Functional Insights into the Adjacent Stem-Loop in Honey Bee Dicistroviruses That Promotes Internal Ribosome Entry Site-Mediated Translation and Viral Infection

Hilda H. T. Au,a Valentina M. Elspass,a Eric Jana

*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada

**ABSTRACT** All viruses must successfully harness the host translational apparatus and divert it toward viral protein synthesis. Dicistroviruses use an unusual internal ribosome entry site (IRES) mechanism whereby the IRES adopts a three-pseudoknot structure that accesses the ribosome tRNA binding sites to directly recruit the ribosome and initiate translation from a non-AUG start site. A subset of dicistroviruses, including the honey bee Israeli acute paralysis virus (IAPV), encode an extra stem-loop (stem-loop VI [SLVI]) adjacent to the intergenic region (IGR) IRES. Previously, the function of this additional stem-loop was unknown. Here, we provide mechanistic and functional insights into the role of SLVI in IGR IRES translation and in virus infection. Biochemical analyses of a series of mutant IRESs demonstrated that SLVI does not function in ribosome recruitment but is required for proper ribosome positioning on the IRES to direct translation. Using a chimeric infectious clone derived from the related cricket paralysis virus, we showed that the integrity of SLVI is important for optimal viral translation and viral yield. Based on structural models of ribosome-IGR IRES complexes, SLVI is predicted to be in the vicinity of the ribosome E site. We propose that SLVI of IAPV IGR IRES functionally mimics interactions of an E-site tRNA with the ribosome to direct positioning of the tRNA-like domain of the IRES in the A site.

**IMPORTANCE** Viral internal ribosome entry sites are RNA elements and structures that allow some positive-sense monopartite RNA viruses to hijack the host ribosome to start viral protein synthesis. We demonstrate that a unique stem-loop structure is essential for optimal viral protein synthesis and for virus infection. Biochemical evidence shows that this viral stem-loop RNA structure impacts a fundamental property of the ribosome to start protein synthesis.

**KEYWORDS** Drosophila, RNA, dicistrovirus, infection, infectious clone, protein synthesis, ribosome, structure, translation, virus

The majority of cellular mRNAs utilize a cap-dependent scanning mechanism to initiate translation. This mechanism involves ~12 core translation initiation factors to recruit the 40S small ribosomal subunit to the 5' cap, followed by 40S scanning to reach the appropriate AUG start codon (1). Alternatively, some viruses initiate translation using an internal ribosome entry site (IRES) mechanism, which is commonly dependent on a reduced subset of canonical factors (reviewed in reference 2). Based on factor requirements and phylogenetic analysis, viral IRESs are classified into four main classes. The type IV IRESs, found in the *Dicistroviridae* family, utilize the most streamlined mechanism to recruit ribosomes without translation factors or initiator Met-tRNA^i^, which has provided a powerful model to study RNA-ribosome interactions that impact translational initiation and ribosome function. In this study, we examine the role of a
novel stem-loop (stem-loop VI [SLVI]) that is 5′ adjacent to a subset of dicistrovirus intergenic region (IGR) IRESs.

Dicistroviruses are nonenveloped, monopartite RNA viruses that are pathogenic to arthropods of agricultural and medical relevance (3). The viral family includes species such as cricket paralysis virus, which is infectious to the model organism *Drosophila melanogaster*, and Taura syndrome virus, which has caused substantial economic losses in the shrimp industry. Additionally, honey bee dicistroviruses, including Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), and acute bee paralysis virus (ABPV), have been implicated in honey bee disease (4, 5). The dicistrovirus genome is comprised of a positive-sense, single-stranded RNA which bears a genome-linked viral protein (Vpg) covalently linked to the 5′ terminus and a poly(A) tail at the 3′ end. The unusual bicistronic arrangement of the genome confers temporal and independent control to effectuate the coordinated synthesis of viral nonstructural and structural proteins through IRES-dependent translational mechanisms (6–8). Both cistrons are translated as polyprotein precursors that undergo subsequent processing by the virally encoded protease to yield mature proteins. The first open reading frame (ORF1), which is expressed under the regulation of the 5′ IRES, encodes viral nonstructural proteins, including a suppressor of RNA-mediated silencing, helicase, 3C-like protease, and RNA-dependent RNA polymerase (RdRp). The downstream ORF2, which is regulated translationally by the IGR IRES, encodes the capsid proteins.

The approximately 180-nucleotide IGR IRES uses a streamlined mechanism to recruit ribosomes independently of canonical initiation factors or initiator Met-tRNA, (9–12). Biochemical and structural studies have revealed unique RNA-ribosome interactions that enable the IGR IRES to engage the ribosome and initiate translation. The IGR IRES adopts a triple-pseudoknot structure comprised of pseudoknot I (PKI), PKII, and PKIII, which fold independently into two domains: PKII and I form a solvent-inaccessible core responsible for ribosome binding, while PKI mediates proper ribosome positioning to establish the reading frame and initiate translation from a non-AUG codon (9–18). Extensive tRNA mimicry within the PKI domain, which encompasses both anticodon- and codon-like elements connected in *cis*, enables the IRES to gain access to the ribosome's tRNA binding sites (16). Moreover, conserved elements, such as SLV, SLVI, and L1.1, of the IGR IRES establish contacts with conserved ribosomal components, including ribosomal protein uS7 and the large subunit L1 stalk, to directly bind and engage the ribosome (19–23). High-resolution cryo-electron microscopy (cryo-EM) structures revealed that PKI is positioned in the ribosomal A site upon initial ribosome binding (22, 24). Intersubunit rotation of the ribosome engages eukaryotic elongation factor 2, which, together with 40S subunit head swiveling, promotes pseudotranslocation of the PKI domain to the P site, thus vacating the A site for delivery of the first aminoacyl-tRNA to the non-AUG initiation codon (23, 25–27). Following a subsequent pseudotranslocation step in which PKI and aminoacyl-tRNA transit into the E and P sites, respectively, translation enters into the elongation cycle utilizing eIF1A and eEF2; however, there are kinetic data that indicate that these initial translocation steps are slow (28). Finally, the IGR IRES translation model is physiologically relevant, as demonstrated using a recently developed dicistrovirus infectious clone (29, 30). IGR IRES-mediated translation initiation is driven exclusively by an RNA structure to bypass the requirements of the canonical initiation pathway, thereby providing a unique and advantageous viral strategy to commandeer the ribosome for viral protein synthesis.

Bioinformatic analyses identified novel conserved features within a subset of dicistroviruses. Sequence alignment of the honey bee (IAPV, KBV, and ABPV) and fire ant (Solenopsis invicta virus 1) dicistroviruses resulted in the discovery of a novel overlapping gene (31, 32). The alternate gene, ORFx, is encoded in the +1 translational reading frame within the 5′-proximal region of the cistron encoding viral structural proteins (31, 32). Initiation of ORFx translation requires key elements within the IAPV tRNA-like PKI domain, including a U-G wobble base pair adjacent to the IRES translation start site (33). Additionally, a hairpin element, designated SLVI, was identified 5′ adjacent to the IGR IRE5s of IAPV, KBV, and ABPV (Fig. 1) (32). The stem-loop structures of IAPV and KBV are
capsid proteins. ORF1 and -2 expression is translationally and temporally regulated by the 5’ IGR IRES bound to the 80S ribosome of *Kluyveromyces lactis*. (A) The *Dicistroviridae* genome is comprised of a positive-sense, single-stranded RNA molecule that bears a 5’ genome-linked viral protein (VPg) and a 3’ poly(A) tail. The genome consists of two open reading frames (ORFs): the upstream cistron encodes viral nonstructural proteins, and the downstream cistron encodes viral capsid proteins. ORF1 and -2 expression is translationally and temporally regulated by the 5’ IRES and the IGR IRES, respectively. The IGR IRES adopts a triple-pseudoknot structure consisting of three pseudoknots (PKI, -II, and -III) which fold independently into two domains. PKII and -III together form the ribosome binding domain, while PKI forms the tRNA mimicry domain to establish the translational reading frame. SLVI located 5’ adjacent to the IGR IRES has been identified in a subset of dicistroviruses, including IAPV, ABPV, and KBV. (B) Structure of the related cricket paralysis virus IGR IRES bound to the 80S ribosome of *Kluyveromyces lactis* facts. The 40S and 60S ribosomal subunits are depicted in yellow and blue, respectively. SLVI of the honey bee dicitroviruses is predicted to be in the vicinity of the E site, near L1.1 of the IRES (shaded in gray). (Modified from reference 24 under a Creative Commons CC BY 3.0 license [https://creativecommons.org/licenses/by/3.0/]). (C) 5’, 3’, and compensatory mutations of SLVI. The mutated nucleotides are depicted in white. For IAPV, the ORF1 stop codon is encompassed in the apical loop of SLVI (bold and red).

FIG 1 Secondary structure of Israeli acute paralysis virus (IAPV) and cognate stem-loop VI (SLVI) mutants. (A) The *Dicistroviridae* genome is comprised of a positive-sense, single-stranded RNA molecule that bears a 5’ genome-linked viral protein (VPg) and a 3’ poly(A) tail. The genome consists of two open reading frames (ORFs): the upstream cistron encodes viral nonstructural proteins, and the downstream cistron encodes viral capsid proteins. ORF1 and -2 expression is translationally and temporally regulated by the 5’ IRES and the IGR IRES, respectively. The IGR IRES adopts a triple-pseudoknot structure consisting of three pseudoknots (PKI, -II, and -III) which fold independently into two domains. PKII and -III together form the ribosome binding domain, while PKI forms the tRNA mimicry domain to establish the translational reading frame. SLVI located 5’ adjacent to the IGR IRES has been identified in a subset of dicistroviruses, including IAPV, ABPV, and KBV. (B) Structure of the related cricket paralysis virus IGR IRES bound to the 80S ribosome of *Kluyveromyces lactis* facts. The 40S and 60S ribosomal subunits are depicted in yellow and blue, respectively. SLVI of the honey bee dicitroviruses is predicted to be in the vicinity of the E site, near L1.1 of the IRES (shaded in gray). (Modified from reference 24 under a Creative Commons CC BY 3.0 license [https://creativecommons.org/licenses/by/3.0/]). (C) 5’, 3’, and compensatory mutations of SLVI. The mutated nucleotides are depicted in white. For IAPV, the ORF1 stop codon is encompassed in the apical loop of SLVI (bold and red).
activity is not known, but hints of its function have been gleaned from in vitro studies using reporter constructs. SLVI acts cooperatively with a partial sequence of IAPV ORF2 (nucleotides 6618 to 6908) to elicit an approximately 2-fold stimulatory effect on IGR IRES activity in vitro (33); however, within the context of a bicistronic reporter construct, fusion of SLVI with the upstream reporter to mimic the natural genomic arrangement of IAPV, such that ORF1 is in frame with the stop codon within SLVI, yielded a consistent decrease in both scanning- and IRES-dependent translation, thus suggesting that SLVI may have a role in coupling translation of ORF1 and ORF2 (33). Furthermore, mutational analysis of the SLVI helical stem showed that its integrity is important for optimal IRES-dependent translation (33). In this study, we obtained biochemical evidence that SLVI does not play a role in ribosome binding but instead contributes to PKI domain positioning in the ribosomal A site. Using a novel chimeric CrPV infectious clone containing the IAPV IGR IRES, we demonstrated that SLVI is important for optimal viral protein synthesis and viral yield. This work sheds light on the functional role of a stem-loop in IRES translation, which likely imparts functions reminiscent of an E-site tRNA-ribosome interaction.

RESULTS

5' and 3' mutations of SLVI disrupt stem-loop formation. We previously explored the functional contribution of SLVI to IRES-dependent translation in vitro using specifically designed mutations that disrupt stem-loop formation (33). The 5' (M1) base substitutions of the helical region of SLVI result in synonymous changes upstream of the natural stop codon of the first viral cistron (Fig. 1C). The 3' mutation (M2) was designed to complement the 5' mutation, which, when introduced simultaneously, would restore the structure of the stem-loop (M1/M2). The effects of these mutations were investigated within two contexts: (i) when the stop codon within SLVI is fused in frame with the upstream reporter to mimic the natural viral genomic arrangement and (ii) as a discrete element in the intercistronic region of a dual-luciferase reporter. Disruption of SLVI reduced IRES-mediated translation within both sequence contexts, thus suggesting that its formation enhances IGR IRES activity in vitro (33).

To verify that SLVI mutations conferred the intended effects in disruption of the helical stem, RNA structural probing was performed on the wild-type and mutant IRESs (Fig. 2). Base pair formation was interrogated using dimethyl sulfate (DMS), which methylates unpaired adenosines and cytosines at the N1 and N3 positions, respectively (34). The sites of modification can be identified by an arrest in the primer extension reaction due to the presence of the adduct. We initially performed primer extension using an oligonucleotide (PrHA40) that annealed downstream of the IRES to assess global changes in IRES structure. For the wild-type IRES, strong arrests in primer extension were observed primarily in sequences that constitute single-stranded regions and loops/bulges of the IRES (Fig. 2A). For example, strong DMS-induced arrests were observed at the unpaired A6576 and the adenosines and cytosines within the loop of SLIII (Fig. 2A). Comparison of the DMS modification profiles for the wild-type and SLVI mutant IRESs did not reveal any dramatic changes, suggesting that the general architecture of the IRES was preserved (Fig. 2A).

To interrogate specific structural changes in SLVI, primer extension analysis was performed using an alternate primer (PrHA190) that annealed internally within the IRES. The extent of DMS modification was assessed across all nucleotides that constitute SLVI, and positions that exhibited high reactivity (>0.5) are indicated on the corresponding secondary structure (Fig. 2B). For the wild-type IRES, treatment with DMS resulted in modification of the two adenosines within the apical loop of SLVI (Fig. 2B, wild type), whereas adenosine and cytosine residues within the helical region showed little or no reactivity toward DMS, indicating that SLVI adopted a secondary structure as predicted. For mutants M1 and M2, DMS modification was detected at residues that are predicted to be unpaired in the secondary structural model, including A6397, A6398, and A6401 for M1 and A6397 and A6398 for M2 (Fig. 2B). Interestingly, for both M1 and M2, reactivities were observed at some adenosines which have the potential to engage in
base pairing with adjacent nucleotides (A6393, A6403, and A6413 for M1 and A6385, A6386, A6388, and A6393 for M2), suggesting that these interactions were absent or occurred transiently (Fig. 2B). The DMS modification profile of the compensatory mutation (M1 + M2) resembled that of the wild type, thus indicating that base-pairing
interactions along the helical stem were likely restored (Fig. 2B, M1+M2). Taken together, DMS probing results indicate that the 5' and 3' mutations resulted in disruption of the SLVI structure, and when they were introduced concomitantly to restore base pairing, SLVI formation was restored.

An intact SLVI contributes to proper positioning of the ribosome. SLVI is located at the extreme 5' end of the IGR IRES near the conserved L1.1 region (Fig. 1). Structural studies indicate that L1.1 interacts with the L1 stalk, functionally mimicking an E-site tRNA (20, 24) (Fig. 1B). Mutations in L1.1 disrupt 60S joining and 80S binding and positioning, suggesting that L1.1, though occupying the E site of the ribosome, can contribute to proper placement of the PKI domain in the ribosomal A site (20, 35). Given the proximity of SLVI to L1.1, we determined the dissociation constants ($K_d$) of 80S ribosomes binding to wild-type and mutant IAPV IRESs by filter binding analysis. RNAs labeled at the 5' end using $^{32}$P were incubated with increasing amounts of 40S and 60S subunits. For the wild-type IAPV IRES, the $K_d$ (4.8 ± 0.6 nM) was consistent with values previously reported for related IGR IRESs (35–37). Mutants M1 and M2, both of which exhibited translational defects, and the compensatory mutation M1+M2, demonstrated robust ribosome binding that was comparable to or exceeded that of the wild-type IRES (Fig. 3); thus, SLVI likely does not have a significant role in ribosome recruitment.

Because SLVI mutants did not exhibit impaired ribosome binding, we next examined whether the defect in IRES translation occurs at a downstream step. To determine if SLVI formation contributes to ribosome positioning on the IGR IRES (i.e., PKI domain in the A site), toeprinting/primer extension was performed on IRES/ribosome complexes. Reverse transcription terminates upon encountering the leading edge of the ribosome, and the resultant cDNA (the toeprint) can be used to infer the specific nucleotides of the query sequence that occupy the ribosomal A and P sites. Initial binding of the IRES to the ribosome positions the tRNA-like PKI domain in the A site (22, 24, 25, 38); toeprinting results can therefore be used to infer A site occupancy by the PKI domain and consequently the reading frame. Toeprinting profiles were determined for wild-

![Graph showing binding affinities of wild-type and mutant IRESs.](image)

**FIG 3** Binding affinities of wild-type and mutant IRESs. The dissociation constants of the wild-type and mutant IRESs were determined by filter binding. Radiolabeled IRES RNAs were incubated with increasing amounts of purified, salt-washed 40S and 60S ribosomal subunits and subsequently applied to a double membrane of nitrocellulose and nylon using a Bio-Dot filtration apparatus. The membranes were dried and subjected to autoradiography.
type or mutant IAPV IRES RNAs bound to purified, salt-washed ribosomes isolated from HeLa cells. As observed previously, ribosome assembly on the wild-type IAPV IRES RNA yielded a discrete toeprint at A6628, 14 nucleotides downstream of the CCU codon constituting PKI base pairing in the ribosomal A site (with the first C designated +1) (Fig. 4A, lane 2, and B) (39). As expected, disruption of PKI base pairing (ΔPKI) or all PKI/II/III basepairing concomitantly (ΔPKI-III) eliminated toeprint A6628, consistent with the established role of PKI in ribosome positioning (9) (Fig. 4A, lanes 4 and 6). For both the 5′ = and 3′ SLVI mutants, the same A6628 toeprint was observed, albeit at reduced intensities (Fig. 4A, lanes 8 and 10). The ability of ribosomes to assemble on mutant M2 was more severely impaired, as supported by the approximately 62% reduction in toeprint intensity (Fig. 4C). A reproducible decrease in toeprint intensity was observed for M1 and M2 that correlated with the IRES translational activities (33), thus indicating that the translational defect observed with SLVI disruption was due to impaired ribosome positioning on the IRES. Additionally, restoration of stem-loop formation (M1+2) was sufficient to rescue the toeprint to approximately 82% of wild-type intensity (Fig. 4A, lane 12, and C), further underscoring the importance of an intact SLVI in optimal ribosome positioning and IRES activity.

A CrPV/IAPV chimeric clone is infectious in Drosophila Schneider line 2 (S2) cells. Having demonstrated that impaired ribosome positioning is a likely cause for the translational defects observed for SLVI mutants, we sought to investigate if and how these defects manifest during virus infection. Due to the lack of an infectious clone for IAPV or other honey bee dicistroviruses which would be amenable to genetic manipulation, we derived a chimeric virus from the related full-length CrPV infectious clone, pCrPV-3, by replacement of the CrPV IGR IRES with that of IAPV (30). The substitution was made such that it included IAPV IRES sequences immediately downstream of SLVI and encompassing PKI base pairing. The CrPV structural protein coding sequence follows immediately downstream of the IAPV IRES (Fig. 5A, SLVI). To verify that mature viral proteins can be expressed and processed, in vitro-transcribed RNA derived from the chimera was incubated in Spodoptera frugiperda (SF21) translation extract in the presence of [35S]methionine-cysteine. Protein expression was monitored alongside the
FIG 5 The chimeric IAPV/CrPV virus is infectious in Drosophila S2 cells. (A) Schematic of the chimeric virus derived from the full-length CrPV infectious clone by replacement of the CrPV IGR IRES with that of IAPV. (−SLVI), chimera lacking SLVI; (+SLVI), SLVI fused in frame with ORF1 such that the stop codon (UAA) is encompassed in the apical loop; (+SLVI)', SLVI resides in the intercistronic region, downstream (Continued on next page)
wild-type CrPV-3 RNA for reference. As shown previously, CrPV-3 resulted in the expression of ORF1 and ORF2 viral proteins (30). For chimera –SLVI, proteins corresponding to the mature viral proteins were also detected, with a profile that reflected that of CrPV-3 (Fig. 5B, lanes 2 and 4). To demonstrate specificity, introduction of a stop codon into ORF2 (A6232T) eliminated the expression of the corresponding viral structural proteins (Fig. 5B, lanes 3 and 5). Given these results, we demonstrated that viral nonstructural and structural proteins can be expressed in vitro from the chimeric clone.

To determine if the chimeric clone is infectious, in vitro-transcribed RNA was transfected into Drosophila S2 cells, and the expression of mature capsid protein VP2 was monitored by immunoblotting at 48 h posttransfection (h.p.t.) (Fig. 5C). As expected, VP2 protein could be detected by immunoblotting following transfection of CrPV-3 and similarly for chimera –SLVI, indicating that the chimeric virus is infectious in S2 cells. Consistent with this, decreased levels of tubulin were observed for both CrPV-3 and chimera –SLVI transfections, further supporting the onset of a productive infection and the induction of cytopathic effects. To confirm that infectious virions were generated, cell lysates were collected and used to reinfect naive cells for viral titers. Results indicate that chimera –SLVI was indeed infectious and the resultant titer was comparable to that of CrPV-3 (Fig. 5D). Together, these results demonstrate that we generated an infectious chimeric virus harboring the IAPV IGR IRES which can be used as a model to study IAPV IRES structure and function in vivo.

To address the role of SLVI under infection, the stem-loop was introduced into the chimera clone such that ORF1 was fused (i) in frame with the stop codon within SLVI (+SLVFused), thus resembling the natural genomic arrangement of IAPV, or (ii) in the intercistronic region immediately downstream of the ORF1 termination codon (+SLVI) (Fig. 5A). For both the +SLVFused and +SLVI chimeras, incubation of the respective RNAs in Sf21 extract yielded observable bands that corresponded to the mature viral structural proteins (Fig. 5E), suggesting that the placement of SLVI in these two constructs did not affect translation of ORF1 and ORF2 in vitro. In contrast, expression of VP2 capsid protein was detected only for chimera +SLVI and not for chimera +SLVFused following transfection of S2 cells (Fig. 5F). To determine if fusion of the stop codon of SLVI with the upstream cistron affects 5’ IRES activity, Western blotting was performed using antibodies raised against the ORF1 viral RNA-dependent RNA polymerase (RdRp) (40). While the expression of RdRp was observed for chimera –SLVI and chimera +SLVI, it was not detectable for chimera +SLVFused, which suggests an impairment in viral replication and/or IRES-dependent translation (Fig. 5F). Further supporting this, lysates of chimera +SLVFused RNA collected at 48 h.p.t. yielded no measurable infectious virions (Fig. 5G). Given that the same chimera can be efficiently translated in vitro, we speculate that fusing the coding region of SLVI with ORF1, which adds 8 additional amino acids to the C terminus of RdRp, results in impairment of RdRp function. Indeed, insertion of a CrPV 3C site to promote cleavage downstream of RdRp was insufficient to rescue defects associated with SLVI fusion (data not shown), thus suggesting that sequence alterations at the C-terminal end of RdRp were likely detrimental. This may be similar to a report demonstrating that deletion of the C-terminal

FIG 5 Legend (Continued)

of the ORF1 stop codon. (B) In vitro-transcribed RNAs derived from the wild-type CrPV infectious clone (CrPV-3) or the chimera lacking SLVI (–SLVI) were incubated in Sf21 extract for 2 h at 30°C in the presence of [35S]methionine-cysteine. A control without RNA was included to monitor background expression. Reactions were resolved by SDS-PAGE and visualized by autoradiography. A stop codon was introduced into ORF2 of each respective clone to demonstrate specificity of viral structural protein synthesis. The identities of the mature viral proteins, annotated based on predicted molecular mass, are indicated. (C) In vitro-transcribed RNAs from pCrPV-3, chimera –SLVI, or their corresponding ORF2 stop mutants were transfected into Drosophila S2 cells. At 48 h.p.t., cells were harvested and the expression of the VP2 viral capsid protein was monitored by Western blotting. (D) Viral titers were measured at 48 h after transfection of CrPV-3 and chimera –SLVI RNAs, as described in Materials and Methods. Shown are the average values from three independent experiments, ± 1 SD. (E) In vitro translation profiles of chimeras +SLVFused and +SLVI in Sf21 extract. (F) The expression of the viral RNA-dependent RNA polymerase (RdRp) and capsid protein (VP2) was monitored by Western blotting at 48 h after transfection of chimera –SLVI, +SLVFused, and +SLVI RNAs. (G) Viral titers were measured at 48 h.p.t. for chimera –SLVI, +SLVFused, and +SLVI RNAs, as described in Materials and Methods. Shown are the average values from three independent experiments ± SDs.
tryptophan residue of mengovirus RdRp (family Picornaviridae) abrogates viral RNA synthesis and the virus’s ability to induce cytopathic effects (41).

**Disruption of SLVI reduces viral yield due to suboptimal IRES translation.** Because fusion of the coding region of SLVI with ORF1 prohibited virus infection, we resorted to using chimera + SLVI to investigate the role of SLVI within the context of the viral genome. To determine if the functional contribution of SLVI to IRES translation can be recapitulated in vitro using the infectious clone, we introduced identical 5′, 3′, and compensatory mutations into chimera + SLVI and monitored protein synthesis in Sf21 extract. While the M1 mutation yielded robust viral protein translation similar to that with chimera + SLVI, the M2 mutation exhibited substantially reduced translation of structural proteins, although nonstructural protein levels were unaffected. Restoration of SLVI structure rescued structural protein expression (Fig. 6A), thus demonstrating that the formation of an intact SLVI is essential for IRES-mediated translation of the ORF2 structural proteins. To examine the kinetics of viral protein synthesis, S2 cells were infected using chimera + SLVI or the respective mutants at a multiplicity of infection (MOI) of 10. Translation of newly synthesized viral proteins was monitored during infection by [35S]methionine-cysteine pulse-labeling at each time point. Mock-infected cells exhibited extensive incorporation of radioactive methionine-cysteine, indicating active translation (Fig. 6B). At 4 h postinfection (h.p.i.), preferential translation of viral nonstructural and structural proteins was observed for chimera + SLVI and its cognate mutants, concomitant with a decrease in overall translation. While the translation profiles were similar for chimeras + SLVI, M1, and M1 + M2, mutant M2 exhibited an observable decrease in viral structural protein levels, which reflected the in vitro activity of the mutant IRES (Fig. 6B) (33). At 6 h.p.i., diminished expression of structural proteins was still observed for M2, although the difference was less distinguishable. Immunoblotting using RdRp and VP2 antibodies showed decreased accumulation of VP2 protein for mutant M2, despite the absence of observable changes in RdRp levels for chimera + SLVI and its derivatives, consistent with the pulse-labeling profiles (Fig. 6B). Additionally, Northern blotting did not reveal substantial changes in the accumulation of viral RNA between the mutant and wild-type chimeras (Fig. 6C). These results altogether suggest that the perturbation in viral structural protein synthesis observed for mutant M2 is attributable primarily to differences in IGR IRES activity. Finally, to determine how the translational defect associated with mutant M2 affects the output of infectious virions, cells were infected with the wild-type chimera (+ SLVI) virus or the cognate M2 mutant virus at an MOI of 1 or 10. Viral titers were measured using lysates collected at 6 h.p.i. For mutant M2, infection at an MOI of 10 yielded an approximate 50% decrease in viral titers compared to those with the wild-type chimera (Fig. 6D). A similar reduction in the yield of infectious virions was also observed for infection at an MOI of 1. Our viral titer results therefore indicate that suboptimal translation of viral structural proteins due to the 3′ mutations of SLVI directly impacts viral infection. Overall, our results reveal the importance of SLVI formation for optimal IRES activity and viral yield.

**DISCUSSION**

Transcripts containing secondary structures must be unwound by the ribosome in order for translation elongation to proceed (42, 43). For the honey bee dicistroviruses, IAPV, KBV, and ABPV, the upstream cistron terminates within SLVI, which is presumably unwound by the ribosome to gain access to the stop codon. The peculiar location of SLVI 5′ adjacent to the IGR IRES prompted us to explore how the context of this structural element influences translation (33). In this study, we derived a chimera from an infectious clone of the related cricket paralysis virus to address if formation of SLVI is important for virus infection. We demonstrated that the chimera is infectious by transfection of the RNA into Drosophila S2 cells, which resulted in the expression of viral nonstructural and structural proteins and yielded virions that could reinfect naive cells. Importantly, disruption of SLVI through 3′ mutations reduced viral protein synthesis by the IGR IRES due to an impairment in ribosome positioning, as supported by toeprint-
FIG 6 Disruption of SLVI formation decreases viral yield. (A) In vitro-transcribed RNAs derived from chimera +SLVI or its cognate mutants were incubated in SF21 extract for 2 h at 30°C in the presence of [35S]methionine-cysteine. Reactions were resolved by SDS-PAGE and visualized by autoradiography. The respective mutations are as indicated in Fig. 1C. The ratio of ORF2 to ORF1 expression was determined by quantification of the bands indicated by asterisks. (B) Drosophila S2 cells were infected with chimera +SLV or its cognate mutants at an MOI of 10. [35S]methionine-cysteine was added 30 min prior to the indicated time points (2, 4, and 6 h.p.i.) to metabolically label newly synthesized proteins. Labeled proteins were resolved by SDS-PAGE and subjected to phosphorimager analysis. The expression of viral RdRp and VP2 was monitored by Western blotting. (C) The accumulation of viral RNA was visualized by Northern blotting at 2, 4, and 6 h after infection with chimera +SLVI and mutant M2 (MOI = 10). (D) Viral titers were measured 6 h after infection with chimera +SLVI and mutant M2 at MOIs of 1 and 10, as described in Materials and Methods.
ing/primer extension analysis. The defect in IRES-mediated translation resulted in a decrease in the viral titer. Together, our results demonstrate that SLVI is an independent structural element that promotes IAPV IRES activity by facilitating proper ribosome positioning on the IRES.

While our results support the importance of SLVI integrity for optimal IRES activity, it is interesting that 5’ and 3’ mutations in SLVI yielded disparate effects on IRES-mediated translation. Consistent with *in vitro* reporter activities (33), the M2 chimera exhibited a greater defect in IRES translation, both in translation extracts and in infected cells (Fig. 6). One potential explanation is that M1 and M2 mutations have differing effects on SLVI stability; however, comparison of the ΔG values of wild-type, M1, M2, and M1+M2 chimeras suggests that there is no strong correlation of SLVI stability with the observed translational activities. Some adenosine residues that have the potential to base pair with adjacent nucleotides are susceptible to DMS modification (Fig. 2B), which may indicate that SLVI is conformationally dynamic within the context of both the M1 and M2 mutations or may be prone to misfolding. Interestingly, for both mutants M1 and M2, DMS modification does not occur symmetrically between the 5’ and 3’ arms of the stem-loop, and greater reactivity was observed in the 5’-proximal region. This result may suggest that SLVI engages in long-range RNA interactions and that the M1 and M2 mutations may promote long-range structural rearrangements rather than destabilizing formation of the stem-loop. The disparity in the defects exhibited by mutants M1 and M2 within the heterologous infectious system lends further support to this. Because mutant M2 exhibited a significant defect, the specific nucleotide substitutions may have perturbed local or distal structural interactions that are intrinsic to IRES activity. Alternatively, it is plausible that M2 mutations may disrupt specific interactions with the ribosome and/or an unknown interacting partner that decreases the efficiency of IRES-mediated translation, although compensatory mutation suggests that restoration of SLVI base pairing is sufficient to rescue IRES activity.

The ribosome has the capacity to unwind the mRNA and mediate strand separation (43). The ribosome’s intrinsic helicase activity is sufficient to unwind a synthetic inverted repeat of 150 nucleotides engineered into the coding region of mRNA, thus suggesting that extensive RNA structure does not arrest an elongating ribosome (44). Because our results suggest that formation of an intact SLVI is required for optimal IGR IRES-mediated translation, its disruption during translation elongation likely reduces IRES activity. However, unwinding of the helical region may also induce sufficient pausing of the elongating ribosome to cause a pileup of ribosomes, thus resulting in a concomitant decrease in ORF1 expression (33). Indeed, this effect was observed *in vitro* in the translation of a construct in which SLVI was fused with the upstream reporter, which lends support to a potential role of SLVI in mediating negative coupling between the two viral cistrons (33). It is provoking to consider that the SLVI may adopt different folded states or bind to a factor that facilitates temporal regulation of nonstructural and structural protein synthesis during infection. Early in infection, expression of ORF1, encoding the viral protease and replicase, likely interferes with SLVI formation and consequently decreases IGR IRES-mediated translation. As infection progresses, the sensitivity of the 5’ IRES to limiting factors may downregulate ORF1 translation to allow SLVI to adopt its folded state. This may stimulate IGR IRES-mediated translation and enhance the expression of structural proteins for viral packaging. Temporal regulation of dicistrovirus infection is not unprecedented and has been observed during cricket paralysis virus infection (6).

Where SLVI is positioned in the IRES/ribosome complex is not known, and currently, there is no structure of the complete IAPV or a related honey bee dicistrovirus IGR IRES. However, given that the IGR IRES binds in the intersubunit space in the conserved core of the ribosome (21, 45), SLVI may interact with specific ribosomal components to facilitate IRES-mediated translation. From the secondary structure of the IRES, SLVI is located adjacent to loop L1.1, which interacts with the L1 stalk of the E site within the IRES-80S complex (20, 21, 45) (Fig. 1). Recent cryo-EM studies suggest that this interaction couples dynamics of the small subunit with movement of the L1 stalk, which
may facilitate translocation of the IRES (25, 26). Interestingly, mutations in L1.1 also resulted in impairment in ribosome positioning (35). Given its proximity to L1.1, we speculate that SLVI likely interacts with ribosomal elements within the vicinity of the E site (Fig. 1B) and may have complementary roles to L1.1. Additionally, it is well established that an E-site tRNA modulates the affinity of aminoacyl-tRNA binding in the A site, and the presence of a deacylated tRNA in the E site contributes to reading frame maintenance (46–49). SLVI may engage in similar interactions with the ribosome and thereby exert allosteric control in PKI positioning in the A site. The availability of a high-resolution structural model of the IAPV IGR IRES will be particularly advantageous in elucidating molecular interactions with the ribosome.

The functional role of SLVI in virus infection has remained elusive due to the lack of an infectious clone of IAPV. In this study, the development of a CrPV/IAPV chimeric clone enabled studies of the role of this structural element in IAPV IRES-mediated translation and virus infection. However, the chimeric infectious clone does not faithfully represent the natural genomic arrangement of IAPV, in which ORF1 is fused in frame with the SLVI stop codon. Because structural proteins are expressed from the same construct in vitro (Fig. 5), fusion of SLVI at the 3′ end of ORF1 likely renders chimera +SLVI fused noninfectious by impairment of RdRp function rather than faulty translation, although this remains to be tested. Due to this limitation, the functional contribution of SLVI to viral RNA replication cannot be sufficiently addressed or discounted. It is possible that the defect associated with SLVI disruption in the chimera is underestimated; should SLVI contribute to replication or other aspects of the viral life cycle, the defect in infectivity may in fact be exacerbated. Furthermore, as SLVI is present in only a small subset of type II IGR IRESs that are infectious to honey bees, it is possible that this structural element may have a species-specific function, such as having a more prominent effect on ribosomes of honey bee origin or modulating a process that is intrinsic to the viral life cycle of honey bee dicistroviruses. Despite the heterologous nature of the CrPV/IAPV infectious clone system, relevant mechanistic and functional insights into the role of honey bee dicistrovirus IGR IRES can still be obtained, and thus, this system serves as an invaluable tool until a bona fide infectious clone of IAPV or another honey bee virus is established.

MATERIALS AND METHODS

Reporter constructs. Monocistronic luciferase reporter constructs containing the wild-type or mutant IAPV IGR IRESs have been described previously (33). Reporter constructs were linearized using SpeI for in vitro RNA synthesis.

Purification of 40S and 60S ribosomal subunits. 40S and 60S ribosomal subunits were purified from HeLa cell pellets (National Cell Culture Center), as described previously (9). HeLa cells were lysed in lysis buffer (15 mM Tris hydrochloride [pH 7.5], 300 mM sodium chloride, 1% [vol/vol] Triton X-100, 6 mM magnesium chloride, 1 mg/ml of heparin), and the supernatant was subjected to brief centrifugation to remove cellular debris. The supernatant was applied to a 30% (wt/wt) sucrose cushion containing 500 mM potassium chloride and centrifuged at 100,000 × g to pellet ribosomes. Ribosomes were resuspended in buffer B (20 mM Tris hydrochloride [pH 7.5], 6 mM magnesium acetate, 150 mM potassium chloride, 6.8% [wt/wt] sucrose, 2 mM dithiothreitol) and subsequently treated with puromycin (2.3 mM final concentration) to dissociate ribosomes from the mRNAs. Potassium chloride was added to a final concentration of 500 mM. The dissociated ribosomes were resolved on a 10 to 30% [wt/wt] sucrose gradient where the peaks corresponding to the free 40S and 60S subunits were detected by measuring the absorbance at 260 nm. The corresponding fractions were collected, pooled, and concentrated in buffer C (20 mM Tris hydrochloride [pH 7.5], 0.2 mM EDTA, 10 mM potassium chloride, 1 mM magnesium chloride, 6.8% [wt/wt] sucrose) using Amicon Ultra spin concentrations (Millipore). The concentrations of the ribosomal subunits were determined by spectrophotometry using the conversions 1 A_{260b} = 50 nM and 1 A_{260c} = 25 nM for the 40S and 60S subunits, respectively. For assembly of 80S ribosomes, 40S subunits are mixed with a 1.5-fold excess of 60S subunits under the buffer conditions described for the respective assays.

RNA structural probing. Ten picomoles of monoclonal tRNA bearing the wild-type or mutant IRESs was briefly heated to 95°C, followed by cooling to 30°C for 20 min in buffer E (20 mM Tris hydrochloride [pH 7.5], 100 mM potassium chloride, 2.5 mM magnesium acetate, 0.25 mM spermidine, 2 mM dithiothreitol) to induce folding. The prefolded RNA was treated with a 1:200 dilution (in 100% ethanol) of dimethyl sulfate (DMS) for 10 min at 30°C in the presence of nonspecific yeast tRNAs. Following incubation, the reaction was terminated by the addition of quench buffer (30% [vol/vol] β-mercaptoethanol, 300 mM sodium acetate [pH 5.0]), and the modified RNAs were recovered by ethanol precipitation (50). Primer extension was performed on DMS-modified RNAs using 5′-end-labeled primer
PHAb40 (AGTCCGGTATTGTGATCTGGAG) or PHAb190 (ACCAGAGTTATTGGAAATTCCCT), and reactions were analyzed by 8% (wt/vol) denaturing gel electrophoresis. Gels were subsequently dried and subjected to autoradiography. For analysis, individual band intensities were quantitated by semiautomated footprinting analysis (SAFA) software and normalized as previously described (39, 51).

**Filter binding.** One picomole of 5'-end-labeled IRES RNA was incubated with increasing amounts of purified 40S, a 1.5-fold excess of 60S, and 25 ng/μl of noncompetitor RNA in buffer E (20 mM Tris hydrochloride [pH 7.5], 100 mM potassium chloride, 2.5 mM magnesium chloride, 0.25 mM spermidine, 2 mM dithiothreitol). The reaction mixtures were incubated for 20 min at room temperature, after which they were applied to a double membrane of nitrocellulose and nylon using a Bio-Dot filtration apparatus (Bio-Rad). The membranes were subsequently dried and subjected to autoradiography.

**Toeprinting/primer extension analysis.** Toeprinting analysis of ribosomal complexes was performed as described previously (9). Fifteen nanograms of monocistronic wild-type or mutant IRES RNA was annealed to primer PrEJ761 (5'-CATGGGGGTATCGATCCTATTTGGAG-3') by slow cooling from 65°C to 37°C in 40 mM Tris (pH 7.5) and 0.2 mM EDTA. Following primer annealing, the RNAs were incubated with 100 nM and 150 nM 40S and 60S ribosomal subunits, respectively. Ribosome positioning was determined by primer extension analysis as described previously (39).

**Construction of the CrPV/IAPV chimeric infectious clone.** The chimeric infectious clone was derived from the full-length CrPV infectious clone (pCrPV-3; GenBank accession number KM974707 [30]) using the Gibson assembly master mix (New England Biolabs), per the manufacturer’s instructions. For construction of chimaera SLVI, sequences harboring the CrPV IGR IRES (corresponding to nucleotides 6025 to 6216 in pCrPV-3) were replaced with nucleotides 6417 to 6617 of the IAPV genomic sequence (RefSeq accession number NC_009025). Chimaera SLVI was derived by replacement of the CrPV IGR IRES with nucleotides 6372 to 6617 of IAPV (RefSeq accession number NC_009025). To generate chimaera SLVIVend, the ORF1 stop codon of pCrPV-3 (AAA at positions 6022 to 6024) was deleted. All constructs were verified by sequencing.

**In vitro transcription and translation.** Purified plasmid of pCrPV-3 or the derived chimeras was linearized using EcoRI36I. RNA was transcribed in vitro using T7 RNA polymerase and purified using an RNeasy kit (Qiagen). The purity and integrity of the RNA were confirmed by denaturing formaldehyde agarose gel electrophoresis. For in vitro translation, 3 μg of purified RNA was prefolded in buffer E (acetae) (20 mM Tris hydrochloride [pH 7.5], 100 mM potassium acetate, 2.5 mM magnesium acetate, 0.25 mM spermidine, and 2 mM dithiothreitol) and incubated in Spodoptera frugiperda (SF21) extract (Promega) in the presence of [35S]methionine-cysteine (Perkin-Elmer). The reactions were performed at 30°C for 2 h and analyzed by SDS-PAGE. The gels were subsequently dried and subjected to autoradiography and phosphorimager analysis (Typhoon, Amersham).

**Cell culture.** Drosophila Schneider line 2 (S2) cells were maintained and passaged at 25°C in Shields and Sang M3 insect medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum.

**RNA transfection.** Three micrograms of in vitro-transcribed RNA derived from the pCrPV-3 or chimeric clones was transfected into 3.0 × 10^6 S2 cells using Lipofectamine 2000 reagent (Life Technologies) per the manufacturer’s instructions.

**Virus infection.** Drosophila S2 cells were infected at the desired multiplicity of infection in minimal phosphate-buffered saline (PBS), with rocking at 25°C. After 30 min, cells were replenished with complete medium and harvested at the desired time point. For metabolic labeling, 250 μCi/ml of [35S]methionine-cysteine (Perkin-Elmer) was added to cells 30 min prior to the end of the period. Cells were washed once in 1× PBS and harvested in lysis buffer (20 mM HEPES, 150 mM sodium chloride, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM tetrapropylphosphosphate, 100 mM sodium fluoride, 17.5 mM β-glycerophosphate, protease inhibitor cocktail [Roche]).

**Western blotting.** Cells were washed once using 1× PBS and harvested in lysis buffer (20 mM HEPES, 150 mM sodium chloride, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM tetrapropylphosphate, 100 mM sodium fluoride, 17.5 mM β-glycerophosphate, protease inhibitor cocktail [Roche]) as described previously (40). Equal amounts of protein lysate (10 μg) were resolved by SDS-PAGE and subsequently transferred onto polyvinylidene difluoride Immobilon-FL membrane (Millipore). Following transfer, the membrane was blocked for 30 min at room temperature in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated overnight with rabbit polyclonal antibody raised against CrPV RdRp (1:5000) or CrPV VP2 (1:5000). Membranes were washed 3 times with TBST and subsequently incubated with anti-rabbit IgG-horseradish peroxidase (1:20,000; GE Healthcare) for 1 h at room temperature.

**Northern blotting.** Total RNA was isolated from S2 cells using TRizol reagent per the manufacturer’s instructions. Five micrograms of RNA was resolved by denaturing agarose gel electrophoresis and subsequently transferred onto a Zeta- Probe blotting membrane (Bio-Rad). Radiolabeled probe complementary to the CrPV viral genome was synthesized using a DecaLabel DNA labeling kit (Thermo Fisher Scientific) and hybridized to the membrane overnight. Radiolabeled bands were visualized by autoradiography and phosphorimager analysis (Typhoon; Amersham).

**Viral titers.** At the desired time point, S2 cells were washed once and harvested in 1× PBS by three freeze-thaw cycles. Naive cells were infected using serial dilutions of the supernatant for 30 min and transferred to a 96-well plate pretreated with concanavalin A (0.5 mg/ml; MP Biomedicals). At 10 h p.i., cells were fixed with 3% paraformaldehyde and incubated in 1× PBS overnight. Cells were permeabilized by methanol treatment and incubated with anti-VP2 antibody (1:500 dilution in 50 mg/ml of bovine serum albumin [BSA]) and subsequently with goat anti-rabbit Texas Red IgG (1:200 dilution in 50 mg/ml of BSA; Life Technologies). Nuclei were stained using Hoechst dye (0.5 μg/ml; Life Technologies). Infected cells were visualized and quantified using a Cellomics Arrayscan HCS instrument.
ACKNOWLEDGMENTS

We thank members of the lab for critical analyses and discussions. This work was supported by the Canadian Institutes of Health Research funds (PJT-148761 and MOP-81244) to E.J. and an NSERC PGs D doctoral fellowship to H.T.A.

REFERENCES

1. Hinnebusch AG, Lorsch JR. 2012. The mechanism of eukaryotic translation initiation: new insights and challenges, p 29–54. In Hershey JW, Sonenberg N, Mathews MB (ed). Protein synthesis and translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Plank TD, Kieft JS. 2012. The structures of nonprotein-coding RNAs that drive internal ribosome entry site function. Wiley Interdiscip Rev RNA 3:195–212. https://doi.org/10.1002/wrna.1105.
3. Bonning BC, Miller WA. 2010. Dicistroviruses. Annu Rev Entomol 55: 129–150. https://doi.org/10.1146/annurev-ento-112408-085457.
4. Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan PL, Briese T, Homig M, Geiser DM, Martinson V, vanEngelsdorp D, Kalkstein AL, Drysdale A, Hui J, Zhai J, Cui L, Hutchison SK, Simons JF, Egholm M, Pettis JS, Lipkin WI. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318:283–287. https://doi.org/10.1126/science.1146498.
5. Genersch E, Aubert M. 2010. Emerging and re-emerging viruses of the dicistroviridae. Virus Res 15:57–64. https://doi.org/10.1016/j.virusres.2010.03.020.
6. Khong A, Bonderoff JM, Spriggs RV, Tammpere E, Kerr CH, Jackson TJ, Kalkstein AL, Drysdale A, Hui J, Zhai J, Cui L, Hutchison SK, Simons JF, Egholm M, Pettis JS, Lipkin WI. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318:283–287. https://doi.org/10.1126/science.1146498.
7. Wilson JE, Powell MJ, Hoover SE, Sarnow P. 2000. Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribo-entry sites of the Dicistroviridae cricket paralysis virus. J Mol Biol 324:889–902. https://doi.org/10.1006/jmbi.2000.4653.
8. Khong A, Bonderoff JM, Spriggs RV, Tammpere E, Kerr CH, Jackson TJ, Willis AE, Jan E. 2016. Temporal regulation of distinct internal ribosome entry sites of the Dicistroviridae cricket paralysis virus. Viruses 8(1):E25. https://doi.org/10.3390/v8010025.
9. Wilson JE, Powell MJ, Hoover SE, Sarnow P. 2000. Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites. Mol Cell Biol 20:4990–4999. https://doi.org/10.1128/MCB.20.14.4990-4999.2000.
10. Moore NF, Reavy B, Pullin JSK, Plus N. 1981. The polypeptides induced in Drosophila cells by dicistronic cricket paralysis virus RNA are regulated by two internal ribosome entry sites. J Virol 37:144–150. https://doi.org/10.1128/JVI.37.1.144-150.1981.
11. Sasaki J, Nakashima N. 1999. Translation initiation at the CUU codon is suppressed by the A site of the ribosome. Cell 102:511–520. https://doi.org/10.1016/S0092-8674(00)00555-6.
12. Nishiyama T, Yamamoto H, Uchiumi T, Nakashima N. 2007. Eukaryotic ribosomal protein RPS25 interacts with the conserved loop region in a dicistroviral intergenic internal ribosome entry site. Nucleic Acids Res 35:1514–1521. https://doi.org/10.1093/nar/gkl1121.
13. Costantino DS, Kieft JS. 2000. Structural basis for ribosome recruitment and manipulation by a viral IRES RNA. Science 314: 1450–1454. https://doi.org/10.1126/science.1133281.
14. Plank TD, Kieft JS. 2012. The structures of nonprotein-coding RNAs that drive internal ribosome entry site function. Wiley Interdiscip Rev RNA 3:195–212. https://doi.org/10.1002/wrna.1105.
15. Schlüter M, Connell SR, Lesicoute A, Giesebricht J, Dabrowski M, Schroer B, Mielek T, Penczek PA, Westhof E, Saphn CM. 2006. Structure of the ribosome-bound parovirus IRES RNA. Nat Struct Mol Biol 13:1092–1096. https://doi.org/10.1038/nsmb1177.
16. Koh CS, Briot AF, Grigorieff N, Korostelev AA. 2014. Taura syndrome virus IRES initiates translation by binding its tRNA-mRNA-like structural element in the ribosomal decoding center. Proc Natl Acad Sci U S A 111:9139–9144. https://doi.org/10.1073/pnas.1406335111.
17. Ayberrythne PD, Koh CS, Grant T, Grigorieff N, Korostelev AA. 2016. Ensemble cryo-EM uncovers inouchworm-like translocation of a viral IRES through the ribosome. Elife 5:e14874. https://doi.org/10.7554/eLife.14874.
18. Fernández JS, Bai XC, Murshudov G, Scheres SH, Ramakrishnan V. 2014. Initiation of translation by cricket paralysis virus IRES requires its translocation in the ribosome. Cell 157:823–831. https://doi.org/10.1016/j.cell.2014.04.015.
19. Muhs M, Hillal T, Mielek T, Skabkin MA, Sanbonmatsu KY, Pestova TV, Saphn CM. 2015. Cryo-EM of ribosomal 80S complexes with termination factors reveals the translocated cricket paralysis virus IRES. Mol Cell 57:422–432. https://doi.org/10.1016/j.molcel.2014.12.016.
20. Murray J, Savva CG, Shin BS, Dever TE, Ramakrishnan V, Fernandez IS. 2016. Structural characterization of ribosome recruitment and translocation by type IV IRES. Elife 5:e13567. https://doi.org/10.7554/eLife.13567.
21. Petrov A, Grosey R, Chen J, O’Leary SE, Puglisi JD. 2016. Multiple parallel pathways of translation initiation on the P.1PV IRES. Mol Cell 62:92–103. https://doi.org/10.1016/j.molcel.2016.03.020.
22. Zhang H, Ng MY, Chen J, Cooperman BS. 2016. Kinetics of initiating polypeptide elongation in an IRES-dependent system. Elife 5:e13429. https://doi.org/10.7554/eLife.13429.
23. Kerr CH, Ma ZW, Jang CJ, Thompson SR, Jan E. 2016. Molecular analysis of the factorless internal ribosome entry site in cricket paralysis virus infection. Sci Rep 6:37319. https://doi.org/10.1038/srep37319.
24. Kerr CH, Wang QS, Keatings K, Khong A, Allan D, Yip CK, Foster LJ, Jan E. 2015. The S′ untranslated region of a novel infectious molecular clone of the dicistrovirus cricket paralysis virus modulates infection. J Virol 89: 5919–5934. https://doi.org/10.1128/JVI.00463-15.
25. Sabath N, Price N, Graur D. 2009. A potentially novel overlapping gene in the genomes of Israeli acute paralysis virus and its relatives. Virol J 6:144. https://doi.org/10.1186/1743-422X-6-144.
26. Firth AE, Wang QS, Jang CJ, Atkins JF. 2009. Bioinformatic evidence for a stem-loop structure S′-adjacent to the IGR-IRES and for an overlapping gene in the bee paralysis dicistroviruses. Virol J 6:193. https://doi.org/10.1186/1743-422X-6-193.
27. Charbonnel H, Khong A, Allan D, Yip CK, Foster LJ, Jan E. 2016. Alternative reading frame selection mediated by a tRNA-like domain of an internal ribosome entry site. Proc Natl Acad Sci U S A 109:E630–E639. https://doi.org/10.1073/pnas.1111303109.
28. Weeks KM. 2010. Advances in RNA structure analysis by chemical probing. Curr Opin Struct Biol 20:295–304. https://doi.org/10.1016/j.sbi.2010.04.001.
29. Jang CJ, Lo MC, Jan E. 2009. Conserved element of the dicistrovirus IGR IRES that mimics an E-site tRNA/ribosome interaction mediates multiple functions. J Mol Biol 387:42–58. https://doi.org/10.1016/j.jmb.2009.01.042.
30. Jang CJ, Jan E. 2010. Modular domains of the Dicistroviridae intergenic internal ribosome entry site. RNA 16:1182–1195. https://doi.org/10.1261/ma.2044610.

January 2018 Volume 92 Issue 2 e01725-17 jvi.asm.org
Uchiumi T, Nakashima N. 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. https://doi.org/10.1093/nar/gkg336.

38. Au HH, Cornilescu G, Mouzakis KD, Ren Q, Burke JE, Lee S, Butcher SE, Jan E. 2015. Global shape mimicry of tRNA within a viral internal ribosome entry site mediates translational reading frame selection. Proc Natl Acad Sci U S A 112:E6446–E6455. https://doi.org/10.1073/pnas.1512088112.

39. Ren Q, Au HH, Wang QS, Lee S, Jan E. 2014. Structural determinants of an internal ribosome entry site that direct translational reading frame selection. Nucleic Acids Res 42:9366–9382. https://doi.org/10.1093/nar/gku622.

40. Garrey JL, Lee YY, Au HH, Bushell M, Jan E. 2010. Host and viral translational mechanisms during cricket paralysis virus infection. J Virol 84:1124–1138. https://doi.org/10.1128/JVI.02006-09.

41. Dmitrieva TM, Alexeevski AV, Shatskaya GS, Tolskaya EA, Gmyl AP, Khitrina EV, Agol VI. 2007. Significance of the C-terminal amino acid residue in mengovirus RNA-dependent RNA polymerase. Virology 365:79–91. https://doi.org/10.1016/j.virol.2007.02.038.

42. Meyer IM, Miklos I. 2005. Statistical evidence for conserved, local secondary structure in the coding regions of eukaryotic mRNAs and pre-mRNAs. Nucleic Acids Res 33:6338–6348. https://doi.org/10.1093/nar/gki023.

43. Takyar S, Hickerson RP, Noller HF. 2005. mRNA helicase activity of the ribosome. Cell 120:49–58. https://doi.org/10.1016/j.cell.2004.11.042.

44. Lingelbach K, Dobberstein B. 1988. An extended RNA/RNA duplex structure within the coding region of mRNA does not block translational elongation. Nucleic Acids Res 16:3405–3414.

45. Spahn CM, Jan E, Mulder A, Grassucci RA, Samow P, Frank J. 2004. Cryo-EM visualization of a viral internal ribosome entry site bound to human ribosomes: the IRES functions as an RNA-based translation factor. Cell 118:465–475. https://doi.org/10.1016/j.cell.2004.08.001.

46. Márquez V, Wilson DN, Tate WP, Triana-Alonso F, Nierhaus KH. 2004. Maintaining the ribosomal reading frame: the influence of the E site during translational regulation of release factor 2. Cell 118:45–55. https://doi.org/10.1016/j.cell.2004.06.012.

47. Blaha G, Nierhaus KH. 2001. Features and functions of the ribosomal E site. Cold Spring Harbor Symp Quant Biol 66:135–146. https://doi.org/10.1101/sqb.2001.66.135.

48. Geigenmüller U, Nierhaus KH. 1990. Significance of the third tRNA binding site, the E site, on E. coli ribosomes for the accuracy of translation: an occupied E site prevents the binding of non-cognate aminoacyl-tRNA to the A site. EMBO J 9:4527–4533.

49. Nierhaus KH. 2006. Decoding errors and the involvement of the E-site. Biochimie 88:1013–1019. https://doi.org/10.1016/j.biochi.2006.02.009.

50. Tijerina P, Mohr S, Russell R. 2007. DMS footprinting of structured RNAs and RNA-protein complexes. Nat Protoc 2:2608–2623. https://doi.org/10.1038/nprot.2007.380.

51. Das R, Laederach A, Pearlman SM, Herschlag D, Altman RB. 2005. SAFa: semi-automated footprinting analysis software for high-throughput quantification of nucleic acid footprinting experiments. RNA 11:344–354. https://doi.org/10.1261/rna.7214405.