

Differential Effects on the Hepatitis C Virus (HCV) Internal Ribosome Entry Site by Vitamin B₁₂ and the HCV Core Protein

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To investigate the role of the hepatitis C virus internal ribosome entry site (HCV IRES) domain IV in translation initiation and regulation, two chimeric IRES elements were constructed to contain the reciprocal domain IV in the otherwise HCV and classical swine fever virus IRES elements. This permitted an examination of the role of domain IV in the control of HCV translation. A specific inhibitor of the HCV IRES, vitamin B₁₂, was shown to inhibit translation directed by all IRES elements which contained domain IV from the HCV and the GB virus B IRES elements, whereas the HCV core protein could only suppress translation from the wild-type HCV IRES. Thus, the mechanisms of translation inhibition by vitamin B₁₂ and the core protein differ, and they target different regions of the IRES.

Hepatitis C virus (HCV) is classified within the genus *Hepacivirus* of the *Flaviviridae* family. All members of the family *Flaviviridae* are small, icosahedral, enveloped viruses that contain a positive-sense RNA genome (2, 5). The genomes share a common organization, in which a single large open reading frame is flanked by 5' and 3' untranslated regions (UTR). The open reading frame encodes a large polyprotein which is processed into individual structural and nonstructural proteins. The family *Flaviviridae* consists of three genera: *Flavivirus*, *Pestivirus*, and *Hepacivirus*. It was suggested that three new proposed members of the *Flaviviridae*, the GB agents (GB virus [GBV] A, B, and C) should be classified in a separate genera within the *Flaviviridae* or in a subgenera of *Hepacivirus* (20).

The genomes of the pestiviruses (viz., bovine viral diarrhoea virus and classical swine fever virus [CSFV]), HCV, and the GB agents have highly structured 5' UTR of similar lengths (341 to 445 nucleotides [nt]) that contain multiple AUG triplets preceding the authentic polyprotein initiation codon. These structured RNA segments function as an internal ribosome entry site (IRES) to initiate polyprotein translation (6, 16, 31). Among these IRES elements, the HCV and the GBV-B IRESs are very similar in terms of proposed structure and function and differ from the IRES elements of the picornaviruses (16). Although there are slight differences in the proposed structures, the IRES elements of HCV, GBV-B, and pestiviruses were classified as type 2 IRES elements (6). The central domain of these IRES elements (domain III) is highly conserved and their predicted secondary structures, first pro-

posed by Brown et al. (3) and modified at a later date (11), are superimposable. The structural integrity of domain III is essential for IRES activity (25, 32). However, a major difference is that the HCV and GBV-B IRES elements contain a stem-loop within domain IV whereas this feature is missing in the pestivirus IRES elements (11). The function of the stem-loop which comprises domain IV of the HCV IRES element is unclear.

There is still some uncertainty over the requirement for the inclusion of the core protein coding sequence for optimal HCV IRES function (for a review, see reference 26), and this point may be refined to question if stem-loop IV is necessary, since the core coding sequence forms part of stem-loop IV. Early studies demonstrated that the inclusion of nt 1 to 32 of the core protein coding sequences was essential for efficient IRES activity (19, 23). A recent study suggested that IRES activity was retained in the absence of stem-loop IV and suggested that it is necessary to avoid the formation of a stable RNA structure downstream of the initiator AUG for optimal HCV IRES activity (24).

A previous study demonstrated that the stability of the stem-loop in domain IV of the HCV IRES was closely related to IRES activity (11). Mutations which led to increased stability of stem-loop IV resulted in a sharp reduction of IRES efficiency. It was suggested that a self-regulatory mechanism may influence HCV translation if a gene product can bind to stem-loop IV and increase its stability (11). However, no evidence has been found to support this hypothesis, although recent studies suggested that the HCV core, NS4, and NS5 proteins can regulate HCV IRES-directed translation (17, 28, 33).

Several small molecules and proteins which can regulate HCV IRES activity have been identified. Polypyrimidine tract binding protein (8), La protein (1), and heterogeneous nuclear ribonucleoprotein L (9) stimulate HCV IRES-directed trans-

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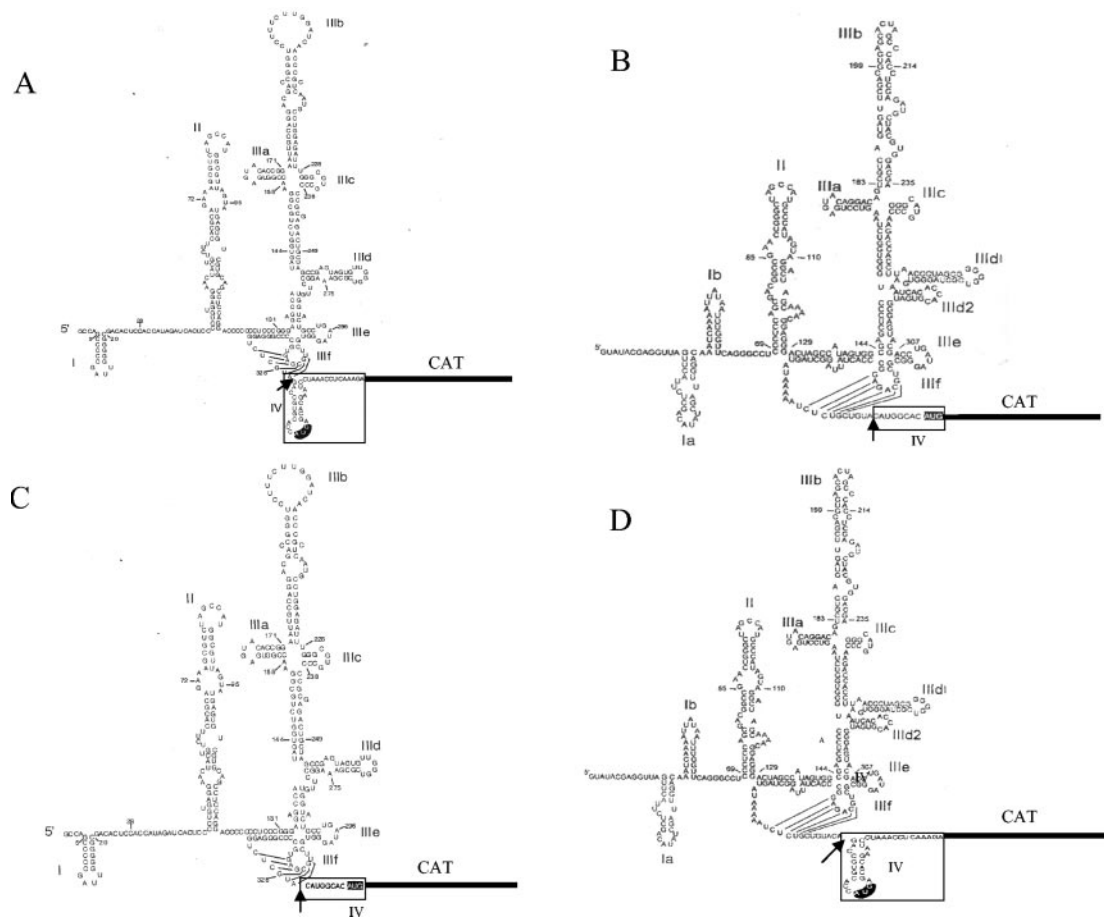


FIG. 1. The construction of two chimeric IRES elements with the reciprocal domain IV/IV(A) of the HCV and CSFV IRES elements. pHCV-IRES-CAT (A) and pCSFV-IRES-CAT (B) represent wild-type HCV and CSFV IRES elements ligated in frame upstream of a CAT gene (18). The shaded AUG in domain IV/IV(A) represents the authentic polyprotein initiation codon. The chimeric IRES element pHCV1-3/CSFV4 (C) contains the HCV IRES domains I to III and the CSFV IRES domain IV(A), and pCSFV1-3/HCV4 (D) contains the CSFV IRES domains I to III and the HCV IRES domain IV. The splice site used to switch the domain IV/IV(A) regions is shown by an arrow, and the respective domain IV regions are outlined.

lation. Two DNA ribonucleases, Dz2 and Dz4, were found to inhibit HCV IRES activity (21). Cyanocobalamin (vitamin B₁₂) was recently demonstrated to selectively inhibit HCV IRES-directed translation (18, 30). Inhibition by vitamin B₁₂ and HCV core and NS5A proteins were demonstrated to be specific for HCV IRES-directed translation and had no effect on translation from CSFV and encephalomyocarditis virus IRES elements (10, 17, 18). Since the stem-loop in domain IV only exists in the HCV and GBV-B IRES elements, this may indicate that stem-loop IV is responsible for any effect which is specific to these IRES elements.

The aim of this study was to examine the role of the stem-loop in the HCV IRES domain IV in the regulation of IRES function. Although the pestivirus IRES elements do not have a recognized domain IV, for convenience we have used the term domain IV(A) (assumed) when referring to the analogous region in the CSFV IRES element. We used two chimeric IRES elements, in which domain IV/IV(A) of the HCV and CSFV IRES elements was exchanged (Fig. 1). To avoid any possible influence on other IRES domains, the splice sites were chosen downstream of a common GUA motif, located downstream of

the pseudoknot structures in both IRES elements. As HCV IRES activity can be influenced by long-range RNA-RNA interactions (13, 15), all IRES elements were used as monocistronic reporter molecules. We examined the effect of two known regulators of the HCV IRES, vitamin B₁₂ and the HCV core protein, to determine the effect on translation from these two chimeric IRES elements.

Two monocistronic IRES plasmids, which reflect the physiological use of viral IRES elements, were used to construct the chimeric IRES elements. pHCV-IRES-CAT directs the expression of chloramphenicol acetyltransferase (CAT) from the HCV IRES (18) and includes the first 27 nt of the core coding sequence to retain the structure of domain IV (Fig. 1A). pCSFV-IRES-CAT contains 375 nt of the CSFV 5' UTR to control expression of the CAT gene (Fig. 1B). To construct the chimeric IRES elements pCSFV1-3/HCV4, which contains the CSFV IRES domain I-III and the HCV IRES domain IV, and pHCV1-3/CSFV4, which contains the HCV IRES domain I-III and the CSFV IRES domain IV(A), the reciprocal domains were substituted downstream of the pseudoknot loop (Fig. 1). This feature, which is highly conserved in the native IRES

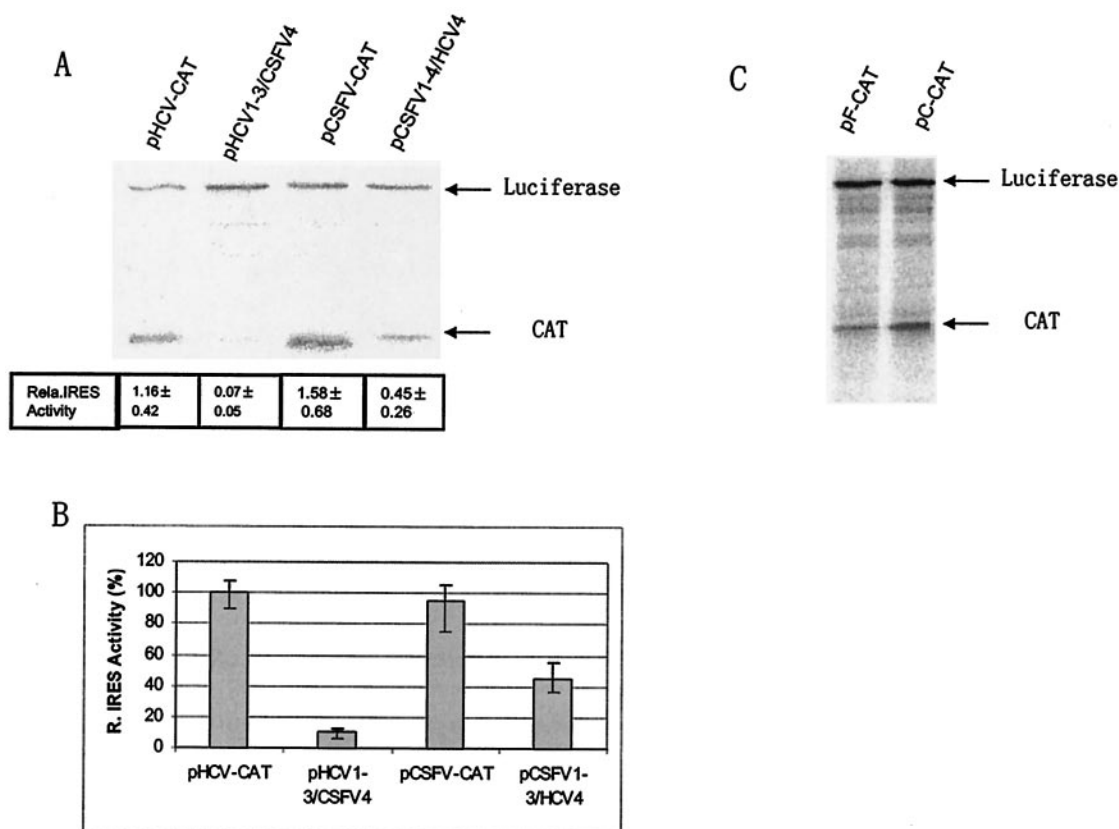


FIG. 2. Analysis of the translation efficiency of each IRES element either in monocistronic (A and B) or bicistronic forms (C). In vitro translation was performed in a RRL reaction. The products from the translation reactions were resolved by SDS-PAGE (A) and quantified by PhosphorImager analysis. The IRES efficiency was defined as the ratio of CAT product to luciferase (relative IRES efficiency = CAT/LUC). The translation efficiency of each IRES element was also determined in HepG2 cells. Capped-luciferase RNA was cotransfected with each IRES RNA to adjust the transfection efficiency. The relative level of CAT in the pHCV-IRES-CAT-transfected cells was normalized to 100% and the relative efficiency of each IRES was calculated (B). The efficiency of pF-CAT-directed translation was also assessed in a bicistronic form and compared against pcCAT (see reference 17).

elements, was retained in pHCV-IRES-CAT and pCSFV-IRES-CAT and the two chimeric IRES elements (Fig. 1C and 1D). The CAT gene was ligated in frame downstream of each IRES element. Compared with wild-type pHCV-IRES-CAT, pHCV1-3/CSFV4 has no stem-loop IV, whereas pCSFV1-3/HCV4 has a stem-loop which contains the authentic initiator AUG and is similar to the HCV IRES domain IV (Fig. 1C and 1D). No other mutations were made that might influence translation efficiency. No other change was noted in the structures of the two chimeric IRES elements as determined by M-fold analysis (data not shown) using the conditions and constraints outlined in the initial publication (3).

Initially, to determine the translational activity of the two chimeric IRES elements, an in vitro translation reaction was performed in a rabbit reticulocyte lysate (RRL) loaded with 80 ng of the respective RNAs. Each reaction contained 50 ng of capped-luciferase RNA as an internal control (18). The translation reactions were carried out for 90 min at 30°C in a reaction volume of 25 μ l. The [³⁵S]methionine-labeled products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantitated by PhosphorImager analysis. The activity of each IRES element was calculated by comparing the level of CAT produced relative to the

expression of luciferase. Compared with the wild-type HCV IRES, the relative activity of pHCV1-3/CSFV4 was reduced to ~5% (Fig. 2A), consistent with virtual loss of IRES function. The translational efficiency of pCSFV1-3/HCV4 was also decreased relative to CSFV IRES, but the IRES was clearly functional (Fig. 2A) and showed an efficiency of approximately 30 to 40% relative to the CSFV and HCV IRES elements, respectively. The experiment was repeated with various amounts of template RNA and the results were similar.

The translational activity of the chimeric IRES elements was then tested in cells. The respective IRES elements and capped-luciferase RNA were cotransfected into HepG2 cells. The cells were harvested 5 h posttransfection and the expression of CAT and luciferase were analyzed as described previously (17). The results of this experiment showed that the wild-type HCV and CSFV IRES elements were translationally efficient as expected (Fig. 2B). The activity of pHCV1-3/CSFV4 was reduced to ~10% of pHCV-IRES-CAT, while the translation efficiency of pCSFV1-3/HCV4 was approximately 40% of that of the wild-type pCSFV-IRES-CAT. To discount the possibility that decreased RNA stability accounted for the reduction in the efficiency of the chimeric IRES elements, the stability of all the RNA species was examined by incubating radiolabeled RNA in

rabbit reticulocyte lysates for up to 90 min followed by acid precipitation and analysis of the recovered counts, but no differences were found (data not shown).

As the chimeric pHCV1-3/CSFV4 IRES only showed low activity, the remainder of the study was restricted to the CSFV1-3/HCV4 chimeric IRES. Although uncapped RNA is not translationally competent in our hands, it was necessary to demonstrate that the chimeric pCSFV1-3/HCV4 IRES functioned as an IRES in a bicistronic vector. Consequently, the HCV IRES in a bicistronic vector, pcCAT (17), was replaced with the chimeric IRES, CSFV1-3/HCV4, in a novel bicistronic vector, pF-CAT, so that the expression of firefly luciferase (LUC) was controlled by a cap-dependent mechanism and the expression of CAT was controlled by the intergenic chimeric IRES, essentially as described previously (17). In vitro-transcribed RNA was added to a rabbit reticulocyte lysate as described above, and the products were analyzed by SDS-PAGE. The results show that the downstream CAT gene was expressed with an efficiency of approximately 40% relative to the HCV IRES and confirmed that the chimera represented a functional IRES (Fig. 2C).

The position of the AUG codon relative to other structural elements of the IRES is likely to be critical since the 40S ribosomal subunit binds directly to these flavivirus elements in such a way that the ribosomal P site is placed in the immediate vicinity of the initiation codon (12). To confirm that the HCV stem-loop in the CSFV1-3/HCV4 chimera is still folded correctly, we performed a toeprinting analysis on the RNA in the presence of ribosomes, as described previously (30). This assay, which results in a strong stop in the product of reverse transcription of the RNA, highlights the 3' position of the RNA that is protected by the ribosome and has been described previously (22, 30) as 15 nucleotides downstream of the AUG codon. The results of this experiment showed that the toeprint of the wild-type HCV IRES and the CSFV1-3/HCV4 chimeric IRES were identical (Fig. 3) and identified a strong stop at U356, 15 nucleotides downstream of the AUG initiation codon. Thus, we can conclude that the HCV IRES domain IV is folded correctly in this chimera and the AUG initiator is in the same context as it is in the HCV IRES.

A previous report demonstrated that vitamin B₁₂ selectively inhibited HCV IRES function, but not CSFV IRES-directed translation, in an in vitro translation system (18). We now wished to determine if an interaction between vitamin B₁₂ and the HCV IRES domain IV is responsible for this translational inhibition. RNA transcribed from pCSFV1-3/HCV4, which contains the HCV IRES domain IV, was incubated with vitamin B₁₂ for 10 min at room temperature and then added to a RRL to initiate the translation reaction. The concentration of B₁₂, 2.5 mM, was previously shown to be optimal, as described previously (18). Consistent with the previous report, the HCV IRES activity was reduced by 60% by prior incubation with vitamin B₁₂, whereas CSFV IRES-directed translation was not affected (Fig. 4A). The effect of vitamin B₁₂ on pCSFV1-3/HCV4 was similar to that of HCV IRES, i.e., translational activity of pCSFV1-3/HCV4 IRES was reduced by approximately 50 to 60%. This level of inhibition is similar to that reported in previous studies (18, 30). These results imply that the HCV IRES domain IV is responsible for translational inhibition by vitamin B₁₂.

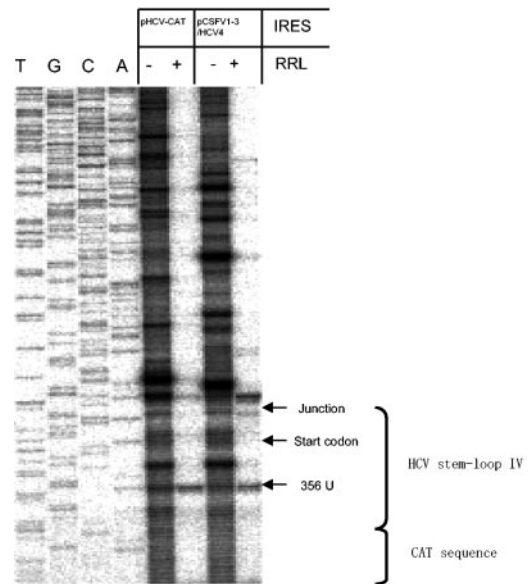


FIG. 3. Toeprint analysis of the HCV IRES and the CSFV1-3/HCV4 IRES after formation of the initiation complex prior to and after incubation in rabbit reticulocyte lysates. The arrows indicate the positions of the start codon for the polyprotein and the ribosomal toeprint at U356. The reference lanes marked T, G, C, and A are dideoxy sequencing reactions of HCV IRES cDNA. Note that the pattern in the HCV and CSFV1-3/HCV4 IRES lanes in the presence of the rabbit reticulocyte lysate is identical in the lower part of the figure, and the pattern only diverges upstream of the HCV domain IV.

To provide additional evidence that vitamin B₁₂ interacts with IRES domain IV, we then examined the effect of vitamin B₁₂ on GBV-B IRES-directed translation. The proposed structure of the HCV and GBV-B IRES elements is similar and each contains a stem-loop which encompasses the polyprotein initiation codon in domain IV (Fig. 1A and 4B). To facilitate this, pGBV-B-CAT, which contains the GBV-B IRES ligated in frame to the CAT gene sequence, was constructed to include the first 27 nt of the GBV-B core gene. Prior incubation with vitamin B₁₂ resulted in a ca. 60% reduction in the level of CAT translation from the GBV-B IRES (Fig. 4C). This result suggests that vitamin B₁₂ does not specifically inhibit HCV IRES activity but also selectively inhibits translation from the IRES of a closely related virus.

It has previously been shown (17, 28, 33) that the HCV core protein can inhibit translation from the HCV IRES. Furthermore, it was shown that the HCV core protein had no effect on CSFV and encephalomyocarditis virus IRES-directed translation in HepG2 cells (17). To determine if the HCV IRES domain IV is also the target for inhibition of translation by the HCV core protein, a recombinant vaccinia virus (RecVV-HCC) was used to express the HCV core protein in HepG2 cells. The cells were infected with RecVV-HCC or a RecVV-HBC to express the hepatitis B virus (HBV) core protein as control and then transfected with the RNA as described previously (17). Cotransfected capped-luciferase RNA was used to adjust transfection efficiency. The cells were harvested 5 h posttransfection, and the levels of CAT and luciferase were analyzed. The IRES activity in RecVV-HBC-infected cells was normalized to 100% (Fig. 5). The HCV IRES activity in the

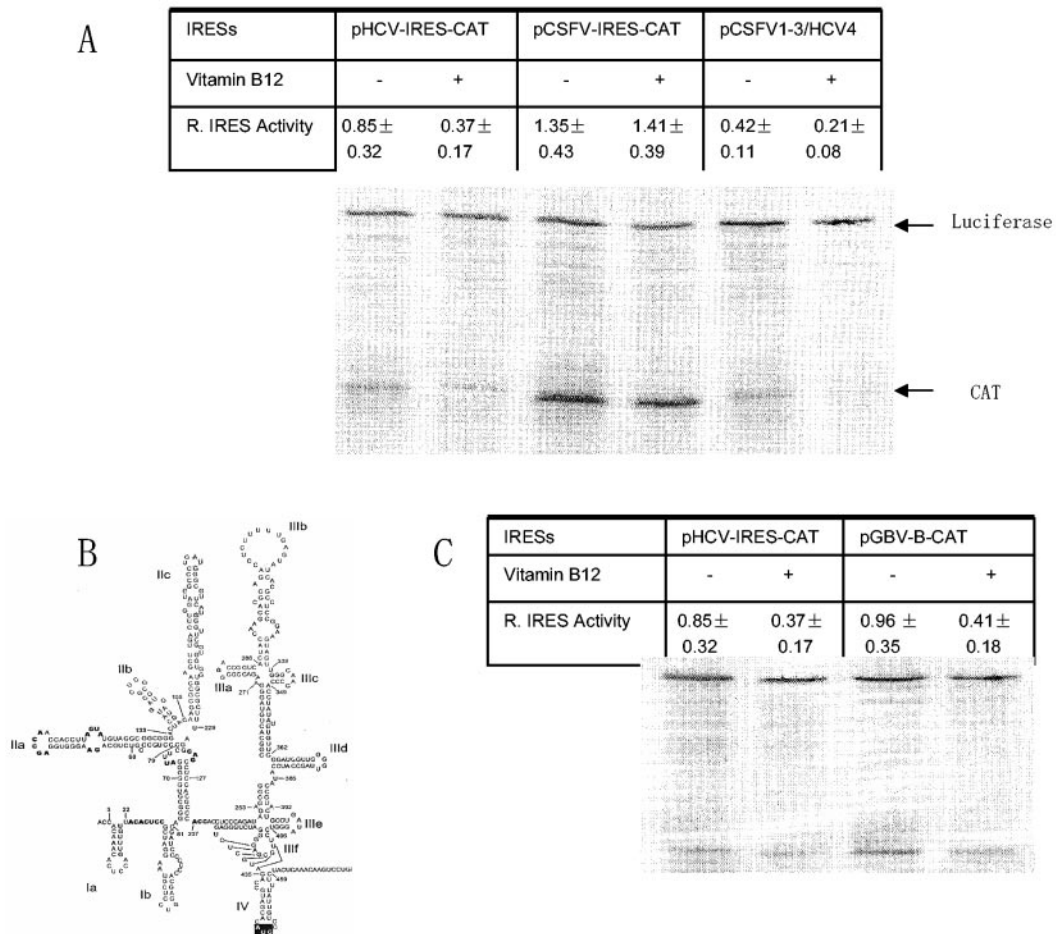


FIG. 4. Vitamin B₁₂ inhibits pCSFV1-3/HCV4- and GBV-B IRES-directed translation. The effect of vitamin B₁₂ on translation directed by these IRES elements was examined by incubating vitamin B₁₂ with the RNA for 10 min at room temperature prior to translation in a RRL. RNA with the same volume of water represented the control. pHCV-IRES-CAT and pCSFV-IRES-CAT translations in the presence and absence of vitamin B₁₂ represented positive and negative controls for inhibition by vitamin B₁₂ (A). The predicted structure of the GBV-B IRES is similar to that of the HCV IRES, including domain IV (B). (C) The effect of vitamin B₁₂ on GBV-B IRES-directed translation.

RecVV-HCC-infected cells was reduced by 51%. The absolute values for CAT and LUC differ from those in Fig. 2, because the transfection efficiency of RecVV-infected cells is higher than that of uninfected cells. However, expression of the HCV core protein had no effect on CSFV IRES-, pCSFV1-3/HCV4-, and GBV-B IRES-directed translations (Fig. 5). Thus, although the primary sequence and structure of the GBV-B IRES is very similar to that of the HCV IRES, this result shows that the HCV core protein regulates HCV IRES-directed translation in a highly specific manner, and furthermore, the target of the core protein is unlikely to be—or is not restricted to—domain IV.

The structure and function of domains II and III in the HCV and CSFV IRES elements are similar (14, 16). The heart of the IRES element is domain III, which is essential for IRES activity. Previous studies showed that domain III recruits the 40S ribosomal subunit and eIF3 (12, 14). However, the proposed structure in the vicinity of the start codon, known as domain IV, is quite different in the HCV and CSFV IRES elements. To examine the role of domain IV in the HCV IRES in translation regulation, the HCV domain IV and the CSFV IRES IV(A)

were exchanged and two chimeric IRES elements were created. No significant change in the predicted structure of domains I, II, and III of the two chimeric IRES elements compared to the structure of the wild-type IRES elements was found by M-fold analysis. The analysis showed that a stem-loop containing the polyprotein start codon in the loop was introduced into the chimeric pCSFV1-3/HCV4 and removed from pHCV1-3/CSFV4.

Compared with the wild-type IRES elements, the pHCV1-3/CSFV4(A) IRES was barely functional while the other, pCSFV1-3/HCV4, showed reduced functional activity in vitro and in cells. This finding is similar to that in a recent report in which the substitution of downstream coding sequences of the HCV and CSFV IRES elements resulted in reduction of IRES activity (7).

We have previously identified two inhibitors of HCV IRES function, viz., vitamin B₁₂ and the HCV core protein (17, 18). Vitamin B₁₂ selectively inhibits HCV IRES-directed translation in RRL but has no effect on CSFV IRES-directed translation. We also showed that vitamin B₁₂ traps 80S ribosomal complexes on the HCV IRES resulting in translational inhibi-

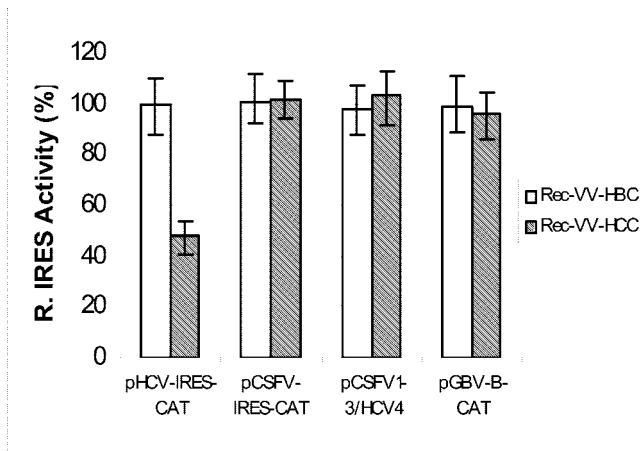


FIG. 5. The HCV core specifically inhibits HCV IRES-directed translation in HepG2 cells. HepG2 cells were infected with RecVV-HBC or RecVV-HCC for 15 h to allow the expression of the HBV and HCV core proteins, respectively. The cells were then cotransfected with the respective IRES elements and capped-luciferase RNA. The levels of IRES-directed translation in HBV core protein-positive cells were normalized to 100% after adjusting for the transfection efficiency as determined by luciferase activity, and the relative IRES efficiencies from HCV core-positive cells were calculated.

tion (30). However, the precise mechanism of this interaction is unclear, although vitamin B₁₂ does not bind directly to the IRES but appears to stabilize the 80S/IRES interaction (30). In this present study, vitamin B₁₂ was shown to inhibit translation directed by HCV, pCSFV1-3/HCV4, and GBV-B IRES elements with similar efficiencies. A common feature of these IRES elements is a stem-loop within domain IV, suggesting that this structure may be necessary for the interaction between vitamin B₁₂, the ribosome, and the IRES. The proposed structure of the CSFV IRES is similar to that of the HCV IRES, and the inclusion of the HCV IRES stem-loop IV in pCSFV1-3/HCV4 that results in vitamin B₁₂ sensitivity suggests that the general mechanisms of initiation are similar. The inhibition of GBV-B IRES-directed translation by vitamin B₁₂ represents yet another feature of this virus that shows similarities with HCV (4, 27, 29). We think it very likely that vitamin B₁₂ binds to an intermediate structure that is formed during the IRES/ribosomal complex interaction and prevents initiation by stabilizing this complex.

In contrast, the HCV core protein employs a different mechanism to suppress HCV IRES-directed translation. The results in this study showed that the HCV core protein specifically inhibited HCV IRES-directed translation in HepG2 cells but had no effect on translation directed by the IRES elements of GBV-B, CSFV, and CSFV1-3/HCV4. As the HCV1-3/CSFV4(A) chimeric IRES was not functional, we were unable to determine if removal of the stem-loop in domain IV from the HCV IRES resulted in elimination of the interaction with the core protein. Previous studies have shown that the HCV core protein binds specifically to the HCV IRES, suggesting that translation inhibition by the core protein results from direct binding (28, 33). This specific interaction signal is unlikely to involve the IRES domain IV.

As vitamin B₁₂ is stored in hepatocytes, vitamin B₁₂ and core

protein suppression of HCV IRES-directed translation may be one mechanism by which the virus has evolved to reduce the level of replication and promote persistence.

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