. **31:**2434–2442.
16. **Odon, O. W., G. Kramer, A. B. Henderson, P. Pinphanichakarn, and B. Hardesty.** 1978. GTP hydrolysis during methionyl-tRNA$_f$ binding to 40S ribosomal subunits and the site of edeine inhibition. J. Biol. Chem. **253:**1807–1816.
17. **Pestova, T. V., and C. U. Hellen.** 2003. Translation elongation after assembly of ribosomes on the Cricket paralysis virus internal ribosomal entry site without initiation factors or initiator tRNA. Genes Dev. **17:**181–186.
18. **Pestova, T. V., C. U. Hellen, and I. N. Shatsky.** 1996. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol. Cell. Biol. **16:**6859–6869.
19. **Pestova, T. V., I. N. Shatsky, S. P. Fletcher, R. J. Jackson, and C. U. Hellen.** 1998. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev. **12:**67–83.
20. **Rueckert, R. R.** 1996. *Picornaviridae*: the viruses and their replication, p. 609–654. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
21. **Sasaki, J., and N. Nakashima.** 1999. Translation initiation at the CUU codon is mediated by the internal ribosome entry site of an insect picorna-like virus in vitro. J. Virol. **73:**1219–1226.
22. **Sasaki, J., N. Nakashima, H. Saito, and H. Noda.** 1998. An insect picorna-like virus, Plautia stali intestine virus, has genes of capsid proteins in the 3′ part of the genome. Virology **244:**50–58.
23. **Spahn, C. M., J. S. Kieft, R. A. Grassucci, P. A. Penczek, K. Zhou, J. A. Doudna, and J. Frank.** 2001. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40S ribosomal subunit. Science **291:**1959–1962.
24. **Spahn, C. M. T., E. Jan, A. Mulder, R. A. Grassucci, P. Sarnow, and J. Frank.** 2004. Cryo-EM visualization of a viral internal ribosome entry site (IRES) bound to human 40S and 80S ribosomes: the IRES functions as an RNA-based translation factor. Cell **118:**465–475.
25. **Wilson, J. E., T. V. Pestova, C. U. Hellen, and P. Sarnow.** 2000. Initiation of protein synthesis from the A site of the ribosome. Cell **102:**511–520.
26. **Wilson, J. E., M. J. Powell, S. E. Hoover, and P. Sarnow.** 2000. Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites. Mol. Cell. Biol. **20:**4990–4999.