Detection of tRNA-like Structure through RNase P Cleavage of Viral Internal Ribosome Entry Site RNAs Near the AUG Start Triplet*

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The hepatitis C virus (HCV) is a flavivirus with a single-stranded RNA genome almost 9600 bases in length. HCV produces a chronic infection that can lead to cirrhosis and liver cancer and is the leading cause of liver transplants in the United States and elsewhere (1).

The 9600-base RNA genome of hepatitis C virus (HCV) has an internal ribosome entry site (IRES) in its first 370 bases, including the AUG start triplet at bases 342–344. Structural elements of this and other IRES domains substitute for a 5′ terminal cap structure in protein synthesis. Recent work (Nadal, A., Martell, M., Lytle, J. R., Lyons, A. J., Robertson, H. D., Cabot, B., Esteban, J. L., Esteban, R., Guardia, J., and Gomez, J. (2002) J. Biol. Chem. 277, 30606–30613) has demonstrated that the host pre-tRNA processing enzyme, RNase P, can cleave the HCV RNA genome at a site in the IRES near the AUG initiator triplet. Although this step is unlikely to be part of the HCV life cycle, such a reaction could indicate the presence of a tRNA-like structure in this IRES. Because susceptibility to cleavage by mammalian RNase P is a strong indicator of tRNA-like structure, we have conducted the studies reported here to test whether such tRNA mimicry is unique to HCV or is a general property of IRES structure. We have assayed IRES domains of several viral RNA genomes: two pestiviral RNA genomes related in structure to HCV, classical swine fever virus and bovine viral diarrhea virus; and two unrelated viruses, encephalomyocarditis virus and cricket paralysis virus. We have found similarly placed RNase P cleavage sites in these IRESs. Thus a tRNA-like domain could be a general structural feature of IRESs, the first IRES structure to be identified with a functional correlate. Such tRNA-like features could be recognized by pre-existing ribosomal tRNA-binding sites as part of the IRES initiation cycle.

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§ The abbreviations used are: HCV, hepatitis C virus; IRES, internal ribosome entry site; CSFV, classical swine fever virus; BVDV, bovine viral diarrhea virus; EMCV, encephalomyocarditis virus; CrPV, cricket paralysis virus; IGR, intergenic region.

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encephalomyocarditis virus, a picornavirus, and in the cricket paralysis virus, an insect virus. Both undergo RNase P cleavage at specific sites. These results strongly suggest that tRNA-like domains may be a general structural feature of IRESs, the first such IRES structure to be identified with a functional correlate. In fact, tRNA-like features could easily be recognized by ribosomal sites as a standard part of all IRES-directed protein synthesis initiation.

EXPERIMENTAL PROCEDURES

Preparation of RNA Transcripts—HCV IRES RNAs were transcribed from Bluescript plasmid pN1–4728, which contains nucleotides 1–4728 of hepatitis C virus under the T7 promoter (a gift from Dr. Stanley Lemon, University of Texas, Galveston). This DNA template was cleaved by the SacII restriction enzyme. When transcribed in vitro, a 32P-labeled RNA spanning bases 1–641 is produced. RNA transcripts of CSFV are from a pGEMII plasmid with an insert containing the CSFV IRES fused to influenza protein NS1, (a gift from Dr. R. J. Jackson, Cambridge University). This plasmid, when linearized with the Ncol restriction enzyme, serves as template for a 32P-labeled RNA containing bases 1–450 of the CSFV genome. BVDV RNA was obtained from transcription of pACCR plasmid BVDV NADL 5Δ 2702, containing the BVDV RNA sequence with a deletion of nucleotides 3094–12147 (a gift from Dr. C. Rice, Rockefeller University, New York). Linearization with ScaI yields a DNA that serves as template for a transcript spanning bases 1–695 of the BVDV genome upon transcription. EMCV IRES RNAs were transcribed from a pGEM plasmid with the EMCV IRES sequence fused to that encoding the N-terminal viral polyprotein of the virus. Restriction with BstXI yields the template for RNA spanning bases 262–834 of the EMCV IRES. This plasmid was a generous gift of Dr. A. Kaminski, University of Cambridge. RNA transcripts of cricket paralysis virus (CrPV) were made from the pEJ4 construct, a kind gift from Dr. E. Jan, Stanford University. This plasmid, when linearized with the Ncol restriction enzyme, encodes an RNA containing bases 1–220 of the CrPV IGR IRES.

All transcription reactions were based on earlier published work from this laboratory (6, 10, 11, (12) (Promega Corp., Madison, WI), was used in all labeled transcription reactions described above, were preheated at 90 °C for 1 min, added to reaction buffer (10 mM HEPES-KOH, pH 7.5, 10 mM MgOAc, 100 mM NH4OAc), and left to cool to room temperature. Cleavage reactions were performed with 4% polyethylene glycol, 20 units of RNasin and RNase P and carried out at 30 °C as described previously (11, 15). Bands were visualized by autoradiography, excised from the gel, and eluted in buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, pH 7.5).

RNase P Cleavage—All substrates for RNase P cleavage, derived from transcription reactions described above, were preheated at 90 °C for 1 min, added to reaction buffer (10 mM HEPES-KOH, pH 7.5, 10 mM MgOAc, 100 mM NH4OAc), and left to cool to room temperature. Cleavage reactions were performed with 4% polyethylene glycol, 20 units of RNasin and RNase P and carried out at 30 °C in a volume of 10 μl for 1 h. The final concentration of the substrates in these reactions was 1.8 nM. Human RNase P (glucosyl gradient fraction, the kind gift of Dr. S. Altman) was used with an excess of enzyme units over substrate (18). At the end of the reactions, samples were subjected to treatment with SDS and proteinase K at 50 °C as described previously (22), and cleavage products were then separated on 4% denaturing polyacrylamide gels and visualized by autoradiography as described (15). For kinetic studies, samples were treated as above and incubated in scaled-up reaction volumes prepared as above. Aliquots of 5 μl were withdrawn at 0, 30, 60, 90, and 120 min, subjected to SDS-proteinase K treatment, and analyzed on polyacrylamide gels as described above. Starting material and product bands were quantified as follows using an Amersham Biosciences Storm Storm PhosphoImager: percent RNase P cleavage = (products)/(starting material + products).

RNA Fingerprinting—RNA fingerprinting, a technique in which RNA molecules are digested to completion with RNase T1 (Calbiochem) and the digestion products are separated in two dimensions by charge and size, has been described previously (23, 24).

Determination of Cleavage Sites—To carry out precise characterization and mapping, it is necessary to use direct sequencing techniques involving internally labeled RNA molecules when the normal internal sequence pattern of an RNA is interrupted. Examples of such events include mapping of RNA splice junctions and lariats; UV-induced covalent RNA-RNA cross-links; RNA editing to produce noncanonical bases, such as inosine; tRNA modification of pre-existing bases; and cleavage by enzymes (such as the RNase P used here) that produce 5′-phosphate and 3′-hydroxyl termini rather than the usual 5′-hydroxyl and 3′-phosphate ends. Thus, direct methods were employed here rather than indirect approaches such as DNA primer extension with reverse transcriptase or partial digestion of end-labeled transcripts.

Oligonucleotides derived from RNase T1 digests were fractionated to yield two-dimensional fingerprints, from which individual oligonucleotides were located by autoradiography and eluted by standard techniques (24). Standard conditions for secondary analysis of the T1 digestion products (with pancreatic RNase A, RNase T2, RNase T2 or 0.4 M NaOH, followed by high voltage electrophoresis on Whatman DE81 or 3MM papers (23)) were used, permitting the oligonucleotides to be identified. Digestion by calf alkaline phosphatase (Promega) was used to identify oligonucleotides containing 5′ terminal phosphate residues produced by RNase P cleavage.

RESULTS

RNase P Cleavage of Pestiviral Transcripts—The 5′ terminal region of the genomic RNA for each of the HCV-type viral RNAs was transcribed as described under “Experimental Procedures,” purified, and digested with RNase P. These reactions yield the results shown in Fig. 1, a 5% sequencing gel electrophoretic profile of HCV (HC), CSFV (CS), and BVDV (BV) IRES substrates without or with RNase P digestion. As shown in Fig. 1, the three HCV-type IRES RNAs are cleaved, CSFV more efficiently than HCV and BVDV less so. Principal cleavage products are indicated by numerals to the right of the lanes showing RNase P-treated RNAs. Fig. 2 depicts kinetics of RNase P cleavage of the HCV
IRES transcript, showing >50% cleavage over a 90-min time course.

Mapping the RNase P Cleavage Site in the CSFV IRES—In the case of CSFV, one larger and two smaller product bands are recovered (Fig. 1) following RNase P cleavage. Enumerated bands were eluted for further analysis. To map the exact location of the HCV and CSFV cleavages, transcripts were digested with RNase P under conditions outlined above and then fractionated on polyacrylamide denaturing gels. Both control transcripts and all digestion products labeled in Fig. 1 were then eluted from the gels, digested with RNase T1, and subjected to two-dimensional RNA fingerprinting analysis (23). Direct characterization of the internally labeled RNA fragments produced by RNase P cleavage in the HCV IRES (Fig. 1) showed that cleavage occurs just beyond the AUG initiator, centering on bases 361–362 (data not shown) as found previously (15).

Fig. 3 shows RNA fingerprinting analysis of CSFV IRES RNase P cleavage products. RNAs eluted from the gel depicted in Fig. 1, center panel, were subjected to exhaustive RNase T1 digestion and two-dimensional RNA fingerprinting. Panel A shows a control fingerprint of the entire 450-base CSFV IRES transcript, whereas Panels B–D show fingerprints of the three RNase P digestion products, CSFV bands 1, 2, and 3, respectively (Fig. 1). Spots numbered 1–7 (Fig. 3) are RNase T1-resistant fragments, characterized by direct methods (23, 24), that are present in the control sequence, absent in band 1, and present in bands 2 and 3 (Table I). It is noteworthy that spot 4, 370UACAUG375, contains the CSFV IRES AUG initiator triplet.

CSFV band 1 (Fig. 3B) contains all T1-resistant RNA oligonucleotides from the control CSFV transcript sequence up to base 359, including the 5' end of CSFV (see legend to Fig. 3). This suggests that cleavage takes place downstream of that position in the IRES sequence. Bands 2 and 3 (Fig. 3, C and D) were found to contain all T1-resistant fragments between bases 370 and 450. Notably, the unique T1-resistant oligonucleotide 363UACAUG368 was missing or significantly reduced in the fingerprint patterns of all of the CSFV RNase P digestion products. Furthermore, bands 2 and 3 (Fig. 3, C and D) were found to contain a novel oligonucleotide, designated spot "X." Upon further secondary analyses, including alkaline phosphatase treatment, spot X was found to have the sequence 5'-pACAUG-3'. RNase P cleavage is well known to yield 5'-phosphate and 3'-hydroxyl termini (16–18); thus it is likely that spot X represents the new 5'-ends created by RNase P.

With respect to band 2, the presence of spot 4 along with spot X suggests that cleavage must have occurred within the "missing" oligonucleotide, 363UACAUG368, precisely between bases 363U and 364A, yielding the T1-resistant oligonucleotide X (364pACAUG368). This cleavage event is shown schematically in Fig. 4 (circled "P" on left).
FIG. 3. RNA fingerprinting analysis of CSFV IRES RNase P cleavage products. RNAs eluted from the polyacrylamide gel depicted in Fig. 1, center panel, were subjected to exhaustive RNase T1 digestion and two-dimensional separation at pH 3.5 (first dimension, right to left) and RNA homochromatography (second dimension, bottom to top) on the basis of base composition and chain length, respectively. Panel A shows the autoradiograph of a control fingerprint pattern of the entire uncleaved CSFV IRES transcript. As explained in the text, spots numbered 1–7 comprise the 3′-proximal oligonucleotides from this RNA; their positions and sequences are listed in Table I. Panel B depicts the RNA fingerprint of band 1 from Fig. 1, center panel; as indicated, this fingerprint pattern lacks spots 1–7 (except for spot 4, which is markedly reduced in intensity). Both patterns contain the 5′ terminus of the CSFV RNA transcript (pppGp), the leftmost spot in each pattern (upper left-hand corner, to the left of spot 7) in A and B. Panels C and D depict RNase T1 fingerprint patterns for CSFV IRES transcript bands 2 and 3 (Fig. 1, center panel), respectively. Both patterns lack the 5′-terminal pppGp spot and reveal a novel spot (spot X; see also Table I). The fingerprint pattern in D lacks spot 4.

With respect to band 3 (Fig. 3D), it was found to contain the same T1-resistant fragments as band 2 (Fig. 3C), with the notable exception of spot 4, which is missing from band 3. As shown in Fig. 4, we conclude that CSFV band 3 is the product of a second RNase P cleavage, which occurs near the first one, this time between bases 370C and 377A (see Fig. 4, circled letter P on right). This second cleavage would also produce a T1-resistant oligonucleotide with the sequence pAUCAG (spot X in Table I). However, in band 3 (Fig. 3D) we conclude that the sequence identity of the spot labeled X is 371pAUCAG. Thus it appears that RNase P makes two cuts, the second seven bases away from the first, at sites near the CSFV IRES initiator AUG triplet that have a very close sequence homology to each other (G/U/C)AUCAGG in each case). This series of events also explains why the fingerprint of CSFV band 1 (Fig. 3B) contains reduced levels of the oligonucleotide 365pAUCAG, the band is actually an unresolved mixture of two sequences, CSFV bases 1–363 and 1–370.

RNase P Cleavage of the BVDV IRES—RNA fingerprinting analysis was carried out on control and RNase P-treated BVDV IRES-containing RNAs transcribed from SacI-treated DNA templates (Fig. 1). Comparison of the resulting patterns and knowledge of the identity of the control T1-resistant fragments (data not shown) allowed us to conclude that all of the T1-resistant oligonucleotides included in this assay (7 bases or longer) from the 5′-end of the BVDV IRES up to base 372 (a total of 8 fragments) were present in BVDV product 1 (Fig. 1, right lane, BV). BVDV product 2 (Fig. 1) was not recovered in sufficient quantities to be included in this T1 mapping survey. Nonetheless, although the BVDV IRES region spanning bases 373–394 (which includes the AUG initiation codon at 386–388) does not contain any RNase T1-resistant fragments of 7 bases or longer, the T1-resistant fragment spanning bases 395–404 was absent from the BVDV product 1 fingerprint pattern, as were all other such T1-resistant oligonucleotides through the 3′-end of the BVDV SacI transcript at base 695 (a total of 11 fragments). We therefore conclude that the primary RNase P cleavage site within the BVDV IRES maps between bases 373 and 394. Because of the similarity of the BVDV and CSFV IRES sequences in this region (see below), we predict that the BVDV IRES RNase P cleavage sites will closely resemble those in CSFV.

**TABLE I**

| Spot | Sequence and position |
|------|-----------------------|
| 1    | 395AAUUAUAUAACAAAAAG  |
| 2    | 411CCACAAAAACACGAG    |
| 3    | 387AAUCAUUAUUCAUG591   |
| 4    | 377CAUAG379           |
| 5    | 347CACG341           |
| 6    | 444CAUCAUGG430        |
| 7    | 377UG431           |
| X    | 365pAUCAG365 or 371pAUCAG375 |

*Fig. 3C.*

*Fig. 3D.*

FIG. 4. CSFV IRES RNase P cleavage domain. The sequence of bases 348–400 of the CSFV IRES is shown with the 5′ and 3′ termini indicated. The two RNase P cleavage sites (each indicated by a circled “P” with a downward pointing arrow) at two closely linked homologous sites (including the sequence labeled “X” as found also in Fig. 3 and Table I) are shown within the CSFV IRES in the immediate vicinity of the AUG start triplet (bold type). The 35 bases in CSFV, indicated in italic type, which span bases 348–382, are identical to their counterpart bases in the BVDV IRES, as indicated in Fig. 7.

**FIG. 2.**—RNase T1-resistant oligonucleotides in the 3′-proximal CSFV IRES domain. As indicated in Fig. 2, the characteristic oligonucleotide spots spanning the 3′ terminal domain of the EcoRI transcript of the CSFV IRES are listed by number. Each sequence is written in the 5′ to 3′ direction (left to right), with the residue number of each 5′ and 3′ terminal base indicated before and after each sequence, respectively. By convention (23, 24), RNase T1-resistant oligonucleotides have 5′-hydroxyl and 3′-phosphate termini (the latter are G residues because of the specificity of the T1 enzyme) unless otherwise noted. As indicated, spot X represents two occurrences of the same five-base sequence, containing 5′-phosphate termini produced by RNase P cleavage. Spot 6 represents the 3′-terminal oligonucleotide of this RNA transcript, which thus has a 3′-hydroxyl end.

**IREs RNase P Cleavage Map Positions**—Fig. 5 summarizes RNase P cleavage locations for all three viral IRES-containing transcripts as identified by RNA fingerprinting and secondary analysis or oligonucleotide survey techniques. The HCV IRES RNase P cleavage site, shown as a circled letter P in Fig. 5,
RNase P Cleavage Sites in Viral IRES RNAs

In this paper we have demonstrated that there are similarly placed recognition elements for the human pre-tRNA processing enzyme, RNase P, in several viral IRES domains including HCV, CSFV, BVDV, CrPV, and EMCV. In the cases of HCV, CSFV, and BVDV, the RNase P-sensitive elements map in close proximity to the AUG initiator triplet. As first pointed out by Nadal et al. (15) concerning human RNase P cleavage of the HCV IRES, it is likely that these events reflect neither the replication cycle of these viruses nor the canonical action of RNase P. Rather, because of the presence of pseudo-knot elements and other structural motifs in IRES RNAs like these, it is more likely that the RNase P cleavage events are reporting the presence of tRNA-like structure. This conclusion is also supported by the results of substrate competition experiments proving that HCV IRES-containing RNAs could inhibit cleavage by RNase P of pre-tRNA substrates (15). We need to ask how RNase P recognizes and cleaves these conserved viral IRES sites. In addition, it is important to know what possible function, regardless of the exact structure and map position of the RNase P recognition elements, the presence of tRNA-like structure may have in IRESs and whether such a feature could be a general requirement for IRES action.

**IRES Sites for RNase P Recognition**—A number of earlier studies have shown that RNase P can often exhibit noncanonical behavior. For example, both bacterial and eukaryotic RNase P enzymes cleave substrates other than pre-tRNAs (17–19). Furthermore, RNase P can cleave the same pre-tRNA simultaneously at two sites near each other (25, 26). Conditions inducing this behavior include substrate mutations as well as ionic or pH variation. The IRES cleavage reactions studied here show signs of noncanonical substrate behavior both by variable cleavage efficiencies (Fig. 1) and, in at least one case, by the presence of two RNase P cleavage sites close together (the CSFV IRES, Fig. 4). In practice, RNase P is known to rely principally on the recognition of tRNA secondary and tertiary structure for its cleavage specificity rather than on particular sequence elements at the point of cleavage (17–19). Fig. 7 shows the schematic diagrams of the HCV, CSFV, and BVDV IRES RNAs represented in their predicted secondary structural folding patterns (11). The RNase P cleavage sites are indicated in context, with the exact bases cleaved in the BVDV IRES still to be...
RNase P Cleavage Sites in Viral IRES RNAs

Fig. 7. Secondary structure maps of the HCV, CSFV, and BVDV IRESs. This figure shows line drawings of the proposed structures (6–12) of each IRES RNA. The RNase P cleavage sites are indicated in context, with the exact bases cleaved in the BVDV IRES yet to be mapped (see “Results”). Stem-loops II and III, major structural features of this IRES class, are indicated. The two pestiviral IRESs, CSFV and BVDV, contain regions of homology as follows. In CSFV and BVDV, the regions highlighted by a thick black outline represent identical 27-base sequences (bases 309–335 in CSFV; bases 324–350 in BVDV). The 5′-proximal portion of this cloverleaf region is highly conserved in a large number of HCV and pestiviral isolates including the 14 bases of HCV from 292 to 305 (black highlighting). Second, as shown by a thick grey line, the 35 bases spanning the AUG start triplet and the RNase P cleavage sites are identical in CSFV and BVDV IRES RNAs. The double-headed arrow on the left (HCV) panel represents a UV-cross-linkable element of local tertiary structure in stem-loop II (11).

mapped. The major IRES structural domains, stem-loops II and III, are also indicated. If there is tRNA-like structure in these IRES RNAs, it might be more highly conserved if it occurred in noncoding sequences upstream from the cleavage sites. In seeking tRNA-like structure in the vicinity of HCV, CSFV, and BVDV RNase P cleavage, the cloverleaf-like regions in stem-loop III, highlighted by a thick black outline in Fig. 7, are obvious candidates. Furthermore, these domains in CSFV and BVDV represent identical 27-base sequences (bases 309–335 in CSFV and bases 324–350 in BVDV; Ref. 27). A third pestivirus, border disease virus, has 26 of these 27 bases conserved as well (28). In addition, the primary sequence of the 5′-proximal portion of this region is highly conserved in a large number of HCV and pestiviral isolates, including the 14 bases of HCV spanning residues 292–305 (Fig. 7, black highlighting in the HCV IRES; Refs. 27 and 29). Future studies correlating RNase P cleavage and protein synthesis in IRES RNAs mutagenized to affect translation in these and other domains will be needed to pinpoint the structural elements involved in RNase P recognition and their relationship to IRES function.

Although Fig. 7 also shows that the two pestiviral IRESs, CSFV and BVDV, contain regions of homology in the initiator AUG-containing region cleaved by RNase P, it is unlikely that these could specify RNase P cleavage (17–19). As shown by a thick grey line in Fig. 7, the 35 bases spanning the start triplet and the RNase P sites are identical in these two IRESs (27). The CSFV version of this sequence, bases 348–382, is depicted in Fig. 4 in italic letters. The equivalent domain of BVDV includes bases 361–395. However, this sequence is unlikely to be a general IRES feature signaling RNase P cleavage because the HCV IRES, which has no significant homology to this sequence (27, 29), is cleaved in a fashion similar to CSFV and BVDV.

In the well studied cases of tRNA mimicry in other viral RNAs (including the 3′ terminal (21) and internal (30) sites of action of tRNA-modifying enzymes), the tRNA elements can usually be recognized by more than one tRNA-specific system. In the case of RNase P cleavage of IRES elements presented here, studies on recognition by appropriate tRNA-binding initiation and elongation factors of protein synthesis, along with tRNA nucleotidyl transferase and appropriate tRNA-modifying activities, may shed additional light (15, 31–33).

If recognition elements are located, as suggested above, in the upstream “cloverleaf” IRES domain in stem-loop III, RNase P would be cleaving to their right or 3′ side. RNase P usually cleaves to the left, on the 5′ or upstream side, of its recognition elements, forming the mature 5′-end of the tRNA involved (16–19). With regard to the polarity of cleavage it is important to note that RNase P activities can cleave in the interior of multimeric tRNA precursor molecules (34) to the left and right of the pre-tRNA elements. In addition, in some cases, the tRNA elements of larger molecules that are RNase P substrates, such as bacterial tmRNA (20), are composed of sequences from both the 5′- and 3′-ends of molecules over 300 bases long. On the other hand, it remains possible that some specificity determinants for the IRES RNase P cleavage reactions reported here could be located downstream from the cleavage sites mapped, although recent studies appear to favor the idea that the region immediately downstream from the HCV IRES initiator AUG triplet must be relatively unstructured (35). Further studies focusing on selected elements in IRES stem-loops II and III should narrow the choice of potential RNase P specificity determinants.

Potential Function of tRNA-like IRES Elements—Regardless of which domains near the AUG start triplets are found to specify the RNase P cleavages mapped here in three viral RNAs, we need to know why such elements are present and whether they have a function. If tRNA-like elements are required for the initiation of translation, for example, we can begin correlating the retention of RNase P cleavability with the IRES function in translation in various mutant IRESs. There is no question that recent studies on IRES domains protected from RNase digestion by various initiation complexes containing ribosomes or ribosomal subunits (6, 10) have shown that extensive additional domains beyond the immediate vicinity of AUG-containing sequences are involved. These results, along with recent studies on the 40 S ribosomal subunit-HCV IRES complexes by cryo-electron microscopy (9), have suggested the potential involvement of the ribosomal E-site.

The initiation pathway for these IRESs, as determined by various published studies, involves the initial formation of a binary complex with 40 S subunits through a 43 S complex with added factors to a full initiation complex with 80 S ribosomes. In our hypothesis, as initiation progresses, the cloverleaf structure shown in Fig. 7, HCV IRES stem-loop III bases 291–323, or their pestiviral equivalents, will move near to the unoccupied ribosomal E-site as the nearby AUG-containing region occupies the P-site. Recognition of the tRNA-like domain of the IRESs by the E-sites of the ribosomes could help to specify accurate initiation by bringing the P-site and the AUG start triplet into close and stable proximity. The IRES domain would then be ejected from the E-site as normal initiation and translation progression.

Our preliminary studies also suggest that this scheme for recognition of tRNA-like elements in HCV and pestiviral IRESs could be extended to other unrelated IRES elements; and thus this step could be a general part of IRES-based translation initiation. In Fig. 6, we showed that the CrPV IGR IRES contains an RNase P-cleavable element. This IRES has recently been found to contain a highly structured RNA domain that maps, like similar elements in HCV-like viral IRESs, immediately upstream from the AUG initiator triplet (36). This CrPV element may bind to a ribosomal site as the first peptide bond forms between amino acids in the P- and A-sites. Furthermore, our preliminary results (not shown) indicate that the IRES structure of EMCV, a representative of picornaviral IRESs, which have a number of protein recognition elements within them (37), harbors a tRNA-like structure. Future work

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with RNase P and other probes for tRNA-like structures should tell how widespread these structures are in IRES and elucidate their role in IRES function.

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