

Structural elements in the internal ribosome entry site of *Plautia stali intestine virus* responsible for binding with ribosomes

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ABSTRACT

***Plautia stali intestine virus* (PSIV) has an internal ribosome entry site (IRES) at the intergenic region of the genome. The PSIV IRES initiates translation with glutamine rather than the universal methionine. To analyze the mechanism of IRES-mediated initiation, binding of IRES RNA to salt-washed ribosomes in the absence of translation factors was studied. Among the three pseudoknots (PKs I, II and III) within the IRES, PK III was the most important for ribosome binding. Chemical footprint analyses showed that the loop parts of the two stem-loop structures in Domain 2, which are highly conserved in related viruses, are protected by 40S but not by 60S ribosomes. Because PK III is close to the two loops, these structural elements were considered to be important for binding of the 40S subunit. Competitive binding analyses showed that the IRES RNA does not bind poly(U)-programmed ribosomes preincubated with tRNA^{Phe} or its anticodon stem-loop (ASL) fragment. However, Domain 3-deleted IRES bound to programmed ribosomes preincubated with the ASL, suggesting that Domains 1 and 2 have roles in IRES binding to 40S subunits and that Domain 3 is located at the ribosome decoding site.**

INTRODUCTION

Internal ribosome entry is a mode of translation initiation in which protein synthesis occurs independently of the 5' mRNA cap (1,2). Tertiary mRNA structures upstream of some coding sequences promote ribosome entry. Such an RNA region responsible for ribosome entry is called an internal ribosome entry site (IRES) (3,4).

The intergenic region (IGR) of the positive-strand RNA virus, *Plautia stali intestine virus* (PSIV), contains an IRES (5). PSIV is a member of the cricket paralysis-like viruses

(CrP-like viruses), which have a dicistronic, positive-stranded RNA genome (6,7). An outstanding feature of IRES-mediated translation initiation is that methionine is not the amino acid used for initiation (6,8,9). Usually, a ternary complex containing eIF2, GTP and initiator methionine transfer RNA (tRNA_i^{Met}) is absolutely required for translation initiation of normal mRNAs (10). However, in PSIV IRES-mediated translation initiation, a pseudoknot (PK) is formed at the initiation site and polypeptide synthesis starts with glutamine (8).

To date, genome sequences of 10 CrP-like viruses have been reported (7,11,12). Sequence alignments and predicted secondary structures of IGR elements from these viruses suggest that they are highly conserved and that they initiate capsid protein translation in a similar manner (13). The IGR elements of CrP-like viruses consist of about 200 nt containing four stem-loops and three PKs. Because IRES-mediated translation initiation does not use methionine, it has been generally believed that the eIF2/GTP/tRNA_i^{Met} ternary complex is not necessary for initiation. Analysis of purified ribosome binding to the cricket paralysis virus (CrPV) IRES shows assembly of an 80S monosome in the absence of eukaryotic translation initiation factors (eIFs) (9). Toeprint analysis shows that a PK is located at the P site of the ribosome (9). This strongly suggests that the ternary complex is not required for IRES-mediated translation initiation. Indeed, an increase in IRES-mediated translation is observed in yeast and mammalian cell lines when eIF2 α phosphorylation is promoted by GCN2, PKR and PKRK kinases and normal translation is suppressed (14,15). In addition, when viral structural protein synthesis is maximal in CrPV-infected cells, eIF2 α is heavily phosphorylated (16). However, molecular details of interactions between the IRES and ribosomes have not been resolved. To approach this question, we have performed *in vitro* binding experiments using PSIV IRES transcripts and high salt-washed ribosomes, and revealed that structurally conserved elements in CrP-like viruses are responsible for its binding to the 40S ribosomal subunit.

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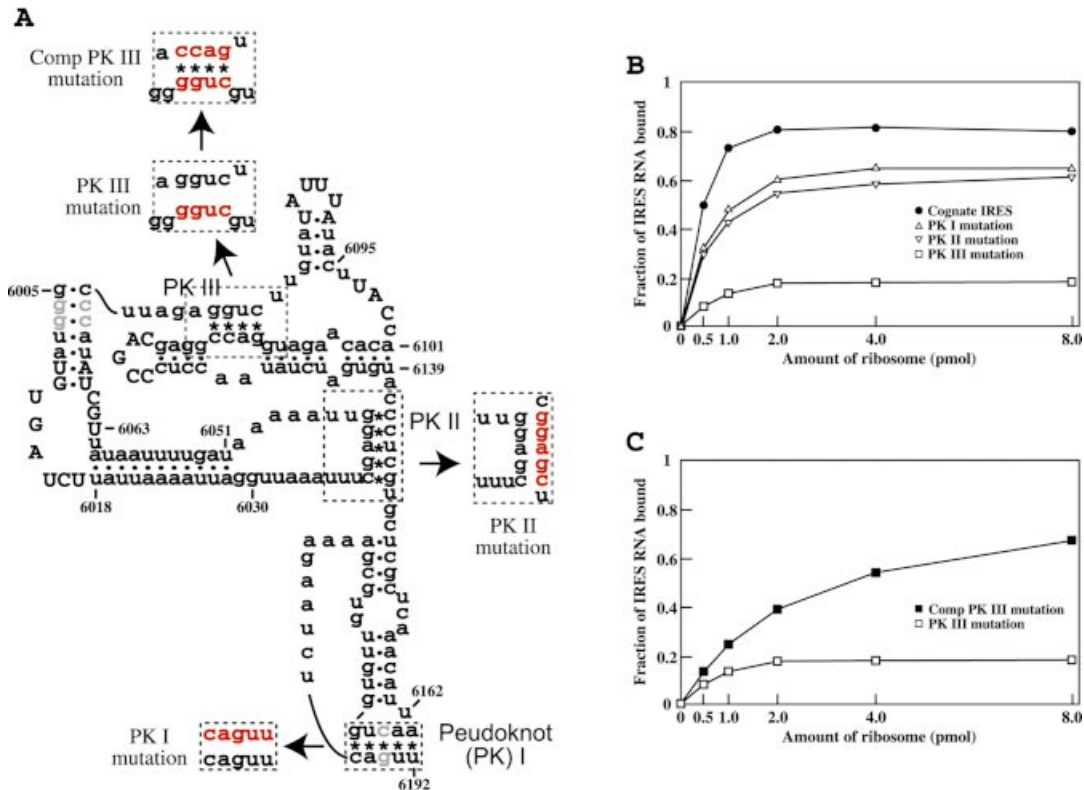


Figure 1. Effect of base pairing in IRES PKs on 80S ribosome binding. (A) Secondary structure model of PSIV IRES RNA. Asterisks and dots indicate base pairing in PKs and helical stems, respectively. Highly conserved nucleotides in CrP-like viruses are capitalized. Half-tones denote nucleotides mutated in these studies to produce the cognate IRES (see Materials and Methods). Nucleotides mutated in PKs, to disrupt base pairing and to restore base pairing by compensatory mutations, are in red. (B) Binding of ³²P-labeled IRES RNAs to 80S ribosomes. The fraction of IRES RNA bound is the ratio of [³²P]RNA retained on the filter to that of the input [³²P]RNA. (C) Effect of the compensatory mutations restoring base pairing in PK III on ribosome binding.

MATERIALS AND METHODS

Plasmid

RNA containing the PSIV IRES, nucleotides 6005–6192 of the PSIV sequence, was synthesized from the dicistronic plasmid pT7CAT-5375 (5) by PCR using primers 5′-GGG-TATGTGATCTTATTAATAAATTAGG-3′ and 5′-AACTGAG-ATTCTTTTCGCACAACA-3′. The underlined nucleotides denote mutations introduced into the PSIV IRES, AC_{6006–6007}→GG and C₆₁₉₀→G. The amplified fragment was ligated with a vector fragment obtained by PCR using pT7Blue (Novagen) as a template and primers 5′-GACTCTAGAGGATCTACTAG-3′ and 5′-TATAGTGAGTCGTATTAGAGCTT-3′. To conserve base pair interactions in the IRES element, two further mutations were introduced into the resultant plasmid, GU_{6070–6071}→CC and G₆₁₆₅→C, by PCR-based mutagenesis, to generate pT7D1-2-3. This plasmid has the sequence GGG immediately downstream of the T7 promoter to facilitate transcription and a *HincII* site at nucleotide 6190. Thus T7 transcription of *HincII*-linearized pT7D1-2-3 produces a cognate IRES element containing all four mutations (Fig. 1A). Variant plasmids containing disrupted or restored base pairs in each PK and truncated IRES sequences were constructed by PCR-based mutagenesis using pT7D1-2-3 as a template. For studies of chemical probes, PSIV nucleotides 5961–6230 were amplified from pT7CAT-5375 with primers having *HindIII* and *EcoRI* sites at their 5′ ends. The amplified

fragment was digested with *HindIII* and *EcoRI*, and ligated into those sites of pT7Blue. For translation activity studies, plasmids that produce capsid–luciferase fusion proteins were constructed. Nucleotides 5951–6241 of the PSIV sequence were amplified from pT7CAT-5375 using primers having *HindIII* and *NcoI* sites at their 5′ ends. The amplified fragments were digested with *HindIII*, phosphorylated and cloned into pT7Blue treated with *HindIII* and *HincII*. Mutations in the IRES were introduced into the resultant plasmid by PCR mutagenesis and replacement of nucleotides was confirmed by sequencing. These plasmids were digested with *HindIII* and *NcoI* to yield 300 bp fragments containing mutated IRESs. The fragments were cloned into *HindIII* and *NcoI* sites of pT7-luc into which had been inserted a luciferase coding sequence (obtained from pSP-*luc+*; Promega) in pT7Blue using *HindIII* and *EcoRI*.

Ribosome isolation

Puromycin-treated and high salt-washed 80S ribosomes were prepared from the posterior part of silkworm silk glands (17). Ribosomal 40S and 60S subunits were prepared from rabbit reticulocyte lysate (Promega) as previously described (18,19).

RNA synthesis and renaturation

Plasmids linearized with restriction enzymes or PCR-amplified fragments containing a T7 promoter were used for transcription. RNAs labeled with ³²P or ³³P were synthesized

and purified as described (20). Non-labeled RNAs were synthesized using a T7 Ribomax large scale RNA production system (Promega). The isolated RNA fragments (in a solution containing 2 mM MgCl₂, 100 mM KCl, 0.2 mM dithiothreitol and 50 mM Tris-HCl, pH 7.5) were heated at 70–75°C for 3 min and then cooled to room temperature.

Filter binding assay

A ribosome sample was incubated with 1 pmol labeled RNA in 50 µl binding buffer (0.5–5 mM MgCl₂, 100 mM KCl, 0.2 mM dithiothreitol and 50 mM Tris-HCl, pH 7.5) at 37°C for 5 min. The mixture was filtered through a nitrocellulose membrane (type HA, 0.45 µm pore size; Millipore). The membrane was washed with 1 ml binding buffer. Radioactivity retained by the filter was counted using a liquid scintillation counter (Aloka). For competitive binding assays with programmed ribosomes, 50 pmol 80S ribosomes were preincubated with 60 µg poly(U), 200 pmol tRNA^{Phe} (Sigma) or 500 pmol of the synthesized anticodon stem-loop (ASL), 5'-GGGGAUUGAAAUC-3', at 37°C for 15 min as described by Iwasaki and Kaziro (21).

Sedimentation analysis

Renatured ³²P-labeled RNAs were incubated with 40S or 60S ribosomes in binding buffer (2 mM MgCl₂, 100 mM KCl and 20 mM Tris-HCl, pH 7.4) at 30°C for 5 min. This mixture then was layered on a 15–30% linear sucrose gradient in binding buffer and centrifuged at 35 000 r.p.m. at 4°C for 3.5 h using an SW 60 rotor (Beckman). Fractions (0.2 ml) were recovered from the bottom of each tube and one-twentieth of each fraction volume was transferred to a solid phase scintillator Ready Cup (Beckman).

Chemical modification and primer extension

Chemical probing of RNAs was done using dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMCT) and hydroxyl radical modification. Preliminary experiments for DMS and CMCT modification were carried out according to the methods of Brunel *et al.* (22), and conditions were optimized for PSIV IRES RNA. Since Mg²⁺ affects RNA folding and tertiary structure (23,24), Mg²⁺ concentrations from 0 to 10 mM were tested and 2 mM Mg²⁺ selected for these experiments, because results were not significantly affected when Mg²⁺ exceeded 2 mM. Prior to chemical modification, 0.4 pmol renatured RNA was incubated at room temperature for 5 min with or without 1.8 pmol rabbit ribosomes. DMS modification was carried out at 20°C for 30 min in a buffer containing 50 mM potassium cacodylate (pH 7.2), 2 mM MgCl₂, 100 mM KCl and 40 mM DMS. CMCT modification was carried out at 20°C for 40 min in a buffer containing 50 mM sodium borate (pH 7.4), 2 mM MgCl₂, 100 mM KCl and 20 mM CMCT. Hydroxyl radical modification was performed as described by Merryman and Noller (25). Two picomoles of renatured IRES RNA was incubated at room temperature for 5 min with or without 6 pmol ribosomal subunits in 21 µl of 96 mM K-HEPES (pH 7.8), 2 mM MgCl₂, 120 mM NH₄Cl and 6 mM DTT. In a separate tube, 4 µl of 50 mM Fe(NH₄)₂(SO₄)₂, 4 µl of 100 mM EDTA, 4 µl of 250 mM ascorbic acid and 4 µl of 2.5% H₂O₂ were mixed sequentially, and immediately 4 µl of the mixture was added to the RNA sample solution. The

reaction was done at 0°C for 10 min, and quenched by adding an equal volume of 1 M thiourea followed by ethanol precipitation.

Modified RNAs were treated twice with phenol-chloroform, precipitated with ethanol and then used for primer extension. Primers complementary to PSIV nucleotides 6230–6211 and 6170–6151 were used for DMS and CMCT probing, and to nucleotides 6170–6151 and 6118–6096 for hydroxyl radical probing. Modified samples were mixed with 0.1 pmol ³²P-labeled primers, and primer extension was carried out at 50°C for 30 min using reverse transcriptase (ReverTra Ace; Toyobo). The resulting cDNA was analyzed on a 6% (w/v) acrylamide sequencing gel.

In vitro translation

RNAs transcribed from linearized plasmids were quantified and translated using a wheatgerm extract (Promega). Luciferase activity in the translation mixtures was measured using the Stedy-Glo Luciferase Assay System (Promega).

RESULTS AND DISCUSSION

Mutational analysis of IRES pseudoknots and ribosome binding

Previous mutational analyses showed that RNA base pairing in PKs I, II and III is essential for IRES translation initiation activity (8,13). To examine the contribution of base pairing in each PK to ribosome binding, base pairs in each PK were disrupted by mutations (Fig. 1A) and ribosome binding was measured. ³²P-labeled cognate and mutated IRES RNAs were incubated with increasing concentrations of 80S ribosomes and ribosome-bound IRES RNA was quantitated by filter binding assays. The amount of ribosome-bound IRES RNA increased with increasing ribosome concentration and reached a maximum at high ribosome concentrations (Fig. 1B). For cognate IRES RNA, 80% of the RNA bound at high ribosome concentrations (Fig. 1B). Mutations disrupting IRES PK base pairing decreased ribosome binding, with mutations in PK III having the greatest effect. At high ribosome concentrations, less than 20% of IRES RNA with mutations in PK III was ribosome bound and about 60% with mutations in PKs I or II was ribosome bound. To confirm the role of base pairing in ribosome binding, compensatory mutations were introduced in disrupted PK III RNA to restore base pairing (Fig. 1A). The PK III construct with compensatory mutations showed an increase in ribosome binding compared to PK III with disrupted base pairing (Fig. 1C), but the binding affinity was not as high as that of the cognate IRES. Similar compensatory mutations to restore base pairing in disrupted PKs I and II had no marked effects on ribosomal binding (data not shown). The modest effects of mutations in both PKs I and II on ribosome binding are consistent with the results of recent binding experiments by Jan and Sarnow (26), in which binding affinity between the CrPV IRES and 40S ribosomes was assayed in a gel shift system. However, mutations in PK III were not tested in the CrPV system. Therefore, these results indicate that the structure of each IRES PK plays a role in ribosome binding, with PK III structure having the most significant role.

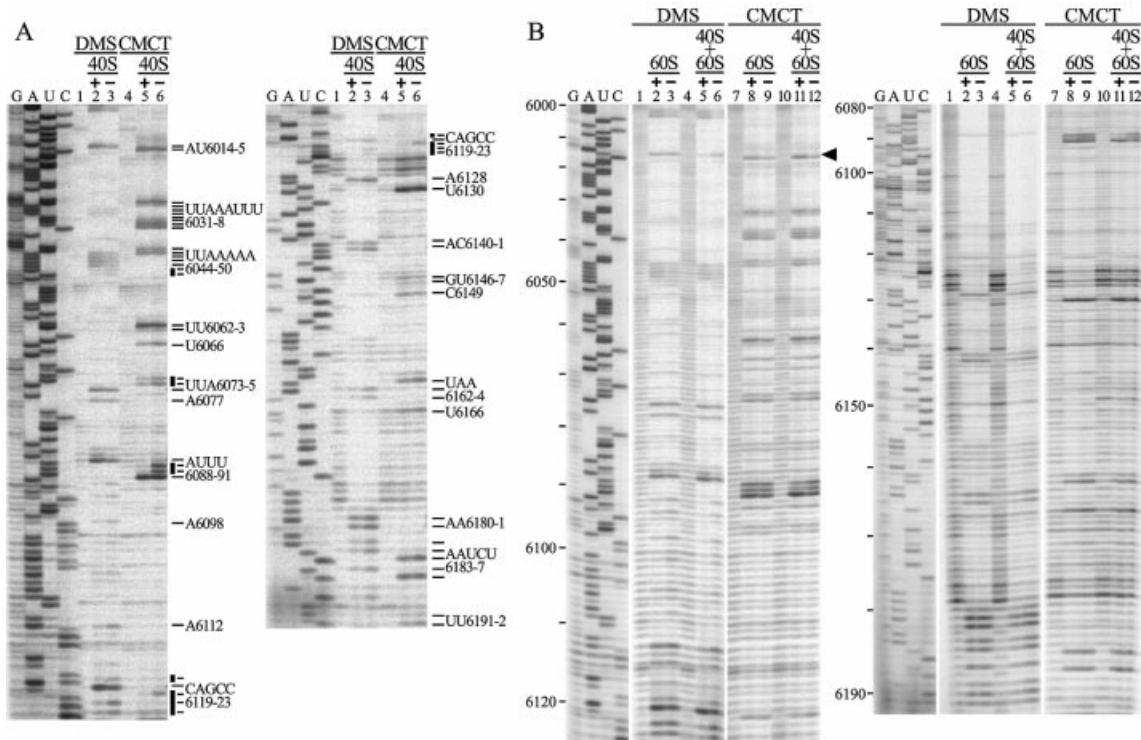


Figure 2. DMS and CMCT probing of the PSIV IRES. To detect modified bases, DMS- and CMCT-modified RNAs were analyzed by primer extension. (A) Modification with DMS (lanes 2 and 3) or CMCT (lanes 5 and 6) in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of 40S ribosomes. Lanes 1 and 4 are the unmodified controls to detect non-specific termination. Bold bars mark IRES nucleotides protected from modification by 40S ribosomes. Nucleotide positions are shown at right. (B) Modification with DMS (lanes 2, 3, 5 and 6) or CMCT (lanes 8, 9, 11 and 12) in the presence of 60S (lanes 2 and 8) or 40S (lanes 5 and 11) ribosomes. Lanes 1, 4, 7 and 10 are the unmodified controls. The arrowhead marks A_{6014} , which was only protected in the presence of both 40S and 60S ribosomes.

Chemical probing of IRES structure

A secondary structure model of the PSIV IRES (Figs 1A and 4A) was generated using the MFOLD program (27) and subsequently updated based on results of PSIV mutation experiments and comparison of CrP-like virus IGR sequences (13). To facilitate discussion, the PSIV IRES structure has been divided into three domains (13): Domain 1 contains PK II and the largest stem-loop, Domain 2 (further divided into Domains 2a and 2b) contains PK III and two stem-loops and Domain 3 contains PK I and one stem-loop. However, the detailed structure of this model has not been confirmed. Therefore, IRES structure was probed using DMS and CMCT to modify IRES RNAs, with and without added ribosomal subunits, and analyzing these RNAs by primer extension for termination at modified bases. DMS reacts with unpaired adenine, cytosine and guanine, although the guanine modification cannot be detected by primer extension, while CMCT reacts with unpaired uracil and guanine (28,29).

Nucleotides that reacted with DMS and CMCT in the absence of added 40S ribosomes (Fig. 2A, lanes 3 and 6) are shown in Figure 4A. Most of these are in single-stranded regions of the predicted model. However, several reactive nucleotides (U_{6066} , A_{6112} , U_{6130} , G_{6146} , $AA_{6163-6164}$, U_{6166} and $UU_{6191-6192}$) are in helical regions. This suggests a re-evaluation of these nucleotides. (i) U_{6066} reacted strongly with CMCT but G_{6011} , the nucleotide to which it is base paired in

the model, was only slightly modified (Fig. 2A), suggesting that these residues are not base paired (Fig. 4A). (ii) A_{6112} was reactive with DMS (Fig. 2A and B) so the A_{6112} - U_{6080} pair may be unstable. (iii) U_{6130} showed strong reactivity with CMCT although in the model it is base paired with G_{6110} . Since neither U_{6082} nor U_{6083} was reactive with CMCT and sequence alignment of CrP-like viruses suggests a single U or G nucleotide between PK III and the stem-loop in Domain 2b (Fig. 4B), G_{6110} may be base paired with U_{6082} rather than U_{6130} (Fig. 4A). (iv) A band corresponding to C_{6149} was detected in the CMCT-modified sample even though CMCT should not react with cytosine. This suggests that the band at C_{6149} is due to non-specific termination. Also, a band at G_{6146} is probably due to non-specific termination since preliminary experiments found that reverse transcription sometimes terminates at nucleotides 6146–6149 when the samples contain 40S ribosomes (data not shown). (v) $AA_{6163-6164}$, U_{6166} and $UU_{6191-6192}$ are in PK I in the model. Since PK I is essential for translation initiation in CrP-like viruses (8,9,30), it is unlikely that these residues remain single-stranded throughout the initiation process. The weak reactivity at these residues in PK I may be due to some IRES RNA molecules having an incompletely folded Domain 3 or to Domain 3 being unstable in these experimental conditions.

Sequence alignments indicate that the CrP-like viruses CrPV, *Rhopalosiphum padi virus* and *Black queen cell virus* form a helical region without an internal bulge in Domain 3

Table 1. Luciferase activity of PSIV IRES variants with mutations in the internal bulge of Domain 3

	Sequence ^a	Relative light units ^b	Relative luciferase activity
Wild-type	CUCAA ₆₁₅₃₋₆₁₅₇ GUGU ₆₁₇₅₋₆₁₇₂	48 445 ± 9843	100%
C ₆₁₇₃	CUCAA ₆₁₅₃₋₆₁₅₇ GUCU ₆₁₇₅₋₆₁₇₂	19 275 ± 1465	39.8%
C ₆₁₇₃ + G ₆₁₅₅	CUGAA ₆₁₅₃₋₆₁₅₇ GUCU ₆₁₇₅₋₆₁₇₂	51 923 ± 3964	107.2%

^aUnderlining indicates mutated nucleotides.^bStandard deviations are also indicated.

(Fig. 4B). In agreement with this model, UCA₆₁₅₄₋₆₁₅₆ and GU₆₁₇₃₋₆₁₇₄ in PSIV Domain 3 did not show obvious reactivity to DMS and CMCT (Fig. 2A). Also, using measurement of translation activity of a capsid-luciferase fusion protein, replacement of G₆₁₇₃ with C decreased luciferase activity to 40% of that of wild-type. Translation activity was completely restored by the compensatory replacement of C₆₁₅₅ with G to restore the base pair (Table 1). Also, in DMS probe studies there was a band corresponding to nucleotide 6173 in the C₆₁₇₃ mutant and this band was missing in RNA with the additional compensatory mutation (data not shown). Thus, the model of internal bulges in Domain 3 should be refined (Fig. 4A).

The DMS and CMCT probes did not detect several nucleotides that were predicted to be in single-stranded regions of the model (Fig. 4A), such as CU₆₀₁₆₋₆₀₁₇, GG₆₀₂₉₋₆₀₃₀, GC₆₀₆₄₋₆₀₆₅, G₆₀₇₆, A₆₁₀₅, A₆₁₂₉, A₆₁₃₅, A₆₁₇₉ and G₆₁₈₂. Therefore, some of these nucleotides may be involved in base pair interactions to form tertiary structures not in the model.

Footprint analysis

To investigate IRES-ribosome binding, DMS and CMCT chemical probing of IRES RNA was done in the presence of ribosomal subunits. Modified 40S ribosome-bound IRES RNA had reduced band intensities at nucleotides 6049-6050, 6073-6074, 6089-6090, 6119 and 6121-6123 (Fig. 2A, lanes 2 and 5 for DMS and CMCT, respectively) compared to band intensities for IRES RNA in the absence of added ribosome (Fig. 2A, lanes 3 and 6 for DMS and CMCT, respectively). This indicates that 40S ribosomes protect these nucleotides from chemical modification. Two of these nucleotides, UU₆₀₈₉₋₆₀₉₀, are in the loop part of the stem-loop structure in Domain 2b, which is conserved in all CrP-like viruses (Fig. 4B). C₆₁₁₉ and GCC₆₁₂₁₋₆₁₂₃ are in the loop part of the stem-loop structure in Domain 2a, which is also highly conserved in CrP-like viruses. In particular, C₆₁₁₉ and C₆₁₂₃ are conserved in all these viruses and, therefore, probably have an important role in 40S ribosome binding. However, A₆₁₂₀ was not significantly protected by 40S ribosomes from chemical modification in PSIV and is replaced by a G in the HiPV sequence, so it probably is not important for 40S ribosome binding.

Similar chemical modification experiments were carried out in the presence of the 60S subunit (Fig. 2B). However, no effect on primer extension profiles was detected. This

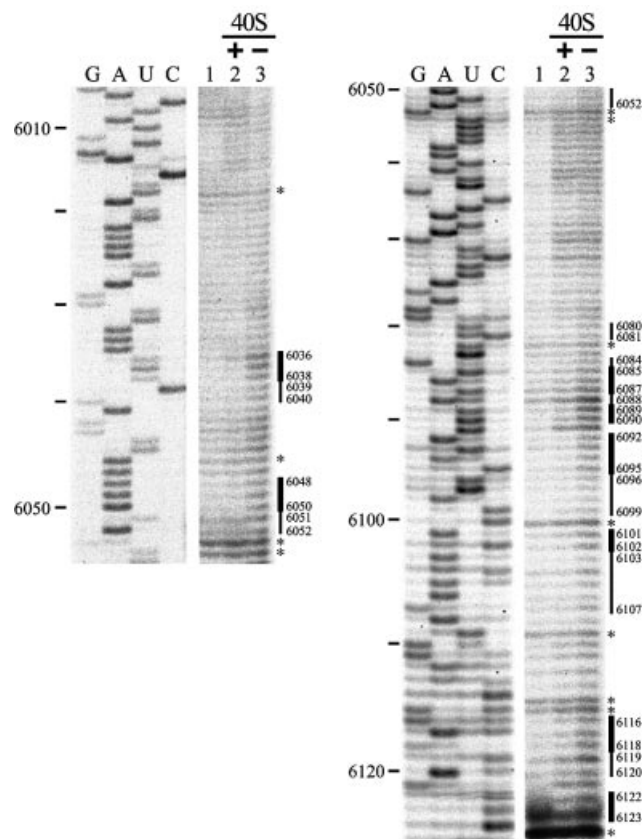


Figure 3. Hydroxyl radical probing of PSIV IRES RNA. The RNA was exposed to hydroxyl radicals in the presence (lane 2) or absence (lane 3) of 40S ribosomes. Lane 1 is the unmodified control, but 40S ribosomes were added to the sample before phenol-chloroform extraction to detect non-specific terminations caused by 18S rRNA as well as the sample in lane 3. Bold bars denote locations of protected residues and narrow bars denote sites of weak protection. Asterisks mark sites of non-specific terminations, as listed in the text. Nucleotide positions of the PSIV sequence are shown at left.

indicates that 60S ribosomes do not interact with single-stranded regions of IRES RNA. When both 40S and 60S ribosomes were included in the incubation mixture, the protection pattern was almost identical to that for 40S ribosomes except that A₆₀₁₄ was also protected (Fig. 2B). A₆₀₁₄ is in the conserved bulge sequence in Domain 1. It was not protected by either 40S or 60S ribosomes alone, but was protected in the presence of both 40S and 60S ribosomes. This suggests that A₆₀₁₄ is in a part of the IRES close to an inter-subunit bridge or that the conformation of the IRES changes in the presence of the 60S ribosomes.

To investigate PSIV IRES ribosomal binding sites further, IRES RNA in the presence and absence of 40S ribosomes was treated with hydroxyl radicals. Since hydroxyl radicals attack ribose in the nucleic acid backbone with little sequence specificity, this is a useful tool for probing interactions between nucleic acids and ligands (31,32). Protection from hydroxyl radical attack was monitored by primer extension. An unmodified control (with 40S ribosomes added to unmodified RNA before RNA isolation) was used to identify non-specific termination caused either by IRES tertiary structure or by the presence of 18S ribosomal RNA (Fig. 3, lane 1). Such non-specific terminations were observed at

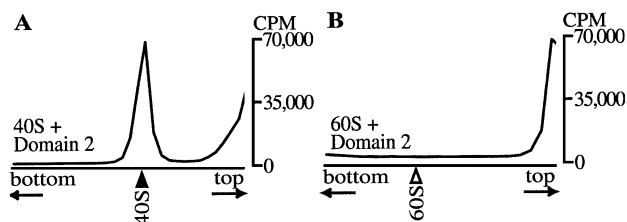


Figure 5. Sedimentation profiles of Domain 2 RNA and ribosomal subunits. ^{33}P -labeled Domain 2 RNA was incubated with purified (A) 40S or (B) 60S ribosomes and centrifuged in sucrose gradients. Fractions were collected and the radioactivity in each fraction was measured. Black and white arrowheads indicate positions of 40S and 60S subunits, respectively, which were monitored by absorbance at 254 nm.

rabbit 40S or 60S ribosomes. Domain 2 RNA co-sedimented with 40S ribosomes (Fig. 5A), but not with 60S ribosomes (Fig. 5B). Therefore, IRES Domain 2 RNA binds 40S but not 60S ribosomes.

Effect of mutations in IRES conserved sequences on translation activity

Protection of the loop sequences in the stem-loop structures in PSIV IRES Domains 2a and 2b by 40S ribosomes from chemical probes suggested that these sequences are important for IRES activity. Therefore, the effects of mutations in these loop sequences on translation activity were studied using a capsid-luciferase fusion protein translation system. The translation activity of a PSIV variant with two mutations in the Domain 2a loop, $\text{C}_{6119}\rightarrow\text{G}$ and $\text{C}_{6123}\rightarrow\text{G}$, decreased to 12% of that of the wild-type, and the activity of a variant with mutations in the Domain 2b loop, $\text{AUUU}_{6088-6091}\rightarrow\text{UAAA}$, decreased to 62% (Table 2). This result for PSIV was different from that reported for CrPV: the same mutation in the CrPV IRES Domain 2b loop sequence as described here for PSIV decreases CrPV IRES translation activity to less than 10% of wild-type (26). Translation activity of the CrPV IRES is measured using dicistronic constructs. On the other hand, that of PSIV was monocistronic. Therefore, we constructed two additional dicistronic uncapped RNAs similar to CrPV using *Renilla* and firefly luciferases. One contained the wild-type PSIV IRES sequence and the other contained a UAAA mutation in the loop sequence in PSIV IRES Domain 2b. The UAAA mutant showed 23% activity of the wild-type. Thus, we considered that the discrepancy of the results in PSIV and CrPV probably came from the difference of constructs, monocistronic and dicistronic. These results indicate that conserved sequences in the loops of the stem-loop structures in Domains 2a and 2b are important for IRES activity. Although the effect of these mutations in PSIV loops on 40S ribosome binding has not been studied, similar constructs in CrPV slightly decrease binding (26).

The single-strand sequence that connects helical segments in Domains 2a and 2b ($\text{UUACC}_{6096-6100}$) is highly conserved among CrP-like viruses (Fig. 4B). Since replacement of $\text{ACC}_{6098-6100}$ with GGG has been shown to abolish translation (13), this region must have an essential role in IRES-mediated translation. However, obvious modifications by the chemical probes DMS and CMCT were not observed at nucleotides 6096–6100 (Fig. 2), suggesting that this region might base pair

Table 2. Luciferase activity of PSIV IRES variants with mutations in the loops of the stem-loop structures in Domains 2a and 2b

	Sequence ^a	Relative light units ^b	Relative luciferase activity
Wild-type	$\text{CAGCC}_{6119-6123}$ $\text{AUUU}_{6088-6091}$	$48\,445 \pm 9843$	100%
Domain 2a	$\underline{\text{GAGCG}}_{6119-6123}$	5689 ± 1687	11.7%
Domain 2b	$\underline{\text{UAAA}}_{6088-6091}$	$29\,834 \pm 3187$	61.6%

^aUnderlining indicates mutated nucleotides.

^bStandard deviations are also indicated.

in the IRES structure. Since the refined structure model (Fig. 4A) suggested that G_{6011} should be single strand, base pair interactions between $\text{GUG}_{6011-6013}$ and $\text{CAU}_{6099-6097}$ were considered. To examine this, mutational analyses were carried out using three variants with mutations to disrupt and restore possible base pairs between these sequences: $\text{ACC}_{6098-6100}\rightarrow\text{UUU}$, $\text{UGUG}_{6010-6013}\rightarrow\text{AAAA}$ and combined mutation of both sequences to restore possible base pair formation. However, recovery of translation activity was not observed using the capsid-luciferase fusion protein translation system (data not shown), suggesting that the $\text{GUG}_{6011-6013}$ and $\text{CAU}_{6099-6097}$ sequences do not interact.

IRES binding to programmed ribosomes

The experiments above identified IRES structural elements involved in 40S ribosome binding and showed that Domain 2 has a crucial role in this binding. However, since no obvious protection in Domain 3 was detected in chemical probe studies, the function of Domain 3 in ribosome binding is unknown. To investigate a possible role for Domain 3, the binding of programmed 80S ribosomes to complete IRES RNA and to truncated IRES RNA, from which Domain 3 was deleted, was studied. Poly(U)-programmed 70S ribosomes are frequently used for translation analysis in prokaryotic systems (33). It is known that tRNA^{Phe} binds to the A, P and E sites of poly(U)-charged prokaryotic 70S ribosomes at high Mg^{2+} concentrations (34) and that the ASL domain of tRNA binds to the decoding site of poly(U)-programmed ribosomes (35,36). Therefore, it was assumed that binding of IRES RNA to 80S ribosomes would be inhibited by poly(U), tRNA^{Phe} or ASL, if the IRES binds to a region at or near the tRNA binding sites of eukaryotic 80S ribosomes *in vitro*.

IRES-ribosome binding was measured by filter binding assays. Preincubation of 80S ribosomes with poly(U) decreased the binding of ^{32}P -labeled complete IRES RNA (containing Domains 1–2–3) to 60–70% of that of controls without poly(U), but did not affect binding of truncated IRES RNA (containing only Domains 1–2) (Fig. 6A and B). When ribosomes were preincubated with tRNA^{Phe} , binding of both IRES RNAs decreased to 45–55% of that of controls without tRNA^{Phe} (Fig. 6A). When ribosomes were preincubated with poly(U) and tRNA^{Phe} together, binding of both IRES RNAs decreased to less than 10% (Fig. 6A). In contrast to the results using tRNA^{Phe} , when only ASL was used for preincubation, binding of both IRES RNAs to ribosomes was not inhibited (Fig. 6B), but this can be explained by the instability of ASL on ribosomes without mRNA. However, preincubation of

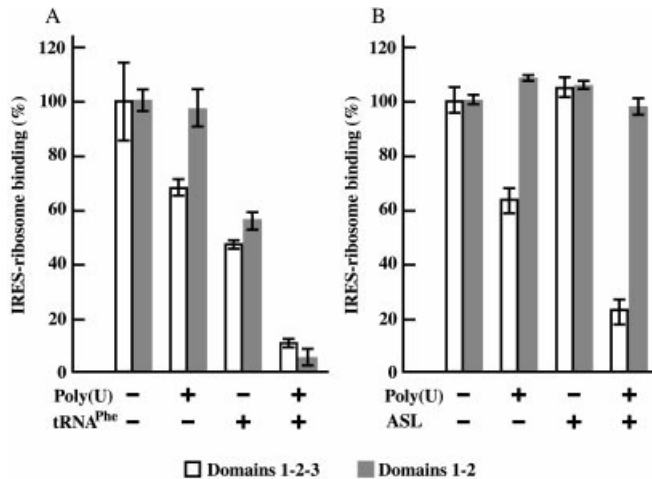


Figure 6. Filter binding analyses of complete IRES RNA (Domains 1–2–3) and truncated IRES RNA (Domains 1–2) binding to programmed 80S ribosomes. (A) Effects of poly(U) and/or tRNA^{Phe} programming on RNA binding. (B) Effects of poly(U) and/or 17mer tRNA^{Phe} ASL programming on RNA binding. ³²P-labeled IRES RNAs were incubated with 80S ribosomes that had been preincubated with poly(U), tRNA^{Phe} and/or ASL. Binding for samples containing 1 pmol RNA and 4 pmol ribosomes [without poly(U), tRNA^{Phe} or ASL] was taken as 100% binding. Results are the average of three independent experiments. Bars represent standard errors.

ribosomes with poly(U) and ASL together decreased binding of complete IRES RNA (Domains 1–2–3) to about 20%, but had no effect on the binding of truncated IRES RNA (Domains 1–2) (Fig. 6B). These results suggest that part of Domain 3 competes with the ASL when IRES RNA binds to poly(U)-charged ribosomes. Binding of truncated IRES RNA (Domains 1–2) to poly(U)-charged ribosomes was also inhibited by tRNA^{Phe} (Fig. 6A). Since the ASL lacks the tRNA molecule acceptor stem and D and T stem-loops, part of Domains 1–2 would occupy the ribosome sites for the tRNA acceptor stem and D or T stem-loop interactions, but not for the ribosome tRNA ASL site. In addition, the filter binding experiments showed that complete and truncated IRES RNAs bind to salt-washed ribosomes with similar affinities (K_d 21–23 nM). From these observations, it appears that Domain 3 does not have a significant role in IRES RNA–ribosome binding.

The studies presented here allow several conclusions regarding the molecular events in IRES-mediated translation initiation. Purified 40S ribosomes interact with the loop sequences in the stem-loop structures in PSIV IRES Domains 2a and 2b. The two loop sequences are highly conserved among CrP-like viruses (Fig. 4B) and mutations in these sequences decreased PSIV IRES translation activity, indicating that these loops have essential roles in IRES function. Mutational disruption of base pair interactions in PK III had deleterious effects on 40S ribosome binding (Fig. 1). Since PK III is near the two Domain 2 loops, PK III may affect the relative positions of these two loops for efficient 40S ribosome binding. Binding of complete IRES RNA (Domains 1–2–3), but not truncated IRES RNA (Domains 1–2), to 80S ribosomes was inhibited by ASL. This suggests that Domain 3 probably interacts with part of the ribosome decoding site. These results imply that Domains 1–2 and Domain 3 have distinct roles in IRES-mediated translation initiation. Since Domain 2 alone can bind 40S ribosomes (Fig. 5A), interactions between 40S

ribosomes and the Domain 2 loops are probably one of the initial steps in IRES-mediated translation initiation. Although when and how 60S ribosomes engage the IRES–40S complex is unknown, PK II may have a role in promoting the interaction of Domain 3 and the ribosome decoding site, presumably in the presence of the 60S ribosomes. CrPV IRES-mediated translation elongation, in the absence of any eIFs, has recently been shown by toeprinting and peptidyl-puromycin formation analysis using purified elongation factors and acylated tRNAs (37). This also means that binding between ribosomes and CrP-like virus IRESs plays a key role for translation initiation.

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