The influence of downstream protein-coding sequence on internal ribosome entry on hepatitis C virus and other flavivirus RNAs

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ABSTRACT

Some studies suggest that the hepatitis C virus (HCV) internal ribosome entry site (IRES) requires downstream 5' viral polyprotein-coding sequence for efficient initiation of translation, but the role of this RNA sequence in internal ribosome entry remains unresolved. We confirmed that the inclusion of viral sequence downstream of the AUG initiator codon increased IRES-dependent translation of a reporter RNA encoding secretory alkaline phosphatase, but found that efficient translation of chloramphenicol acetyl transferase (CAT) required no viral sequence downstream of the initiator codon. However, deletion of an adenosine-rich domain near the 5' end of the CAT sequence, or the insertion of a small stable hairpin structure ($\Delta G = -18$ kcal/mol) between the HCV IRES and CAT sequences (hpCAT) substantially reduced IRES-mediated translation. Although translation could be restored to both mutants by the inclusion of 14 nt of the polyprotein-coding sequence downstream of the AUG codon, a mutational analysis of the inserted protein-coding sequence demonstrated no requirement for either a specific nucleotide or amino acid-coding sequence to restore efficient IRES-mediated translation to hpCAT. Similar results were obtained with the structurally and phylogenetically related IRES elements of classical swine fever virus and GB virus B. We conclude that there is no absolute requirement for viral protein-coding sequence with this class of IRES elements, but that there is a requirement for an absence of stable RNA structure immediately downstream of the AUG initiator codon. Stable RNA structure immediately downstream of the initiator codon inhibits internal initiation of translation but, in the case of hpCAT, did not reduce the capacity of the RNA to bind to purified 40S ribosome subunits. Thus, stable RNA structure within the 5' proximal protein-coding sequence does not alter the capacity of the IRES to form initial contacts with the 40S subunit, but appears instead to prevent the formation of subsequent interactions between the 40S subunit and viral RNA in the vicinity of the initiator codon that are essential for efficient internal ribosome entry.

Keywords: cap-independent translation; CSFV; GBV-B; pestivirus; RNA structure

Hepatitis C virus (HCV) is classified within the genus *Hepacivirus* of the family *Flaviviridae*. Other members of this family of enveloped, positive-strand RNA viruses include the classical flaviviruses, pestiviruses, and the recently discovered GB viruses. The genomes of these viruses all encode a single polyprotein that is co- and posttranslationally cleaved to yield 11 or more viral proteins (for reviews see Houghton, 1996; Rice, 1996; Thiel et al., 1996).

The 5' nontranslated RNA (NTR) segments of the HCV, pestivirus, and GB virus-B (GBV-B) genomes range from 341 to 445 nt in length. The sequences of these 5' NTRs contain several short regions sharing a high percentage of sequence identity (Han et al., 1991; Muerhoff et al., 1995; Rijnbrand & Lemon, 2000). More importantly, the 5' NTRs of these viruses can be folded into very similar structures, each consisting of several complex stem-loops and an RNA pseudoknot (Brown et al., 1992; Wang et al., 1995; Honda et al., 1996a; Sizova et al., 1998). These highly structured RNAs contain an internal ribosome entry site (IRES) that is capable of directing the ribosome to initiate translation at an internal position on the viral RNA that is distant from

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the 5' end (Tsukiyama-Kohara et al., 1992; Wang et al., 1993; Poole et al., 1995; Rijnbrand et al., 1997, 2000; Chon et al., 1998). With the exception of small hairpin structures located within the most 5' segment of the 5' NTR (domain I), most of the predicted RNA stem-loop structures are essential for IRES directed translation (Wang et al., 1994, 1995; Rijnbrand et al., 1995, 1997, 2000; Honda et al., 1996b; Chon et al., 1998; Kamoshita et al., 1997). Pestova et al. (1998) have shown that the 40S ribosomal subunit can directly interact with RNA sequences within the HCV IRES. This is likely to be an important early step in the initiation of translation on the viral RNA.

Controversy exists as to whether the HCV protein-coding sequence downstream of the IRES is essential for IRES function. Reynolds et al. (1995) suggested that up to 32 nt of the HCV capsid-coding sequence are absolutely required for efficient IRES activity. Further evidence that HCV IRES function might be dependent on specific sequence downstream of the initiator AUG codon was suggested by the sharply reduced IRES activity noted *in vitro* upon introduction of 13 silent nucleotide mutations between positions 9 and 42 of the HCV open reading frame (Honda et al., 1996b). The possible importance of the first 21 nt of the HCV coding region for internal ribosome entry was further supported by the observation that chimeric polioviruses containing the HCV IRES in lieu of the poliovirus IRES required at least 21 nt of HCV capsid protein-coding sequence to be replication competent (Lu & Wimmer, 1996). A frameshift and internal deletion in the capsid sequence of the poliovirus-HCV chimera resulted in a reduced plaque size (Lu & Wimmer, 1996), suggesting a role for the encoded amino acid sequence. However, despite these observations, efficient IRES activity has been noted with reporter constructs containing between 3 (i.e., only the AUG codon) and 8 nt of HCV-coding sequence placed between the IRES and a downstream sequence encoding a reporter protein (Tsukiyama-Kohara et al., 1992; Wang et al., 1993; Rijnbrand et al., 1995).

Available evidence suggests that the HCV polyprotein initiation codon is located within the loop of a small stem-loop (stem-loop IV) that is highly conserved among HCV strains (Smith et al., 1995; Honda et al., 1996a). Mutations that increase the stability of stem-loop IV negatively modulate IRES-mediated translation even though the presence of this structure is not required for IRES activity (Honda et al., 1996a). These results can be explained by the absence of scanning of the 40S ribosome subunit prior to its interaction with the initiator AUG codon (Honda et al., 1996a; Reynolds et al., 1996; Rijnbrand et al., 1996, 1997), and the requirement that stem-loop IV must melt to accommodate binding of the ribosome at the site of the AUG codon. Given these observations, it is intriguing to speculate that conflicting interpretations of the requirements for viral

polyprotein-coding sequence downstream of the IRES may be due to the stable folding of some heterologous reporter sequences placed immediately downstream of the initiation codon. Like mutations that increase the stability of the stem-loop IV structure (Honda et al., 1996a), stable RNA structure assumed by heterologous sequences fused directly at the AUG codon could also hinder binding of the 40S subunit and prevent translation. Thus, a reasonable hypothesis is that there may be no requirement for specific HCV sequence downstream of the AUG codon, but only a requirement for the lack of stable RNA structure immediately downstream of the IRES.

Here, we describe the translational activities of the IRES elements of HCV, classical swine fever virus (CSFV), and GBV-B when placed in the context of downstream RNA sequences encoding different reporter proteins. Our results indicate that the presence of stable secondary structure immediately downstream of the translation initiation codon is, in fact, detrimental to translation directed by these IRES elements, but does not impair the initial binding of viral RNA to the 40S ribosomal subunit.

RESULTS

A conserved A-rich domain near the 5' end of the polyprotein-coding sequences of HCV, pestiviruses, and GBV-B

The IRES elements of CSFV and GBV-B are predicted to fold into structures that are very similar to that of HCV (Honda et al., 1996a; Rijnbrand & Lemon, 2000). These IRES elements also contain several stretches of nucleotides with a high level of sequence identity, and thus are likely to function by a similar mechanism. This makes it reasonable to assume that any RNA elements downstream of the AUG codon that are essential for HCV IRES activity are likely to be present in the coding sequences of these other viruses. To search for common features in the 5' polyprotein-coding sequences of these viruses, we aligned the 5' nucleotide sequences of the HCV, pestivirus, and GBV-B open reading frames (Fig. 1A). This revealed the presence of an A-rich domain in each of these viral RNAs, although the domain varies in its distance downstream of the AUG initiator codon in each virus. The domain is 17–18 nt in length, and its 5' border is located between 12 (HCV) and 39 nt (GBV-B) from the AUG codon. It contains a percentage of adenosine residues ranging from 55% in GBV-B to 83% in BVDV and CSFV. The frequencies of guanosine and uridine residues are correspondingly lower in this region (0–18% and 0–12%, respectively).

Two reports have suggested that the HCV IRES functions well when fused directly at the initiator AUG to sequence encoding either CAT or firefly luciferase (FLuc; Tsukiyama-Kohara et al., 1992; Wang et al., 1993). We

FIGURE 1. Alignments of the 5'-terminal regions of the HCV, pestivirus, and GBV-B polyprotein-coding sequences and selected reporter sequences. **A:** Alignment of the 5'-termini of the open reading frames of HCV-H (Inchauspe et al., 1991), BVDV-Osloss (De Moerloose et al., 1993), CSFV-Brescia (Moormann et al., 1990), Border disease virus (BDV) X818 (Becher et al., 1998), and GBV-B (Muerhoff et al., 1995). Gaps introduced to optimize the sequence alignment are indicated by (-); nucleotides that are identical to the HCV sequence are indicated by (.). The conserved A-rich domain is boxed. **B:** Alignment of the 5' ends of the HCV-H coding sequence (Inchauspe et al., 1991), HCV+8-CAT (Rijnbrand et al., 1995), and FLuc reporter sequences. HCV-derived sequence in HCV+8-CAT is underlined.

have also noted efficient internal entry when the CAT sequence is fused at nt 8 of the HCV polyprotein-coding sequence (Rijnbrand et al., 1995). Thus, we searched the 5' CAT and FLuc reporter protein-coding sequences for the presence of a similar A-rich domain. There is no significant nucleotide identity between these sequences and the HCV polyprotein-coding sequence. However, we noted the presence of an A-rich domain near the 5' end of both reporter sequences (Fig. 1B). The A-rich domains in the CAT or FLuc sequences could potentially substitute for the viral A-rich domain when these reporter protein sequences are placed at or close to the initiation codon, if, in fact, the A-rich domain is required for efficient internal initiation of translation.

The A-rich domain contributes to efficient translation of the CAT reporter sequence

To determine whether the A-rich domain in the CAT sequence contributes to efficient IRES activity when this reporter sequence is fused directly to the 5' NTR of HCV, we created a modified CAT reporter sequence ($\Delta 5'$ ACAT) in which the A-rich domain was deleted (Fig. 2A). We then constructed a series of dicistronic plasmids in which the modified CAT sequence was fused to the HCV sequence 3 (AUG), 14 or 32 nt downstream of the HCV 5' NTR (Fig. 2A). These plasmids were used to program coupled in vitro transcription/translation reactions, and the IRES activity for each construct was determined by comparing the amount of CAT produced relative to the RLuc expressed from the upstream cistron. The IRES activity in transcripts from each of the plasmids was compared with that of HCV+32-CAT (arbitrarily set at 100%), in which the wild-type CAT sequence is fused to the 5' 32 nt of the HCV open reading frame. CAT and RLuc translation products were readily detected in each reaction (Fig. 2B). However, the con-

struct containing the deletion of the A-rich domain and no HCV polyprotein-coding sequence downstream of the AUG codon (HCV+3- $\Delta 5'$ ACAT) translated with only 28% of the activity of HCV+32- $\Delta 5'$ CAT, which contains the natural HCV A-rich domain. Thus, the A-rich domain contributes to IRES activity when the CAT sequence is fused directly at the initiator AUG codon. However, the insertion of 14 nt of HCV coding sequence between the IRES and the modified $\Delta 5'$ CAT reporter sequence resulted in a substantial restoration of IRES activity, even though it did not restore the presence of an A-rich domain downstream of the IRES (Fig. 2A). This construct translated at 74% of the efficiency of the HCV+32-CAT construct (Fig. 2B). These results indicate that a downstream A-rich domain, like that which is naturally present in the polyprotein-coding sequences of these viruses, may enhance the activity of the HCV IRES, but is not absolutely required for IRES-dependent translation.

Influence of the HCV polyprotein-coding region on IRES-directed translation

The above results demonstrate that a change in the most 5' nucleotide sequence encoding the reporter proteins can have a significant effect on the efficiency of translation directed by the HCV IRES in dicistronic RNAs such as those shown in Figure 2. Previous reports suggest that reporter protein sequences can be assigned to one of two categories. Some appear to translate well with no or only minimal HCV coding sequence placed between the IRES and the reporter sequence, such as FLuc and CAT (Tsukiyama-Kohara et al., 1992; Wang et al., 1993; Rijnbrand et al., 1995), whereas others demonstrate a strong requirement for HCV coding sequence, such as influenza 'NS, secretory alkaline phosphatase (SAP), or the poliovirus polyprotein (Reynolds et al., 1995; Lu & Wimmer, 1996). However, few direct

FIGURE 2. Influence of the A-rich domain on IRES-directed translation of CAT. **A:** Schematic presentation of dicistronic expression plasmids containing the wild-type and A-rich domain deletion mutant CAT sequence fused to variable lengths of the HCV polyprotein sequence. Solid box: CAT sequence; open box: HCV 5' NTR; cross-hatched box: HCV polyprotein-coding sequence; stippled box: RLuc sequence; T7: T7 RNA polymerase promoter; TΦ: T7 RNA polymerase transcription terminator. The "gguaacc" sequence represents a *KpnI* restriction site facilitating the fusion of the CAT and HCV sequences. **B:** SDS-PAGE analysis of the products of in vitro coupled transcription-translation reactions programmed with the indicated plasmids. Protein products were quantified by PhosphorImager analysis, and the IRES activity determined for each plasmid relative to that of HCV+32-5'ΔCAT (100%) by determining the amount of CAT produced in relation to the RLuc product translated from the upstream cistron. The positions of CAT and RLuc proteins are indicated.

comparisons of IRES activity have been made with different reporter protein sequences in any one set of experiments. Therefore, we determined the relative translational efficiencies of dicistronic transcripts containing the HCV IRES and either the CAT or SAP reporter sequences fused to the HCV sequence 3 (AUG), 14 or 32 nt downstream of the HCV IRES (Fig. 3A). Results of in vitro translation reactions demonstrated that IRES-mediated translation of CAT was efficient and, as suggested by the results presented previously in Figure 2, does not require any HCV capsid sequence beyond the AUG initiation codon (Fig. 3B). In fact, compared with HCV+32-CAT, the translational activity of the IRES was consistently higher in constructs containing 3 or 14 nt of the HCV polyprotein-coding sequence (138% and 121% for HCV+3-CAT and HCV+14-CAT, respectively; Fig. 3B). In contrast, the translational ef-

iciency of the SAP constructs showed a different pattern. Transcripts containing 32 nt of the polyprotein-coding sequence were translated most efficiently, and had a relative translational activity almost three-fold that of the HCV+3-SAP transcripts (Fig. 3B). The inclusion of 14 nt of the HCV coding sequence (HCV+14-SAP) resulted in only a slight increase over the translational activity of HCV+3-SAP. These trends were consistently reproduced in independent experiments.

These results confirm that the IRES requirement for HCV coding sequence is conditional, and dependent upon the specific reporter protein sequence placed downstream of the AUG codon. It is interesting to note that the SAP sequence does not contain an A-rich domain near its 5' end, and that efficient IRES-mediated translation of this protein was dependent upon the inclusion of the natural HCV A-rich domain.

FIGURE 3. Influence of downstream protein-coding sequence on the IRES-mediated translation of CAT, hpCAT, and SAP. **A:** Schematic showing the plasmids used to assess the influence of different reporter sequences and RNA secondary structure on translation. See legend to Figure 2A for details. **B:** SDS-PAGE analysis of *in vitro* translation products, with translation activities relative to the HCV+32 plasmid (100%) calculated as in Figure 2B. **C:** Predicted folding of the hairpin structure that was introduced between the HCV capsid and CAT sequences in Figure 2A. The predicted stability of this hairpin is -18 kcal/mol. **D:** Translation activity of the IRES in the context of CAT (white bars) or hpCAT (black bars) reporter protein sequences in transfected BT7-H cells. Reporter protein expression was determined by enzymatic assay, and translation activities calculated relative to the HCV+32 plasmid (100%) as in the *in vitro* experiments.

Secondary structure immediately downstream of the AUG codon impairs IRES-directed translation

The results presented in Figures 2B and 3B indicate that the nature of the reporter gene determines the requirement for HCV coding sequence. This could be the result of the accidental introduction of RNA structure in close vicinity to the AUG initiator codon when some reporter sequences are fused to the IRES (Honda et al., 1996a). To assess this hypothesis, we inserted a moderately stable hairpin structure (-18 kcal/mol; Fig. 3C) between the HCV IRES and the CAT reporter sequence (hpCAT reporter; Fig. 3A). *In vitro* translation reactions programmed with dicistronic constructs containing the hpCAT reporter sequence and 3 (AUG), 14, or 32 nt of HCV coding sequence revealed a pattern similar to that observed with SAP constructs (Fig. 3B). The IRES in transcripts containing 32 nt of the HCV capsid-encoding sequence (HCV+32-hpCAT) translated the hpCAT product approximately fourfold more

efficiently than the IRES in the HCV+3-hpCAT transcripts (Fig. 3B). Transcripts containing 14 nt of the HCV capsid sequence (HCV+14-hpCAT) translated the hpCAT protein with about 1.5-fold the efficiency of HCV+3-hpCAT. These differences in relative translational activity were confirmed in repeat experiments.

The dicistronic CAT and hpCAT constructs were transfected into BT7-H cells to determine whether the inclusion of a hairpin structure downstream of the IRES would have a similar effect on translational activity *in vivo*. Expression of RLuc from the upstream cistron of the T7 transcripts that are produced in the cytoplasm of these cells served as a control for transfection efficiency, and the relative translational activities of the constructs was calculated from the enzymatic activities of the reporter proteins expressed in these cells. The results were very similar to those observed in the previous *in vitro* translation reactions (Fig. 3D). In transfected cells, the CAT protein was most efficiently translated from transcripts in which no HCV protein-coding sequence was placed between the IRES and

the reporter sequence, whereas the modified hpCAT reporter was most efficiently translated from the construct containing 32 nt of capsid-coding sequence downstream of the AUG codon.

These results argue strongly that the presence of stable RNA sequence immediately downstream of the AUG codon has an important negative effect on translation directed by the HCV IRES. The introduction of a hairpin structure at the 5' end of the CAT sequence strongly reduced its translation from dicistronic transcripts and altered its requirement for intervening HCV protein-coding sequence. The HCV coding sequence placed downstream of the IRES in the HCV+32-hpCAT construct is likely to have enhanced translation by facilitating the interaction of the 40S ribosome subunit with the RNA at the site of the initiation codon. However, it was important to consider the possibility that insertion of the HCV coding sequence may have relieved a repression of translation related to a reduced ability of the hpCAT RNA to bind to the 40S ribosome subunit, or to undergo normal protein elongation following the initiation of translation.

Insertion of a hairpin structure downstream of the initiator AUG does not inhibit the binding of IRES RNA to 40S ribosomal subunits, nor ribosome movement during translation

Pestova et al. (1998) have shown that HCV 5' NTR sequences lacking domain IV (including the initiator AUG codon) retain the ability to bind directly to purified 40S ribosome subunits, indicating that this region of the viral RNA is not required for the formation of a binary complex with the 40S ribosome subunit. This observation makes it unlikely, but does not rule out the possibility that insertion of the hairpin structure immediately downstream of the AUG codon may have reduced the capacity of the RNA to bind to the 40S subunit. To formally evaluate this possibility, we carried out filter binding assays to determine the ability of RNA containing the hairpin to bind to purified 40S ribosome subunits. Because the largest difference in translation initiation was observed between Rluc-HCV+3-CAT and Rluc-HCV+3-hpCAT (Fig. 3D), we tested [³²P]-labeled RNA probes encompassing the entire 5' NTR and a short segment of the CAT-coding sequence from these constructs in these assays. To include a control RNA lacking an intact IRES and thus devoid of 40S subunit-binding activity, we created a related mutant RNA deficient in domain III IRES sequence (HCV+3ΔIII). The results of these filter binding assays indicated that the 40S subunit-binding activity of the HCV+3hp RNA was indistinguishable from that observed with the HCV+3 probe over the range of 40S ribosomal subunit concentrations tested (Fig. 4). As expected, there was no significant binding of the HCV+3ΔIII RNA. These re-

FIGURE 4. Binding of the HCV IRES to purified 40S ribosomal subunits is not impaired by the insertion of the hairpin structure immediately downstream of the AUG initiator codon in hpCAT, as determined in a filter binding assay. The ratio of bound to unbound RNA is indicated as a function of the concentration of purified 40S ribosomal subunits in each binding reaction. RNA transcripts evaluated in the assay include HCV+3ΔIII, which lacks an active IRES due to the removal of domain III sequences (◆), HCV+3 (□), and HCV+3hp (△).

sults confirm that the introduction of the hairpin structure immediately downstream of the AUG in hpCAT does not compromise the ability of the IRES to bind to the 40S subunit, and eliminates this as an explanation for the decrease in translational activity associated with the insertion of the hairpin.

To further demonstrate that the hairpin is not inhibitory to ribosome movement along the RNA, we constructed monocistronic plasmids containing the CAT and hpCAT reporter sequences that lacked both the upstream RLuc sequence and the 5' 278 nt of the HCV 5' NTR (Fig. 5A). Transcripts produced from these constructs lack much of the IRES sequence, and initiate translation by a 5'-dependent ribosome scanning mechanism (Honda et al., 1996a). These constructs varied in the efficiency with which they produced CAT when used to program *in vitro* translation reactions (Fig. 5B). However, in contrast to the dicistronic constructs in which translation was mediated by the HCV IRES, the variation in translational activity did not correlate with the presence or absence of the hairpin nor the length of HCV coding sequences included. The translational differences observed in this experiment are likely to reflect the different context of the AUG codon in individual constructs. These results confirm that the insertion of the hairpin sequence specifically inhibits IRES-mediated translation initiation, and does not interfere with later steps in protein synthesis.

The HCV IRES has no requirement for a specific nucleotide or amino acid coding sequence downstream of the initiator codon

The experiments described above confirm that HCV protein-coding sequence is required for efficient IRES-

FIGURE 5. The hairpin insertion in hpCAT does not influence the efficiency of scanning-dependent translation of CAT. **A:** Schematic showing the 5' deletion mutants. "ΔIRES" indicates the 5' truncated HCV 5' NTR that lacks IRES activity. For other details, see the legend to Figure 2A. **B:** SDS-PAGE analysis of the products of in vitro translation reactions programmed with the plasmids depicted in **A**. The IRES activity of each construct was calculated relative to that of ΔIRES+32-CAT as in Figure 2B.

mediated translation of some but not all reporter sequences. To determine whether this requirement relates to the nucleotide sequence of the downstream RNA or the amino acid sequence of the protein it encodes, we assessed the effect of frameshift mutations placed within the HCV coding sequence in HCV+32-hpCAT. As shown in Figure 3B, the 32 nt of HCV protein-coding sequence downstream of the IRES is essential for efficient translation of this reporter protein. To determine whether this sequence is acting at the RNA or protein level, a guanosine residue at position +346 in the HCV genome was removed in combination with the insertion of a cytidine at the fusion with the hpCAT sequence in RLuc-HCV+32-hpCAT (Fig. 6A, Sh-hpCAT). Amino acids 2–11 of the Sh-hpCAT reporter thus differ from those in the HCV+32-hpCAT reporter protein. When these constructs were used to program in vitro translation reactions, the activity of the IRES in the frameshift mutant was similar to that of the wild-type sequence (Fig. 6B, 156% of HCV+32-hpCAT). This indicates that the ability of the inserted HCV sequence to enhance IRES activity is related to its nucleotide sequence and not dependent on the protein it encodes.

Reynolds et al. (1995) reported that nt 4–33 of the HCV protein-coding sequence were specifically required for optimal translation of a downstream reporter protein, and that it could not be converted to its complementary sequence without loss of HCV IRES activity. To determine whether this was the case in the context of the +32-hpCAT reporter protein, we constructed a series of additional mutants in which different segments of the HCV coding sequence in pRLuc-HCV+32hpCAT were converted to their complementary sequence (Fig. 6A; C5–21, C22–28, C29–35). Each of these three mutants translated with efficiencies that were similar to or greater than that of the wild-type construct, indicating that no specific nucleotide sequence is required for efficient HCV IRES activity (Fig. 6B). These data provide strong evidence in favor of the hypothesis that the only specific requirement is

FIGURE 6. The influence of changes in the 5' terminal capsid-coding sequence on the ability of the HCV IRES to mediate translation of the hpCAT reporter. **A:** Schematic showing the nucleotide (boxes) and amino acid sequences of the mutated spacer sequences placed between the initiation codon and the downstream hpCAT sequence in dicistronic reporter plasmids. For details, see the legend to Figure 2A. Nucleotides that are complementary to the wild-type HCV sequence are shown in lower case; a (–) indicates a deleted nucleotide. To prevent the formation of an in-frame stop codon in mutant C22–28, an A-to-C conversion was introduced at position 28 of the reading frame. **B:** SDS-PAGE analysis of the products of in vitro translation reactions programmed with the plasmids depicted in **A**. The IRES activity of each construct was calculated relative to that of HCV+32-hpCAT (100%) by determining the amount of hpCAT produced in relation to RLuc.

for a lack of stable secondary RNA structure immediately downstream of the IRES.

The GBV-B and CSFV IRES elements also have a requirement for nonstructured RNA downstream of the IRES

To determine whether the GBV-B and CSFV IRES elements require downstream cognate viral protein-coding sequence for optimal activity, we constructed sets of dicistronic expression plasmids containing the GBV-B or CSFV IRES and either CAT or hpCAT reporter sequences, similar to those described above for HCV. In the GBV-B genomic sequence, the A-rich domain is located between 39 and 56 nt downstream of the AUG codon (Fig. 1A). Accordingly, we constructed GBV-B plasmids that contained either 3 (AUG), 14, 39, or 63 nt of the GBV-B polyprotein-coding region between the entire 5'NTR of GBV-B and the CAT or hpCAT sequence (Fig. 7A). As with HCV, the CAT protein was translated efficiently *in vitro* when the CAT coding sequence was fused directly at the AUG codon (Fig. 7B; GBB+3-CAT). No significant increase in translation of the reporter protein followed the insertion of GBV-B

protein-coding sequence downstream of the IRES (compare GBB+63-CAT with GBB+3-CAT in Fig. 7B). However, as with the HCV dicistronic plasmids, GBV-B IRES activity was exquisitely dependent upon the inclusion of an intervening segment of viral protein-coding sequence when the CAT reporter sequence was replaced by the structured hpCAT sequence. The inclusion of 14 nt of GBV-B sequence increased translation two-fold, while the inclusion of 39 or 63 nt resulted in a ninefold increase in translation of the hpCAT reporter protein (Fig. 7B). Thus, the requirement for unstructured RNA immediately downstream of the initiator codon that is demonstrated by the HCV IRES appears to be shared also by the GBV-B IRES.

One important difference in the structures that have been predicted for the HCV, GBV-B, and pestiviral IRES elements is the lack of a stem-loop containing the AUG initiation codon in the pestiviruses (stem-loop IV in HCV and GBV-B; Honda et al., 1996a; Sizova et al., 1998). To determine whether the apparent absence of RNA structure in the vicinity of the initiator codon lessens or enhances the requirement of the pestivirus IRES for unstructured downstream sequence, we created an additional series of dicistronic plasmids containing the CSFV IRES and the CAT or hpCAT reporter protein sequences. These contained either 4, 14, 32, or 55 nt of the CSFV N^{pro} coding sequence placed between the IRES and the reporter sequence (Fig. 8A). When these constructs were used to program *in vitro* translation reactions, the results were very similar to those obtained with the HCV (Fig. 3B) and GBV-B (Fig. 7B) constructs. CAT was efficiently translated in the absence of any intervening CSFV protein-coding sequence downstream of the IRES (Fig. 8B; CSFV+3-CAT), and the structured hpCAT sequence generated no detectable product when fused directly to the CSFV IRES (Fig. 8B; CSFV+3-hpCAT). A minimal amount of product was observed with the inclusion of 14 nt of protein-coding sequence downstream of the IRES. This was increased 10-fold or more by the inclusion of 32 or 54 nt of CSFV polyprotein sequence (Fig. 8B). These results indicate that the CSFV IRES is extremely sensitive to the presence of structured RNA placed immediately downstream of the AUG initiation codon.

FIGURE 7. Influence of downstream RNA secondary structure on the activity of the GBV-B IRES. **A:** Schematic representation of the DNA cassette that was used to construct dicistronic plasmids to determine the influence of downstream viral polyprotein-coding sequence on GBV-B IRES activity. The nucleotide sequence of the putative GBV-B capsid protein-coding region is depicted, with the A-rich domain enclosed within a box. Arrows indicate the points of fusion between GBV-B and CAT or hpCAT sequences. For additional details, see the legend to Figure 2A. **B:** SDS-PAGE analysis of the products of *in vitro* translation reactions programmed with the plasmids depicted in **A**. The IRES activity of each transcript was calculated relative to that of GBB+63-CAT, in order to facilitate comparison between the different constructs.

DISCUSSION

Previous reports concerning the role of the 5' polyprotein-coding sequence in the initiation of translation by the HCV IRES have been contradictory. Whereas some investigators concluded that the most 5' sequence encoding the capsid protein has an important role in translation (Reynolds et al., 1995; Honda et al., 1996b; Lu & Wimmer, 1996), others have demonstrated efficient IRES activity in transcripts containing no more than 8 nt of the HCV coding sequence and, in some cases, no more than the AUG codon

FIGURE 8. Influence of downstream RNA secondary structure on the activity of the CSFV IRES. **A:** Schematic representation of the DNA cassette that was used to construct dicistronic plasmids to determine the influence of downstream viral polyprotein-coding sequence on activity of the CSFV IRES. The nucleotide sequence of the CSFV N^{pro} proteinase-coding region is depicted, with the A-rich domain enclosed in a box. Arrows indicate the points of fusion between CSFV and CAT or hpCAT sequences. For additional details, see the legend to Figure 2A. **B:** SDS-PAGE analysis of the products of *in vitro* translation reactions programmed with the plasmids depicted in **A**. The IRES activity of each construct was calculated relative to that of CSFV+55-CAT.

alone (Tsukiyama-Kohara et al., 1992; Wang et al., 1993; Rijnbrand et al., 1995). The IRES elements of GBV-B and the pestiviruses have not been studied as intensively. However, Chon et al. (1998) recently reported that there was only a 21% difference in the translation of transcripts in which the FLuc sequence was fused directly to the AUG codon of BVDV, or placed downstream of the entire N^{pro} coding sequence.

Here, we have shown that dicistronic transcripts containing no HCV coding sequence other than the AUG codon translated with greater efficiency than transcripts in which either 14 or 32 nt of the capsid sequence were placed between the IRES and the CAT sequence (Fig. 3B). These results confirm the early observations of Tsukiyama-Kohara et al. (1992) and Wang et al. (1993). On the other hand, we have also shown that efficient translation of the SAP sequence requires the inclusion of approximately 32 nt of the downstream coding sequence (Fig. 3B). These results confirm those reported by Reynolds et al. (1995). The conflicting requirements for coding sequence that are apparent with these two reporter proteins indicate that the requirement for coding sequence is conditional, dependent on the nature of the translated sequence, and not an intrinsic characteristic of the IRES element.

Reynolds et al. (1995) suggested that the HCV IRES was able to translate the FLuc sequence efficiently in the absence of HCV coding sequence because of a coincidental homology between the nucleotide sequence at 5' end of the HCV open reading frame and that of FLuc. This prompted us to look more closely at the 5' segment of the CAT sequence that is translated efficiently in the absence of the HCV polyprotein-coding sequence (Rijnbrand et al., 1995, 1996). The only feature we found these sequences to share in common is the presence of an A-rich domain near their 5' ends, a feature that is also present in the FLuc sequence (Fig. 1B). A more extensive search among related viruses revealed a similar nucleotide sequence in the most 5' polyprotein-coding segments of both the pestivirus and GBV-B genomes (Fig. 1A). This domain is approximately 18 nt in length, and contains >50% adenosine residues. Although located at varying distances from the 5' terminus of the coding sequence, the presence of similar A-rich domains in each of these RNAs suggested an important role in the viral life cycle. Because this region of the genome encodes different amino acid sequences, and even different proteins in these viruses (e.g., N^{pro} in the pestiviruses, and a capsid protein in HCV and GBV-B), it is unlikely that the shared presence of an A-rich domain reflects a conserved protein function.

The deletion of the A-rich domain from the CAT sequence significantly reduced the ability of the HCV IRES to direct its translation (Fig. 2B), suggesting that this domain does play a role in translation initiation. However, we found that the translational activity could be substantially restored by the inclusion of at least 14 nt of the HCV capsid-coding sequence upstream of CAT, even though this did not reconstitute an A-rich domain within the transcript. Thus, although the A-rich domain in the CAT sequence contributes to its ability to be translated by the IRES, these results indicate that it plays no essential role in IRES-mediated translation. We also found this to be the case with the A-rich domain within the GBV-B and CSFV genomes, as we consistently observed efficient translation of reporter proteins from transcripts that lacked this domain (Figs. 6B and 7B). These results are consistent with studies of pestivirus RNA replicons, as Meyers and Thiel (1995) described a CSFV RNA that replicated efficiently despite the absence of the A-rich domain. We conclude that although the A-rich domain may facilitate translation in some RNAs, it is not essential for IRES-mediated translation.

Because these results suggested that there is no specific sequence required downstream of the IRES for efficient translation, we considered the possibility that the conditional requirement for polyprotein-coding sequence reflects a need for unstructured RNA immediately downstream of the IRES, and that different 5' reporter sequences may vary in the extent to which

they form stable RNA structure (Honda et al., 1996a). Substantial evidence indicates that there is no scanning of the 40S ribosome subunit prior to its interaction with the authentic initiator AUG codon on HCV RNA (Honda et al., 1996a; Reynolds et al., 1996; Rijnbrand et al., 1996). These data thus indicate that stem-loop IV, which contains the initiation codon within its loop segment, must melt to accommodate binding of the ribosome at the site of the AUG codon. Mutations that minimally stabilize this structure, and that do not hinder the passage of a scanning 40S ribosome subunit, significantly inhibit IRES-mediated translation (Honda et al., 1996a). It is possible that the coding sequences of some reporter proteins may either interact with the stem-loop IV sequence in a manner that stabilizes structure in this region, or alternatively form stable structure immediately downstream of the AUG that is similarly antagonistic to the interaction with the 40S subunit. In either case, stable structure could interfere sterically with the interaction of the RNA with the 40S subunit.

Secondary structure predictions for this region of the HCV genome indicate the presence of a relatively unstructured region encompassing the A-rich domain, flanked by two hairpin structures: stem-loops IV (Smith et al., 1995; Honda et al., 1996a) and V (Smith & Simmonds, 1997). A similar lack of secondary structure is predicted for the A-rich domains of pestiviruses and GBV-B, consistent with the concept that an unstructured RNA sequence just downstream of the start codon may be required for these viral IRES elements. Selective pressure against secondary structure in this region may explain the relative bias towards adenosine residues, as a predominance of adenosine residues would reduce the potential to form stable stem-loop structures.

To assess the impact of secondary RNA structure downstream of the IRES, we constructed dicistronic plasmids in which a stable hairpin structure was placed at the 5' end of the CAT sequence. With each of the IRES elements examined (HCV, GBV-B, and CSFV), translation was sharply reduced when this hairpin was placed immediately downstream of the initiation codon, but could be restored by the insertion of viral polyprotein-coding sequence between the AUG and the hairpin (Figs. 3B, 7B, and 8B). These experiments confirm that IRES function is extremely sensitive to stable structure immediately downstream of the initiator AUG, and add to previous studies indicating that stabilization of stem-loop IV is detrimental to translation (Honda et al., 1996a). Computer folding of the SAP sequence that was employed as a reporter by Reynolds et al. (1995) suggests that it may form an extended, complex stem-loop structure at its 5' end (Fig. 9A). The results presented in Figure 3B suggest that such a structure would be strongly inhibitory to translation, if it were fused directly at the AUG initiator codon of HCV. This is likely to be the reason that Reynolds et al. (1995) found it necessary to place HCV coding sequence upstream of the SAP sequence in order to obtain efficient translation of this reporter protein. Analysis of the $\Delta 5'$ ACAT sequence, which translated poorly as shown in Figure 2B, similarly suggests the presence of relatively stable RNA structure at its 5' terminus (data not shown).

Although Reynolds et al. (1995) suggested that a specific nucleotide sequence was required downstream of the initiator codon for efficient IRES activity, our results argue strongly against this conclusion. We did not document a decrease in translational activity when the complementary RNA sequence was substituted for the polyprotein-coding sequence placed between the IRES

FIGURE 9. Computer-assisted predictions of RNA folding. **A:** Predicted structure of the SAP RNA transcript. The 5' terminus of the SAP sequence is marked by a circle. RNA structure was predicted using the RNA draw program V1.1 from the Vienna package. **B:** Predicted RNA structure within the XL40-372.Arec transcript. The AUG codon is indicated in boldface.

and the reporter sequence, in those transcripts containing a reporter protein (hpCAT) that was poorly translated in the absence of any intervening sequence (Fig. 6). These results are contradictory to those of Reynolds et al. (1995), who found that this RNA segment had to be in the sense orientation to promote translation. However, computer folding of the sequence surrounding the translation initiation codon of their mutant, XL40-372.ARec (Reynolds et al., 1995), suggests the formation of a novel hairpin structure containing the AUG initiator codon (Fig. 9B). Such a structure would be expected to be strongly inhibitory to HCV IRES activity (Honda et al., 1996a).

In summary, our results suggest that there is no strict requirement for a specific nucleotide sequence immediately downstream of the IRES elements of HCV, GBV-B, or CSFV, but rather a requirement for a lack of stable RNA structure in this region. Current concepts of the mechanism by which these IRES elements initiate internal entry of the ribosome involve a direct interaction of the RNA with the 40S subunit that can occur in the absence of any soluble protein translation factors (Pestova et al., 1998). In the course of this interaction, the ribosome becomes positioned at the AUG codon in the absence of any scanning on the viral RNA (Honda et al., 1996a; Reynolds et al., 1996; Rijnbrand et al., 1996, 1997; Pestova et al., 1998). Stable structure immediately downstream of the AUG is likely to inhibit this interaction, resulting in a negative effect on cap-independent translation. The results of filter binding assays (Fig. 4) indicated that the hairpin structure inserted into hpCAT did not interfere with the ability of the IRES RNA to bind to purified 40S ribosome subunits. This suggests that the repression of translation by stable RNA structure located just downstream of the initiator codon results from inhibition of a subsequent step in the internal entry of the ribosome, perhaps one that allows the correct positioning of the initiation codon within the ribosomal P-site (Pestova et al., 1998). Consistent with the 11–15 nt footprint of a ribosome that extends on either side of the initiation codon, the insertion of unstructured viral RNA sequence downstream of the AUG codon that results in the repositioning of such stable structure in a 3' direction more than 15 nt relieves this steric repression, and allows the initial RNA–ribosome interaction to proceed.

MATERIALS AND METHODS

Enzymes, oligonucleotides, and bacterial strains

Restriction endonucleases, DNA and RNA polymerases, and T4 DNA ligase were supplied by New England Biolabs, Gibco BRL, and Promega. Oligonucleotides were purchased from Eurogentec, Inc., and Genosys. *Escherichia coli* JM109 was used as the bacterial host.

Dicistronic reporter plasmids containing the HCV IRES

DNA fragments containing the entire HCV (Hutchinson strain) 5' NTR and either 3 (AUG only), 14, or 32 nt of the HCV capsid-coding sequence were obtained by polymerase chain reaction (PCR) and cloned into the plasmid pRL-GBV-B+3-CAT (Rijnbrand et al., 2000), resulting in pRL-HCV+3-CAT, pRL-HCV+14-CAT, and pRL-HCV+32-CAT, respectively (Fig. 3A). T7 RNA polymerase transcription of these plasmids results in a dicistronic RNA, in which the *Renilla* luciferase (RLuc) coding sequence is the upstream cistron and the bacterial CAT coding sequence forms the downstream cistron. The translation of the CAT sequence is dependent on the HCV IRES that is placed within the intercistronic domain. A T7 RNA transcription terminator is located 3' of the CAT coding sequence. An additional series of expression plasmids was constructed in which the CAT reporter sequence was replaced by either the sequence encoding SAP or CAT open reading frames modified by PCR-based strategies. The SAP coding sequence, flanked by *KpnI* and *BglII* restriction sites, was amplified by PCR and cloned into the HCV +3, +14, and +32 constructs between the *KpnI* and *BamHI* restriction sites. Modifications of the CAT reporter sequence were constructed by PCR mutagenesis. These included a mutant that lacks the sequence CGAGAAAAAATCAC that is present at the 5' end of the wild-type CAT sequence (indicated by the prefix $\Delta 5'$ ACAT). In a second mutant, the CAT sequence was fused to a short upstream sequence that is able to form a stable hairpin structure ($\Delta G = -18$ kcal/mol) at the 5' end of the CAT open reading frame (indicated by the prefix hpCAT) by inserting the sequence GCCGAGCTCTCGGCGGTACGT downstream of the *KpnI* restriction site at the start of the CAT sequence (Fig. 3C). PCR fragments containing specific mutations within the first 35 nt of the HCV open reading frame were substituted for the analogous sequences within the hpCAT reporter constructs. The resulting plasmids contain either a frameshift mutation or the sequence complementary to the native HCV sequences. These specific constructs are described in the text and figures. Recombinant DNA techniques were carried out using standard procedures (Sambrook et al., 1989). The nucleotide sequences of the mutagenized PCR fragments were verified by DNA sequence analysis of the resulting plasmids.

Monocistronic reporter plasmids

pRL-HCV+3-CAT, pRL-HCV+14-CAT, pRL-HCV+32-CAT, pRL-HCV+3-hpCAT, pRL-HCV+14-hpCAT, and pRL-HCV+32-hpCAT were digested with *SacII* and *StuI* restriction enzymes. The ends were polished using T4 DNA polymerase and the DNA was religated. The resulting plasmids (p Δ IRES+3-CAT, p Δ IRES+14-CAT, p Δ IRES+32-CAT, p Δ IRES+3-hpCAT, p Δ IRES+14-hpCAT, and p Δ IRES+32-hpCAT) lack the upstream RLuc reading frame and nt 1–278 of the HCV 5' NTR.

Reporter plasmids containing the GBV-B or CSFV IRES elements

Plasmids pRL-GBB+3-CAT, pRL-GBB+14-CAT, pRL-GBB+38CAT, and pRL-GBB+63-CAT were described previously

(Rijnbrand et al., 2000). The *KpnI*-*Bam*HI CAT fragment in these constructs was replaced by the hpCAT fragment to create pRL-GBB+3-hpCAT, pRL-GBB+14-hpCAT, pRL-GBB+38-hpCAT, and pRL-GBB+63-hpCAT. PCR fragments containing the entire CSFV-C strain 5' NTR in combination with 4, 14, 32, or 55 nt of the CSFV-C open reading frame from pPRC129 (Moormann et al., 1990) were cloned into the *XhoI* and *KpnI* restriction sites upstream of the CAT and hpCAT reporter sequences in pRLuc-HCV+3-CAT and pRLuc-HCV+3-hpCAT.

In vitro translation

Proteins were expressed in a coupled in vitro transcription-translation reaction carried out in rabbit reticulocyte lysate (TNT; Promega). The protein products were analyzed by SDS-PAGE and quantitated using a PhosphorImager (Molecular Dynamics). The relative translation efficiency (RTE) of mutant constructs was determined by calculating the ratio of the CAT products in relation to the amount of RLuc produced in these reactions.

DNA transfection of BT7-H cells and preparation of cell lysates

Nearly confluent monolayers of BT7-H cells (Whetter et al., 1994) grown in 35-mm dishes were transfected using FuGENE 6 (Boehringer-Mannheim) as follows. In a polypropylene tube, 100 μ L OptiMEM (Gibco-BRL) and 6 μ L of FuGENE reagent were mixed and incubated for 10 min at room temperature. QIAprep Spin (Qiagen) purified plasmid DNA (4 μ g) was added, mixed, and incubated for 15 min. Finally, 100 μ L of this mixture was added directly to the cell culture medium (2 mL). At 20–24 h following transfection, the cells were washed twice with PBS and harvested with a plastic scraper into 1 mL TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) and centrifuged for 2 min at room temperature. The cells were resuspended in 125 μ L of 0.25 M Tris-HCl, pH 8.0, and lysed by three cycles of freeze/thawing.

Determination of luciferase expression

Immediately following the third freeze/thaw cycle, cellular debris was removed from cell lysates by centrifugation. RLuc activity was measured in 20 μ L of cell lysate using reagents from the Dual Luciferase Reporter Assay System (Promega). Prior to determining CAT activity, the lysate was heated for 10 min at 60°C to inactivate endogenous acetylase activity. Ten microliters of the lysate were incubated in a total volume of 125 μ L of a reaction mix containing 200 μ g/mL *n*-butyryl Coenzyme A, 250 mM Tris-HCl, pH 8.0, and 10 μ L D-threo[dichloroacetyl-1,2-¹⁴C]chloramphenicol (50–60 mCi/mmol) for 12 h at 37°C. Activity was measured by liquid scintillation counting following xylene phase extraction.

RNA binding to 40S ribosome subunits

RNA filter binding assays were carried out as described previously (Kieft et al., 2001). Monocistronic [³²P]-CTP labeled transcripts were synthesized from plasmids created by swap-

ping the appropriate fragments of pRLuc-HCV+3-CAT and pRLuc-HCV+3-hpCAT and pWT-CATT (Rijnbrand et al., 1995). Transcription templates were generated by PCR amplification of the region between the T7 promoter and the 5' terminal 56 nt of the CAT gene.

ACKNOWLEDGMENTS

We thank M.T. Chapa for expert technical assistance and E.R. Surriga and S. Smith for sequencing of plasmid DNA. This work was supported in part by grants from the National Institute of Allergy and Infectious Disease (U19-AI40035), and the Texas Advanced Technology Program (004952-025). RR is the recipient of the American Liver Foundation Blowitz-Ridgeway Liver Scholar Award.

Received March 9, 2000; returned for revision April 14, 2000; revised manuscript received January 3, 2001

REFERENCES

- Becher P, Orlich M, Thiel HJ. 1998. Complete genomic sequence of border disease virus, a pestivirus from sheep. *J Virol* 72:5165–5173.
- Brown EA, Zhang H, Ping L, Lemon SM. 1992. Secondary structure of the 5' nontranslated regions of hepatitis C virus and pestiviruses genomic RNAs. *Nucleic Acids Res* 20:5041–5045.
- Chon SK, Perez DR, Donis RO. 1998. Genetic analysis of the internal ribosome entry segment of bovine viral diarrhoea virus. *Virology* 251:370–382.
- De Moerloose L, Lecomte C, Brown-Shimmer S, Schmetz D, Guitt C, Vandenberg D, Allaer D, Rossius M, Chappuis G, Dina D. 1993. Nucleotide sequence of the bovine viral diarrhoea virus Osloss strain: Comparison with related viruses and identification of specific DNA probes in the 5' untranslated region. *J Gen Virol* 74:1433–1438.
- Han JH, Shyamala V, Richman KH, Brauer MJ, Irvine B, Urdea MS, Tekamp-Olson P, Kuo G, Choo QL, Houghton M. 1991. Characterization of the terminal regions of hepatitis C viral RNA: Identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. *Proc Natl Acad Sci USA* 88:1711–1715.
- Honda M, Brown EA, Lemon SM. 1996a. Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. *RNA* 2:955–968.
- Honda M, Ping LH, Rijnbrand RCA, Amphlett E, Clarke B, Rowlands D, Lemon SM. 1996b. Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. *Virology* 222:31–42.
- Houghton M. 1996. Hepatitis C viruses. In: Fields BN, Knipe DM, Howley PM, eds. *Virology*. Philadelphia: Lippincott-Raven. pp 1035–1058.
- Inchauspe G, Zebedee S, Lee DH, Sugitani M, Nasoff M, Prince AM. 1991. Genomic structure of the human prototype strain H of hepatitis C virus: Comparison with American and Japanese isolates. *Proc Natl Acad Sci USA* 88:10292–10296.
- Kamoshita N, Tsukiyama-Kohara K, Kohara M, Nomoto A. 1997. Genetic analysis of internal ribosomal entry site on hepatitis C virus RNA: Implication for involvement of the high ordered structure and cell type-specific transacting factors. *Virology* 233:9–18.
- Kieft JS, Zhou K, Jubin R, Doudna JA. 2001. Mechanism of ribosome recruitment by hepatitis C IRES RNA. *RNA* 7:194–206.
- Kozak M. 1990. Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. *Proc Natl Acad Sci USA* 87:8301–8305.
- Lu HH, Wimmer E. 1996. Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus. *Proc Natl Acad Sci USA* 93:1412–1417.

- Meyers G, Thiel H-J. 1995. Cytopathogenic of classical swine fever virus caused by defective interfering particles. *J Virol* 69:3683–3689.
- Moormann RJ, Warmerdam PA, van der Meer B, Schaaper WM, Wensvoort G, Hulst MM. 1990. Molecular cloning and nucleotide sequence of hog cholera virus strain Brescia and mapping of the genomic region encoding envelope protein E1. *Virology* 177:184–198.
- Muerhoff AS, Leary TP, Simons JN, Pilot-Matias TJ, Dawson GJ, Erker JC, Chalmers ML, Schlauder GG, Desai SM, Mushahwar IK. 1995. Genomic organization of GB viruses A and B: Two new members of the Flaviviridae associated with GB agent hepatitis. *J Virol* 69:5621–5630.
- Pestova TV, Shatsky IN, Fletcher SP, Jackson RJ, Hellen CUT. 1998. A prokaryotic-like mode of cytoplasmatic eukaryotic ribosome binding to the initiation codon during internal translation of hepatitis C virus and classical swine fever virus RNAs. *Genes & Dev* 12:67–83.
- Poole TL, Wang C, Popp RA, Potgieter LND, Siddiqui A, Collet MS. 1995. Pestivirus translation initiation occurs by internal ribosome entry. *Virology* 206:750–754.
- Reynolds JE, Kaminski A, Carroll AR, Clarke BE, Rowlands DJ, Jackson RJ. 1996. Internal initiation of translation of hepatitis C virus RNA: The ribosome entry site is at the authentic initiation codon. *RNA* 2:867–878.
- Reynolds JE, Kaminski A, Kettinen HJ, Grace K, Clarke BE, Carroll AR, Rowlands DJ, Jackson RJ. 1995. Unique features of internal initiation of hepatitis C virus RNA translation. *EMBO J* 14:6010–6020.
- Rice CM. 1996. Flaviviridae: The viruses and their replication. In: Fields BN, Knipe DM, Howley PM, eds. *Virology*. Philadelphia: Lippincott-Raven. pp 931–959.
- Rijnbrand R, Abell G, Lemon SM. 2000. Mutational analysis of the GB virus B internal ribosome entry site. *J Virol* 74:773–783.
- Rijnbrand R, Bredenbeek P, van der Straaten T, Whetter L, Inchauspe G, Lemon S, Spaan W. 1995. Almost the entire 5' non-translated region of hepatitis C virus is required for cap-independent translation. *FEBS Lett* 365:115–119.
- Rijnbrand R, Lemon SM. 2000. IRES mediated translation in hepatitis C virus replication. *Curr Top Microbiol Immunol* 242:85–116.
- Rijnbrand R, van der Straaten T, van Rijn P, Spaan WJM, Bredenbeek PJ. 1997. Internal entry of ribosomes is directed by the 5' noncoding region of classical swine fever virus and is dependent on the presence of an RNA pseudoknot upstream of the initiation codon. *J Virol* 71:451–457.
- Rijnbrand RC, Abbink TE, Haasnoot PC, Spaan WJ, Bredenbeek PJ. 1996. The influence of AUG codons in the hepatitis C virus 5' nontranslated region on translation and mapping of the translation initiation window. *Virology* 226:47–56.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Sizova DV, Kolupaeva VG, Pestova TV, Shatsky IN, Hellen CU. 1998. Specific interaction of eukaryotic translation initiation factor 3 with the 5' nontranslated regions of hepatitis C virus and classical swine fever virus RNAs. *J Virol* 72:4775–4782.
- Smith DB, Mellor J, Jarvis LM, Davidson F, Kolberg J, Urdea M, Yap PL, Simmonds P. 1995. Variation of the hepatitis C virus 5' non-coding region: Implications for secondary structure, virus detection and typing. The International HCV Collaborative Study Group. *J Gen Virol* 76:1749–1761.
- Smith DB, Simmonds P. 1997. Characteristics of nucleotide substitution in the hepatitis C virus genome: Constraints on sequence change in coding regions at both ends of the genome. *J Mol Evol* 45:238–246.
- Thiel HJ, Plogemann PGW, Moennig V. 1996. Pestiviruses. In: Fields BN, Knipe DM, Howley PM, eds. *Virology*. Philadelphia: Lippincott-Raven. pp 1059–1073.
- Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A. 1992. Internal ribosome entry site within hepatitis C virus RNA. *J Virol* 66:1476–1483.
- Wang C, Le SY, Ali N, Siddiqui A. 1995. An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. *RNA* 1:526–537.
- Wang C, Sarnow P, Siddiqui A. 1993. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome binding mechanism. *J Virol* 67:3338–3344.
- Wang C, Sarnow P, Siddiqui A. 1994. A conserved helical element is essential for internal initiation of translation of hepatitis C virus RNA. *J Virol* 68:7301–7307.
- Whetter LE, Day SP, Elroy-Stein O, Brown EA, Lemon SM. 1994. Low efficiency of the 5' nontranslated region of hepatitis A virus RNA in directing cap-independent translation in permissive monkey kidney cells. *J Virol* 68:5253–5263.