

HEPATITIS C VIRUS CORE PROTEIN ACTS AS A *TRANS*-MODULATING FACTOR ON
INTERNAL TRANSLATION INITIATION OF THE VIRAL RNA

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Running title: Modulation of HCV IRES efficiency by core protein

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Translation initiation of hepatitis C virus (HCV) RNA occurs through an internal ribosome entry site (IRES) located at its 5' end. As a positive-stranded virus, HCV uses the genomic RNA template for translation and replication, but the transition between these two processes remains poorly understood. HCV core protein (HCV-C) has been proposed as a good candidate to modulate such a regulation. However, current data are still the subject of controversy in attributing any potential role in HCV translation to HCV core protein. Here we demonstrate, using centrifugation on sucrose gradients, that HCV-C displays binding activities toward both HCV IRES and 40S ribosomal subunit. To gain further insight into these interactions, we investigated the effect of exogenous addition of purified HCV-C on HCV IRES activity by using an *in vitro* reporter assay. We found that HCV IRES-mediated translation was specifically modulated by HCV-C provided *in trans*, in a dose-dependent manner, with up to five-fold stimulation of the IRES efficiency upon addition of low amounts of HCV-C, followed by a decrease at high doses. Interestingly, mutations within some domains of the IRES, as well as the presence of an upstream reporter gene, both lead to changes in the expected effects, consistent with the high dependence of HCV IRES function on its overall structure. Collectively these results indicate that the HCV core protein is involved in a tight modulation of HCV translation initiation, depending on its concentration, and they suggest an important biological role of this protein in viral gene expression.

Hepatitis C virus (HCV) is the primary causative agent of non-A, non-B hepatitis,

frequently associated with high rates of progressive liver disease, leading to cirrhosis and hepatocellular carcinoma (1). Since its identification in 1989 (2), appreciation of the significant worldwide impact of chronic HCV infection has grown as approximately 170 million people, *i.e.* 3% of the world's population, are infected with HCV. HCV is an enveloped positive-stranded RNA virus whose genome of about 9600 nucleotides (nt) encodes a polyprotein that is processed by both host and viral proteases to yield individual structural and nonstructural HCV proteins (3).

HCV, along with pestiviruses and GB virus B (GBV-B), are members of the *Flaviviridae* family that utilize a cap-independent mechanism to initiate translation of their viral RNA which starts at the AUG codon (position 341 for HCV), following ribosome recognition of the upstream internal ribosome entry site (IRES) (4). HCV IRES is a *cis*-acting element, highly folded into a complex secondary and tertiary structure consisting of several stem-loop domains which has been shown to be crucial for its functionality (reviewed in (5)). Of the four domains delineated within the HCV 5' untranslated region (5' UTR) only the first seems not to be part of the IRES. The minimal sequence necessary for IRES activity extends approximately from nt 42 through to a short stretch (12-30 nt) downstream of the initiation codon (6). However, the 3' border of the IRES element remains controversial due to conflicting data on the requirement of some viral coding sequences between the AUG codon and the reporter gene for its efficiency. The following explanation has been proposed to reconcile the different reports: the HCV IRES-dependent translation negatively correlates with the stability of the putative stem-loop IV involving base pairing between the extreme 3' end of the HCV 5'

UTR and the 5' proximal open reading frame following the initiation codon, located in the loop of that hairpin structure (7). Several studies have demonstrated a significant enhanced expression of different reporters when part of the viral coding sequence is retained (6, 8-10), although no specificity in that coding sequence seems to be required, as demonstrated with chimeric constructs harbouring either CSFV or HCV coding sequences (11).

The mechanism of the internal initiation of HCV IRES, due to its extreme sensitivity to secondary structure in the vicinity of the initiation codon, resembles to the situation described in prokaryotic systems rather than that observed for picornavirus RNAs (12). First, there is formation of a binary complex between the basal part of domain III of the IRES (loop III_d) and the 40S ribosomal subunit, followed by binding of the eIF3 factor to the apical domain III_b (13, 14). This complex is further stabilized by the apical loop of domain II, independently of any other canonical translation initiation factors, leading to the terminal 80S ribosomal assembly by binding of the 60S subunit, and allowing translation initiation (12) of the viral RNA. However, it has been suggested that *trans*-acting non-canonical factors either of cellular or of viral origin interact with HCV RNA in the stem-loop region IV, tightly modulating translation (for a review see (5)). It appears, by analogy with picornaviruses, that interaction of the HCV IRES with cellular factors has been more studied than the effect of HCV-encoded proteins on the HCV translation. Owing to its particularity of promoting persistent infection, HCV has been suggested to possess a self-modulating mechanism to maintain a low level of replication. Therefore, it can be assumed that viral protein(s) might modulate RNA balance between the following three key steps of the viral life cycle: translation, replication, and packaging. Some of these proteins have been proposed to influence HCV IRES activity, such as core protein (15, 16) NS4A and NS4B (17), and NS4B and NS5A (18). However, there are conflicting results, mostly those concerning the HCV core protein, which still appears to be a good candidate that might interact with HCV IRES for the following reasons. First, it is supposed to be present into the infected cell with E1 and E2 glycoproteins after decapsidation as a component of the nucleocapsid, and also to be the first protein synthesized in that cell due to its most N-terminal position in the HCV polyprotein. Second, in addition to its role as structural protein in assembly and morphogenesis to form viral nucleocapsid, HCV

core protein is involved in a wide range of biological activities such as cell transformation (19), apoptosis (20), dysregulation of lipid metabolism (21), modulation of immunological functions (22), regulation of cell signalling (23) and of cellular transcriptional promoters (24), for reviews (25-27). Therefore, it is still the subject of debate as to whether HCV core protein acts as a translational regulator. Some controversial reports assert that the core protein-coding sequence modulates HCV IRES function, acting through a long-range RNA-RNA interaction that is either nonspecific (28) or specific, as described between nt 24-38 within the HCV 5' UTR and nt 428-442 of the core coding sequence (29). In contrast, other studies indicate that HCV core protein reduces the efficiency of HCV IRES translation by binding to the IRES either (i) through more than two of the four identified clusters of basic amino acid (aa) residues (15), or (ii) through two different aa sequences of core protein described as follows: aa 34-44 (16) and aa 1-20 as well as the corresponding synthetic peptide (30). In either case, a downregulation of the IRES activity was reported.

In this study, to gain insight into the potential role of the HCV core protein in influencing the efficiency of the HCV IRES-directed translation, we further examined the effect of the wild-type purified HCV core protein on the expression of reporter genes in an *in vitro* system. We found that the core protein provided *in trans* specifically modulates the HCV IRES activity in a dose-dependent manner. These findings suggest that HCV core protein may play a role in the regulation of viral gene expression, which is discussed as one possible molecular mechanism facilitating viral persistence.

MATERIALS AND METHODS

Plasmid constructs - Enzymes used for cloning and modifying DNA were from New England Biolabs and Roche Applied Science. Bicistronic vectors were derived from the pIRF vector, previously described (10). Briefly, this plasmid contains the CMV and T7 promoters for transcription, the Firefly luciferase (FLuc) gene, followed by the HCV 5'-UTR sequence and the *Renilla* luciferase gene (RLuc), in the pcDNA3.1Zeo (+) vector (Invitrogen). In this system, the upstream reporter FLuc (control) is translated in a cap-dependent fashion, whereas the downstream reporter RLuc (assay) is under the control of the HCV IRES. The constructs are described in Fig. 1. Vectors containing different

HCV IRES sequences were obtained by inserting in the intercistronic region of pIRF, in place of the original HCV-1a IRES, HCV IRES sequences amplified from human patient sera by RT-PCR performed on corresponding HCV RNAs and digested with *Bam*HI and *Pst*I enzymes. HCV IRES of genotype 1b was described in (31), and mutated IRESes of genotype 1a as depicted in Fig. 1, have been reported in previous studies: Q12 and Q22 in (10), and Q511 and Q519 in (32). Heterologous IRES sequences were amplified with appropriate primers containing *Bam*HI and *Pst*I sites (i) from pCITE vector (Novagen) for encephalomyocarditis virus (EMCV) IRES (586 nt) (33), (ii) from pA/C-4 (a gift from Dr. C. Wychowski) for classical swine fever virus (CSFV) IRES (Alfortville strain) (373 nt) (34), and (iii) from pSV CAT BiP Luc (kindly provided by Dr. P. Sarnow) for human immunoglobulin heavy chain-binding protein (BiP) IRES (220 nt) (35)

Monocistronic vectors consist of (i) vm FLuc, containing the FLuc gene alone, and cloned into pcDNA3.1, and (ii) vm IRLuc, the second plasmid concomitantly used with vm FLuc which includes the RLuc gene expressed under the different IRES sequences described above, inserted upstream of it.

The constructs for expression of HCV and GBV-B core proteins were obtained by inserting into pT7-7(6His) vector (36) the following respective *Nde*I-*Pst*I-digested fragments, previously amplified by PCR: (i) a 507-bp fragment corresponding to amino acids 1-169 for HCV-1b core protein (EMBL accession number D89872) encoded by the plasmid pCMV-C980, a gift from Dr. K. Shimotohno (37), and (ii) a 354-bp fragment corresponding to amino acids 1-118 for GBV-B (geneBank accession number AF179612) encoded by the plasmid pGBB, a gift from Dr. J. Bukh. (38). The correct orientations and sequences of all these constructs were confirmed by nucleotide sequencing.

Purification of core proteins and 40S ribosomal subunit - Core-(6 His) fusion proteins were produced in the inclusion bodies and purified as described in (39). Briefly, *E. coli* strain BL21 SI (Invitrogen) was transformed with the plasmids encoding respective core proteins of HCV and GBV-B. Transformants were grown at 37°C in Luria broth medium without sodium chloride (NaCl), and expression of recombinant fusion protein was induced by adding NaCl to a final concentration of 200 mM. Thereafter, two extractions were performed with 6 M urea in the

presence of 10 mM β -mercaptoethanol, and the proteins were purified on a Ni-TA-agarose column (Qiagen) by elution with 250 mM imidazole, and submitted to a step of reverse phase HPLC. After lyophilization, each of the proteins was dissolved in 20 mM Tris-HCl, pH 7.4, 1 mM DTT buffer, containing or not 0.1% N-dodecyl-maltoside (DM). The overall conformation and the oligomeric properties of the core proteins in solution were investigated using circular dichroism, analytical centrifugation, intrinsic fluorescence measurements and proteolysis with endoproteinase Glu-C. The results indicated that core proteins produced aggregate in the absence of detergent, but behave as soluble alpha-helical folded proteins in the presence of mild detergent. The folded form displayed an exchange between monomer and dimer state (S. Boulant *et al.*, submitted).

HIV-1 IIIB p24 core was from Advanced Biotechnologies, Inc. (Tebu, France), and consisted of the native viral protein obtained from HIV-I IIIB infected HUT76 cells, purified by immunoaffinity and solubilized in phosphate-buffered saline pH 7.2.

40S ribosomal subunit was prepared by zonal centrifugation as previously described, using free polysomes (40).

In vitro transcription - Plasmids were linearized at the *Not*I site located immediately downstream of the gene of interest. The linearized sequences were purified by Qiaquick PCR purification kit (Qiagen) and served as templates for *in vitro* transcription of uncapped RNAs from the T7 promoter using large-scale RNA production T7 RiboMAX system (Promega). After 90-min incubation at 37°C followed by treatment with RQ1 DNase (Promega) for 15 min at 37°C, the RNAs synthesized were extracted and purified using the RNeasy kit (Qiagen). Then, their content was determined by UV spectrophotometry, and their integrity confirmed by sodium dodecyl-sulfate (SDS) agarose gel electrophoresis.

³²P-labeled HCV IRES RNA was obtained from vm IRLuc plasmid linearized with *Pst*I, by *in vitro* transcription and treatment with T4 polynucleotide kinase (Promega) in presence of [γ ³²P] adenine triphosphate (3000 Ci/mmol, Amersham), under standard conditions.

Western blot analysis - Protein analysis by Western was performed with standard protocols after migration on a 12.5 % SDS-polyacrylamide gel. After transfert to a nitrocellulose membrane

(Amersham), HCV core protein was probed with primary monoclonal anti-core antibody (19D96D6; gift of Dr. C. Jolivet-Reynaud) (1: 10,000), and HRP-conjugate goat anti-mouse monoclonal antibody (1: 3,000) as secondary reagent, then revealed by ECL + detection procedure (Amersham).

Sucrose gradient centrifugation analysis of 40S ribosomal complexes - Purified 40S ribosomal subunit (5 pmoles), purified HCV core protein 1b (2.5 pmoles) and ³²P-radiolabeled HCV IRES RNA (2.5 pmoles) were incubated according to different combinations in a final volume of 100 µl of gradient buffer (20 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 20 mM β-mercaptoethanol, and 0.1% dodecylmaltoside) for 15 min at 30°C. The reactions were then layered over 5-20% (w/v) linear sucrose gradient and centrifuged in a Beckman SW 60 swing bucket rotor at 50,000 rpm for 1 h at 4°C. Fractions (400 µl) were collected from the bottom of the tube, and the absorbance profile at OD₂₈₀ was monitored. Alternatively, the total radioactivity in each gradient fraction was estimated in a Packard Tri-Carb counter, and the presence of core protein determined after 10% (v/v) trichloroacetic precipitation, followed by SDS-PAGE and Western blotting.

In vitro translation and luciferase assay - *In vitro* translations in micrococcal nuclease-treated rabbit reticulocyte lysates (Promega) were performed according to the supplier's instructions, after 1 h incubation at 30°C, in a final volume of 10 µl containing 200 ng RNA, with the addition, where appropriate, of 1 µl of core protein in increasing amounts varying from 10 to 1000 ng. When starting from plasmid DNA constructs, the TNT coupled reticulocyte lysate system (Promega) was primed with 200 ng of linearized DNA and increasing concentrations of core protein for transcription-translation with T7 RNA polymerase as indicated by the manufacturer in a 10 µl reaction mixture for 60 min at 30°C. DNA and RNA amounts were in the translation linear response range for corresponding batch of lysate.

Luciferase activities were assayed on 6 µl of the reaction mixture by using dual-luciferase reporter assay system (Promega). The FLuc and RLuc values were expressed relative to the control transcript without addition of HCV-C protein, which was assigned a value of 1 (100% efficiency). Errors were calculated as the standard deviation of three calculated IRES activities and expressed as percentages of the average activity.

RESULTS

HCV core protein interferes with the formation of the HCV IRES-40S ribosomal subunit primary complex - As an initial examination of the impact of HCV core (HCV-C) protein on IRES-directed translation, we investigated the potential interaction of that viral protein with HCV IRES-40S complex. Because the HCV IRES specifically binds to the 40S ribosomal subunit, even in the absence of any initiation factors, it is likely that additional factor(s) may modulate internal initiation of translation, interfering with IRES-40S assembly. We addressed this possibility for HCV core protein, taking into account that it has already been reported to interact with HCV IRES (as mentioned above) and with the ribosome (41). To this end, we analyzed the potential interaction between the three constituents: HCV IRES, 40S ribosomal subunit, and HCV-C protein, by ultracentrifugation on sucrose gradients of the complexes resulting from different combinations between them (Fig. 2). After characterization of the constituents taken individually, their putative association with each other was assessed, either by counting radioactivity for IRES or by immunoblotting for core protein. IRES RNA-core association was confirmed (Figs. 2B and 2C-b) in fractions 2-6, suggesting the formation of complexes slightly entering the gradient. Moreover, a complete co-sedimentation of 40S subunit with HCV-C protein was observed (Fig. 2C-b). The sedimentation pattern of IRES plus the 40S subunit indicated that the HCV IRES was recovered in two regions, in addition to the top of the gradient (Fig. 2B): fractions 7-9 corresponding to the association with 40S, and fractions 2-5 which might result from interaction of IRES with ribosome element(s), due to the background noted in the distribution of the optical density profile at 280 nm (Fig. 2A). Accordingly, it has recently been reported that HCV IRES exhibited a strong affinity for the ribosomal S5 protein that prevented the recruitment of the 40S subunit (42). Therefore, it appears in this experiment, that any event which might impair the observed unexplained binding of IRES in fractions 3-6, could improve IRES-40S interaction. This happened in the last step, with the addition of purified core protein as shown in Figs. 2B and 2C-d, with a shift in IRES distribution, corresponding to an enhanced association of IRES-40S in fractions 7-9. Taken together, these data indicate that HCV core protein displays binding activity

towards both HCV IRES and the 40S ribosomal subunit. Moreover, they suggest that HCV-C may help IRES RNA-40S assembly.

HCV core protein stimulates HCV IRES activity in a dose-dependent manner - The results reported above prompted us to elucidate which regulatory mechanism, if any, would modulate HCV IRES efficiency through HCV core protein. We first used an *in vitro* coupled transcription-translation TNT system. All HCV IRESes used contained the first thirty nucleotides of the viral coding sequence, as previously described (10), between the HCV initiation codon and the *Renilla* luciferase (RLuc) reporter. The core protein of genotype 1b was expressed in a prokaryotic system and purified as described in Materials and Methods. In this study, we used aa 1-169 of the protein, which is similar in size to the mature protein as suggested earlier by Santolini *et al.* (41). The TNT mix was primed with 200 ng of bicistronic DNA reporter vector vb IRF-1b containing the genotype 1b HCV IRES (31), and supplemented, when appropriate, with increasing amounts of purified recombinant HCV core protein diluted in buffer with or without dodecyl-maltoside (DM). This mild detergent was expected to avoid core aggregation as the protein has the propensity to multimerize *via* an interacting domain mapped within its amino-terminal hydrophobic region (aa 36-102) (43, 44). As an equilibrium between both dimeric and multimeric forms is suspected in the infected cell (43), we were interested in testing their respective effects on HCV IRES activity.

Luciferases activities were measured using the Stop and Glo dual luciferase assay system (Promega) and were plotted against HCV-C concentrations (Fig. 3A, left). The results showed modest enhancement of RLuc activity (shaded bars) at increasing concentrations (10, 100, and 1000 ng) of core protein. However, the activity observed for FLuc, expressed in a cap-dependent manner, was almost the same (striped bars). Therefore, the effect of core addition on HCV IRES efficiency could be evaluated by comparing the ratio of RLuc activity relative to that of FLuc, with the ratio obtained with the same vector in the absence of core protein, arbitrarily considered to be 1 (Fig. 3B, left). A gradual increase was observed upon the addition of HCV-C protein to the translation reactions, suggesting an impact of the viral protein on the HCV IRES-mediated translation. This effect is dose-dependent, since RLuc expression was the highest in the presence of 1000 ng of core protein, with a

relative IRES efficiency of 1.84 when diluted in buffer with DM (Fig. 3B, left; solid bars). When diluted in the absence of DM (open bars), *i.e.* under conditions favoring its aggregation, core protein was less potent, with a 1.45-fold increase at 1000 ng, suggesting that the multimeric state may impact on its effect.

In a second step, (i) considering the well-documented structure of HCV IRES as well as its diverse interactions with cellular factors (for a review see (5), and (ii) since it has been reported that the context of an IRES element may be important for its activity (45), it was tempting to determine whether HCV-C protein influences the IRES function according to its context within the plasmid reporter vector used. We therefore reproduced the same experiment as described above with monocistronic vectors vm FLuc and vm IRLuc. As shown in Fig. 3A, right; shaded bars), RLuc activities regularly increased and were higher than in a bicistronic context, whereas those obtained for FLuc were not affected in the presence of any amount of core protein (striped bars). Therefore, a gradual enhancement of HCV IRES relative efficiency was observed in proportion to core addition, with an RLuc/FLuc ratio above that obtained in a bicistronic context, up to three-fold in the presence of 1000 ng of HCV-C protein. These results suggest easier accessibility of the HCV IRES in the monocistronic context. Moreover, values obtained for core dilution in the absence of DM (open bars) were still below those obtained with dilution in DM (solid bars), with an RLuc/Fluc ratio of 1.72 *vs* 3 in the presence of 1000 ng of core protein. We did not succeed in gathering relevant data (either increase or decrease in HCV IRES activity) with a larger amount of core protein (such as 5000 ng), even in greater reaction volume.

HCV core protein acts on HCV IRES activity at the translational level - The TNT system was convenient for roughly assessing the potential impact of HCV-C protein on the corresponding IRES efficiency. However, our next goal was to specify this effect, taking into account the two following points. First, the HCV core protein has been implicated in interactions with DNA molecules, (1) due to its overall high basic charge through non specific binding to negative DNAs, and (2) through regulation of transcriptional host and viral promoters (24, 46). Moreover, a cellular promoter activity has been recently depicted within the HCV IRES (47). Consequently, it was considered important to

exclude these putative interfering mechanisms, in order to clarify whether the modulation of the IRES activity by the core protein, observed above, occurs at the translational level. Second, when the transcription-translation coupled TNT system is used, the estimation of IRES RNA template interacting with core protein, and so their stoichiometric ratio, is not available. In this regard, further experiments were performed starting with RNA instead of DNA, restricting the observed events to the translation, and allowing quantitation of RNA. Rabbit reticulocyte lysate reaction mixture was programmed with corresponding RNAs *in vitro* transcribed either from bi- or monocistronic reporter plasmids containing HCV IRES and supplemented with increasing amounts of core protein. Similarly to what was observed with TNT system, changes in luciferases activities were observed only for RLuc, although the FLuc activity was almost the same upon any addition of HCV core protein (data not shown). Therefore, the effect of core on HCV IRES efficiency could be estimated through the activity of RLuc relative to FLuc (Fig. 4). As RLuc expression is directly under the control of HCV IRES, and the only one to vary upon supplementation with core protein, this reflects the effective influence of HCV-C on translation efficiency. A dose-dependent effect was observed on HCV IRES activity, but was slightly different in the two systems. In the bicistronic system, (Fig. 4A, solid bars) a two-fold maximal enhancement of translation (1.73) was achieved with 100 ng of HCV-C, though in the monocistronic system (Fig. 4B, solid bars) a stimulation up to five-fold (4.9) was induced by the addition of 1000 ng of core protein. One possible explanation lies in the fact that in a bicistronic context added HCV-C protein may rapidly become saturating due to the blockage resulting from the presence of the upstream cistron. As first underlined with the TNT system, these observations are consistent with the idea that in the absence of any upstream reporter gene, HCV IRES is more accessible to factors that putatively affect its function. Unlike assays performed with the TNT system, amounts greater than 1000 ng of HCV-C protein could be tested, which means at least up to 5000 ng to remain available in monitoring luminometric activities. Interestingly, a decrease in HCV IRES efficiency was observed at such high concentrations, from 4.9 to 2.8 in the monocistronic system (Fig. 4B), and almost an inhibition from 1.73 to 0.96, in the bicistronic context (Fig. 4A), strongly arguing in favor of a

tight modulation of HCV IRES activity by HCV core protein, according to its concentration. Moreover, as previously noted, the activation observed in this last series of assays was more effective when aggregation of core protein was prevented; compare solid (+DM) and open bars (-DM) in Fig. 4. However, in the presence of 5000 ng of core protein, this difference is less important, probably due to the fact that at high concentration, even if diluted with detergent, HCV-C protein tends to oligomerize.

HCV IRES-directed translation is specifically modulated by HCV-C protein compared to heterologous viral core proteins - To determine whether the purified recombinant HCV-C protein was capable of a specific modulation of HCV IRES translation, we studied *in vitro* translation of reporter transcripts vb IRF-1b and vm IR-1b plus vm FLuc as described above in rabbit reticulocyte lysates, but replacing the HCV-C protein by the GBV-B core protein or the HIV-1 p24 core protein, as described in Materials and Methods, whose size and affinity for RNA are close to those of the HCV core protein. These proteins were added to the translation reaction mixture in increasing amounts ranging from 10 to 1000 ng. In contrast to the modulatory effect observed for HCV-C protein, none of the heterologous core proteins tested had any effect on HCV IRES activity, in either a bi- or monocistronic context, and even at the highest concentration (Fig. 5). These data suggest that the observed modulation of HCV IRES-directed translation is specific to HCV-C protein.

HCV core protein specifically modulates HCV IRES activity compared to that of heterologous IRESes - Alternatively, due to results obtained with heterologous core proteins, it was tempting to determine whether or not the modulating effect of HCV-C protein on IRES-directed translation was restricted to HCV IRES. For this purpose, the impact of HCV-C was assessed on different heterologous IRESes: two viral IRESes (i) EMCV IRES, belonging to the *Picornaviridae* family, and (ii) CSFV IRES, belonging to the same *Flaviviridae* family as HCV, and BiP, one cellular IRES. HCV IRES in each bi- and monocistronic plasmid reporter, was replaced by one of the other IRESes, and the resulting RNA transcripts were used to program *in vitro* reactions as described above, in the absence or presence of increasing amounts of HCV-C protein (Fig. 6). Unlike what was observed for HCV IRES, luciferases activities obtained with

bicistronic RNA containing EMCV IRES behaved differently upon addition of HCV-C protein. FLuc activity dramatically decreased approximately six-fold in the presence of increasing amounts of HCV-C (see Fig. 6A, left, striped bars). In contrast, RLuc activity did not change significantly, (shaded bars). Consequently, it can be assumed that the apparent activation of the EMCV IRES relative efficiency (RLuc/FLuc), up to 7.85-fold, as depicted in figure 6B, left, was irrelevant. Taking into account that an inhibitory effect of EMCV IRES on capped-RNAs translation has been described previously, presumably resulting from the ability of the J-K region within the IRES RNA to sequester cellular factors needed for cellular mRNA translation (48), this surprising result could be explained as follows: rather than faithfully reflecting an actual role of HCV-C in EMCV translation activity, the observed effect might result from facilitation of the sequestration of cellular factors in the vicinity of EMCV IRES inserted between luciferase genes, thus being detrimental to cap-dependent FLuc translation.

In contrast to EMCV, translation reactions programmed with transcripts containing CSFV or BiP IRES in the bicistronic context displayed luciferases activities with tendencies, upon addition of HCV-C protein, similar to those observed with the HCV IRES *i.e.* no variation in FLuc activity in comparison to RLuc activity, which varied in amplitude according to the IRES considered (data not shown). Thus, due to stabilization of FLuc activity, the variations in CSFV and BiP IRES efficiencies could be assessed through their relative RLuc activity (Fig. 6B, left). Accordingly, for CSFV IRES, the enhancement was approximately two-fold (1.93) on addition of 100 ng of HCV-C protein, and then tended to a plateau (1.80-fold) between 100 and 1000 ng of recombinant protein. The increase observed at 100 ng of core protein, close to that of 1.84 depicted in Fig. 3B, left, for HCV IRES activity in the same conditions, could be explained by a possible interaction between HCV-C protein and the loop IIIId domain of CSFV IRES in the presence of a small amount of HCV protein. The loop IIIId domain is well conserved among both HCV and pestiviruses (49), and its secondary structure is the most efficient to interact with HCV core protein (50). Such an interaction may become irrelevant at higher concentration, leading to a plateau. Unlike CSFV IRES, a slight 0.7-fold decrease was obtained for BiP IRES activity (Fig. 6B, left). Noteworthy, luminometric measures were unsuccessful in the presence of 5000 ng of

HCV core protein. Therefore, subsequent analyses performed with heterologous IRESes were limited to the addition of 1000 ng of HCV-C protein. Moreover, for each IRES considered, the relative similarity of the curves obtained with and without DM (Fig. 6C left, compare B and C), although efficiency was slightly lower in the absence of DM, suggests the lack of actual influence of HCV-C in either state, aggregated or not, on heterologous IRESes.

In the case of monocistronic systems, patterns for luciferase activities for each of the heterologous IRESes tested were comparable to those obtained in a bicistronic context, except for EMCV IRES (Fig. 6A, right). In that case, RLuc activity still stabilized around 40×10^6 light units (shaded bars), whereas that for FLuc dramatically decreased upon addition of core protein (striped bars), rendering the estimation of the EMCV IRES relative activity in those conditions inappropriate to reflect any actual modulation of its efficiency by HCV core protein. The apparent stimulation observed in the presence of HCV-C protein in this monocistronic context (Fig. 6B, right), which was less than that noted in a bicistronic context, could be explained by facilitation of the primary tendency of EMCV IRES free of any upstream reporter gene to sequester cellular factors, thus dramatically reducing the cap-dependent translation. However, increasing amounts of HCV-C protein might be responsible when accumulated around EMCV IRES, for partial impairment of its attraction of cellular factors to the benefit of capped FLuc RNA, which therefore becomes translatable, leading to a plateau. Unlike EMCV, owing to stabilization of FLuc activity whatever the amount of added HCV-C protein, the respective variations in efficiency of CSFV and BiP IRESes could be investigated through relative RLuc activity (Fig. 6B, right). For CSFV IRES, a four-fold stimulation (4.14) occurred upon the addition of 100 ng of HCV-C, and a ratio of 4.09 was obtained with 1000 ng. Such an increase could be explained by the same mechanism as in the bicistronic system, *i.e.* by binding of HCV-C protein to the IRES IIIId domain, though somehow facilitated in the monocistronic context. Furthermore, the tendency to reach a plateau at 1000 ng of HCV-C might result from a saturating nonspecific impact of core protein. Such a common behavior of EMCV and CSFV IRESes at high concentrations of HCV-C protein underlines its non specificity. Moreover, in a monocistronic context, no effect on BiP IRES activity was observed upon addition of HCV-C protein (Fig.

6B, right). This was expected because BiP IRES is unrelated to HCV IRES. In addition, less structuring of its sequence, owing to its shortness, might account for absolute lack of susceptibility to HCV core protein. IRES activities were substantially lower when HCV-C protein was aggregated (buffer - DM) than when diluted in DM (Fig. 6C, right). Profiles were almost identical for EMCV and CSFV IRESes, indicating an apparent increase in their efficiency as HCV-C protein concentration increased. These results are not completely ambiguous, and suggest that in aggregated form HCV-C core protein might exert a nonspecific stimulatory effect even at low concentrations (see discussion). Altogether, these data are in favor of a specific interaction of HCV-C protein with homologous IRES and reinforce the importance of the context of the IRES tested for its efficiency.

Modulation of HCV internal initiation of translation by the viral core protein is tightly dependent on the overall integrity of the HCV IRES structure - To further assess the extent of such specific interference of HCV-C protein in HCV IRES-directed translation, we examined its impact on HCV IRES variants. To this end, we used the HCV-1a IRES as contained in pIRF vector (vb IRF-1a) and various natural mutants previously isolated and described in Materials and Methods and in Fig. 1. HCV-1a IRES differs from HCV-1b by only a few changes which are clustered at four loci (UGA, nt 11-13 instead of GAU, GA at nt 34-35 instead of AG, C204A, and G243A) (51). Mutations in the variants studied were located as follows: Q519 (C18T) in domain I, Q511 (G76A) in domain II, Q22 (G266A) in domain III_d, and Q12 (G301C) between domains III_e and III_f.

Like HCV IRES 1b, in both bi- and monocistronic contexts, luciferase activities obtained for each reporter gene exhibited the same tendency for HCV IRES 1a and corresponding variants, *i.e.* gradual variations for IRES-dependent RLuc activities and almost stable values for FLuc translation (data not shown). Therefore, the respective efficiencies of these different IRESes could be drawn as shown in Fig. 7. In a bicistronic context, maximum relative activity of HCV IRES 1a upon addition of increasing amounts of HCV-C-1b protein was about that observed with IRES 1b *i.e.* 1.93 *vs* 1.84 with 100 ng of core protein in the monomeric state (Fig. 7A, left), and 1.69 *vs* 1.76 in the multimeric state (Fig. 7B, left). However, this activity drastically decreased to 0.83 with 1000 ng

of HCV-C protein in buffer with DM, and to 0.55 in the absence of DM. This suggests a less stringent effect of HCV-C 1b on HCV IRES 1a than in the closest homologous system (HCV-C 1b/HCV IRES 1b) for which a decrease in activity was observed only with amounts of HCV-C higher than 1000 ng.

We further assessed the activity of mutated IRES 1a in order to determine whether some variations in overall structure of the HCV IRES 1a might either restore the relative efficiency obtained with IRES 1b, or emphasize the difference observed between the two genotypes. That purpose was based on the following observations: (i) the HCV IRES whole conformation is fully required for its efficiency (52), (ii) the HCV IRES-40S association, the primary step for HCV translation initiation, requires an intact III_d loop to allow 40S subunit binding to IRES RNA (14), and (iii) mutations in the bulge loop III_d, mostly those affecting the G content, are deleterious in the HCV-C/IRES interaction (T. Shimoike, personal communication). Patterns of curves obtained with vb Q511 and vb Q519 mutants were almost identical to that of vb 1a with less activity (Fig. 7A, left), still slightly lower in buffer without DM (Fig. 7B, left). However, both vb Q22 and vb Q12 mutants gave different results, with a gradual inhibition of the IRES function on addition of HCV-C core protein, to 0.73 for vb Q22, and to 0.27 for Q12. When HCV-C protein was diluted in buffer without DM, data obtained with both of these mutants were not significantly different from those obtained with DM, suggesting that HCV-C protein not exerts a non specific effect on the activity of these IRESes.

In a monocistronic context (Fig. 7AB, right), HCV 1a IRES was as much stimulated as IRES 1b (3.69 *vs* 3.61) in the presence of 100 ng of monomeric HCV-C. However, this effect rapidly decreased to 2.45 after addition of 1000 ng of HCV-C protein, instead of continuing to increase as observed for IRES-1b (Fig. 4B). The vm Q519 mutant behaved exactly the same, which was not surprising due to location of the mutation (C18T) in the first structural domain I, which is not considered to belong to the HCV IRES sequence (6). Similarly, the vm Q511 mutant exhibited approximately the same efficiency in the presence of HCV-C protein as that of vm 1a and vm Q519 IRESes, except for a slightly smaller decrease with 1000 ng of HCV-C protein (3.43 *vs* 2.45 and 2.51), tending to a plateau. G76A mutation responsible is outside the region involved in RNA/RNA and RNA/protein(s)

interactions. In the presence of the multimeric HCV-C protein (Fig. 6B, right) these mutants reacted differently with higher stimulation of both mutants vm Q511 and vm Q519 than vm 1a with respectively 100 ng and 1000 ng of core protein, suggesting the importance of the form of HCV-C interacting with HCV IRES, leading to the possibility of an unexpected interaction with core when aggregated.

The variants vm Q22 and vm Q12 behaved differently in that context. A surprising initial phase of activation was observed with 10 and 100 ng of HCV-C, up to a factor approaching 2.5, followed by near inhibition, with a factor <1 (0.75 and 0.85, respectively) in the presence of 1000 ng of core protein. These results might be explained during the first phase, by interaction between HCV-C protein and IRES, helping IRES to display a conformation recognizable by the translation machinery (40S subunit). In the second phase, at higher concentration of core protein, the IRES conformation might be unsuited to subsequent positive interaction with 40S subunit due to a saturating mechanism, leading to partial inhibition of IRES activity. Such an interpretation is emphasized by examination of data obtained with the same mutants in a bicistronic context (Fig. 7A and B, left). Whatever the concentration of HCV-C, near inhibition of the corresponding IRES was observed, probably accounting for its inaccessibility by core protein. Data obtained in the presence of HCV-C protein diluted in buffer without DM (Fig. 7B, right) appeared to be identical to those with DM, suggesting that in the case of IRES harboring Q22 and Q12 mutations, thus important structural changes, HCV core protein is expected to act as a helper rather than a specific modulator of translation initiation.

DISCUSSION

Owing to conflicting data on the modulatory mechanism of HCV IRES translation initiation by HCV core protein, we attempted in this study to clarify the impact of increasing amounts of a purified HCV-C recombinant protein provided *in trans*, on the expression of a reporter gene in an *in vitro* system. From data obtained, four major conclusions can be drawn. First, HCV-C protein substantially modulates HCV IRES function in a dose-dependent manner. Second, the effects observed occur at the translational level. Third, they are specific to both interacting partners: HCV IRES and HCV-C protein. Fourth, the modulatory effects concern the fidelity of translation initiation, being tightly dependent on

the overall structural integrity around the AUG initiating codon. Moreover, even though non specific, a non-negligible impact of HCV-C protein could be observed on EMCV or CSFV IRES activity. This effect might be explained, being amplified with the aggregated form of HCV-C protein, by molecular attraction between basic domain(s) of HCV-C protein and positively charged RNA sequences which would confer on HCV core protein a kind of chaperone role towards some cellular factors gathered around the IRES. HCV core protein has recently been described to exhibit a chaperone activity towards nucleic acids (53), but such activity has not yet been tested on HCV IRES.

Our study strongly supports a relevant biological role for HCV core protein in IRES efficiency, showing it to have a tight modulatory effect, depending on its concentration, not restricted to inhibition as previously reported by others. Although the interpretation of such a discrepancy remains unclear, it might result from several experimental features such as (i) the choice of reporter system and, more importantly, as demonstrated here, the importance of its use in a mono- or bicistronic context, (ii) the method of purification and storage (*i.e.* the use of detergent in buffer) for recombinant HCV-C protein tested, as well as (iii) the ratio between core protein and IRES RNA interacting with each other. Concerning the first point about the relative proximity of IRES and core protein at the translation level in assessing HCV IRES efficiency, our results point to its importance through the comparative use of bi- and monocistronic systems. Increased relevance in a monocistronic context can be easily justified due to the 5' position of the IRES regulatory sequence within the HCV genome, without any other upstream coding sequence, which would be expected to disrupt the overall structure, thus, hampering interaction with cellular and/or viral factors. This clearly appears in Table 1 in which the relative efficiencies of IRESes studied without any addition of HCV core protein, taken as 1 (100% efficiency when calculation of relative RLuc activity), are depicted in both contexts. This is emphasized (i) for EMCV IRES (1.4 in bicistronic context vs 19.7 in monocistronic context) due to its ability to sequester cellular factors, as mentioned above, and (ii) for BiP IRES (0.4 vs 6), owing to its small size. Moreover, this is confirmed when considering the impact of HCV-C on the IRES efficiency placed in a monocistronic context, which is approximately twice (2.8 with homologous system HCV-C

1b/IRES 1b, and 1.9 with HCV-C 1b/IRES 1a system) that in the situation in which IRES is inserted between the two reporter genes.

In this report we limited our study to an *in vitro* assay for which, obviously, we cannot ascertain the native conformation of the HCV-C protein and RNAs used. Nevertheless, concerning the recombinant core protein produced, a lot of precautions were taken to ensure a high degree of purity (39) and satisfactory solubilization by adding 1% dimethyl-maltoside to buffer. The modulation of HCV IRES function we observed, which depended on the amount of HCV-C protein, argues in favor of an RNA-protein interaction, rather than an RNA-RNA interaction. Binding of HCV core protein to HCV 5' end RNA has already been described, specific (15, 50, 4) or not (41), in order to elucidate the encapsidation mechanism, without really attempting to assess its involvement in translation, excepted for Shimoike *et al.* (15). No mechanism would seem to be favored over any other. Nonetheless, it can be assumed that both events might be distinguished owing to their order of appearance within the life cycle. Accordingly, it has been reported (i) that the multimerization state of HCV core protein, which is common to both its membrane-bound and free forms, might occur at a later stage of the viral cycle (43), and (ii) that HCV-C protein appeared more active on IRES efficiency in the monomeric or dimeric state than when multimerized (the present study), reinforcing the possibility of sequential and multiple involvement of HCV core protein in the infected cell.

Concerning the conformation of the different HCV IRESes used, we are confident of their integrity owing to the following well-known structural/functional features of HCV IRES: (i) the adoption of an autonomous folding of the 5' UTR structure depending on the ionic environment (55), and (ii) the prokaryotic-like mechanism of HCV translation initiation which does not require most of the eukaryotic initiation factors (eIFs) (12). Accordingly, HCV IRES can be considered not to be hampered in its function in an acellular system, compared to *in vivo*, despite the low cellular factor content of rabbit reticulocyte lysates. Moreover, the use of HCV IRES 1a mutants revealed a satisfactory sensitivity of the assay used to investigate their respective efficiencies as described above. The conclusions obtained are in agreement with (i) recent work reporting the strict requirement of intact loop III_d to allow primary interaction between HCV IRES and 40S subunit (14), and (ii) the interference of HCV core protein in the binary

IRES-40S complex assembly as suggested in Fig. 2. In addition, as already well documented, our results confirm a better efficiency of the HCV IRES 1b than that of genotype 1a, implying a further selection between variants in the infected cell when coinfection with several genotypes has occurred.

Furthermore, an advantage which deserves to be underlined in our *in vitro* assay is the possibility of determining the exact ratio between the RNA of interest (here different IRESes) and core protein potentially interacting with each other. This ensures high data reproducibility, which could not be achieved *in vivo* where interaction of the partners, even after their calibrated input into the cell, depends on parameters such as transfection efficiency and functional features specific to the recipient cell. However, owing to the lack of an efficient cellular system for producing HCV, we do not have any idea of the relative proportion of HCV RNA IRES and HCV core protein interacting with each other. Recently, an average value of 25 pg (ranging from 7 to 56 pg per cell) has been estimated in single hepatocytes by means of laser capture microdissection (56). Moreover, an average value of 33 viral genomes (ranging from 7 to 64 RNA molecules) is currently accepted per productively infected hepatocyte (57), thus enabling determination of the ratio of HCV-C protein to RNA potentially able to interact in one infected cell. Consequently, the relatively high proportion of 24×10^6 molecules of core protein for one of viral RNA genome (IRES extremity) was found (24×10^6 C/1 IRES) (Table 2), arguing for a biological impact of HCV core protein in the infected cell. When calculated in our assays, proportions varied from one molecule of HCV core protein to 500 for one of RNA when supplementation was carried out with 10 ng (1 C/1 IRES) to 5000 ng (500 C/1 IRES) of HCV-C protein respectively (Table 2). The ratio was thus significantly 4×10^4 -fold lower, even with the highest concentration of core protein tested (5000 ng), than that expected in the cell. In other words, a rather small amount of HCV-C protein (100 molecules) is sufficient in our system to exert a 5-fold stimulation of one molecule of HCV IRES, although a little more (5 times) HCV-C protein, which is still little compared with its cellular availability for one molecule of HCV RNA, is already enough to diminish this effect nearly two-fold. This is indicative of a certain quality of the HCV core protein used in this work, and of the relevance of its sharp modulatory effect on HCV IRES efficiency, excluding any artefactual IRES-

core protein interaction due to saturation of RNA with core protein. Obviously, HCV core protein detected in the cell interacts not only with HCV IRES but is also involved in other cooperative functions owing to its broad cell distribution (25-27).

Based on the poliovirus model for which nonstructural proteins (NS) 2A and 3CD, with the aid of cellular factors, regulate coordination between translation and replication (58), the two mechanisms being unable to take place simultaneously (59), HCV core protein has been proposed to play a role in viral persistence by downregulating the translation process to the benefit of replication (15, 16, 30). Accordingly, a model has been proposed by Zhang *et al* (16), suggesting a specific arrest of the HCV genomic RNA translation by core binding to the IRES, when all viral proteins are sufficiently synthesized for replication, then allowing initiation of the negative-strand synthesis. However, such a scheme proposes that core protein at cellular high levels impairs HCV IRES, without addressing the possibility that lower levels may have a different effect. In view of our results, an alternative, albeit complementary pathway of viral life cycle events is possible, considering the importance of the core concentration for its modulatory effect. Why might HCV-C protein not play a stimulatory role at an early stage of the infection, keeping in mind that in the infected cell (i) it remains close to viral RNA after decapsidation, and (ii) it is the first protein expressed from polyprotein? Thus, it would seem extraordinary that this protein might exert an inhibitory effect on the subsequent expression of other viral proteins rather than promote their accumulation to initiate further viral life cycles, ensuring viral propagation inside the infected cell. According to this new scheme, the events leading to transition from translation to replication might be as follows: a first step would involve HCV-C protein for active translation, in order to produce each of the individual viral proteins to a level consistent with the formation of replication and assembly complexes at endoplasmic reticulum (ER) membranes. Later on, when the process is sufficiently under way, and the concentration of core protein tends to saturation, its own modulatory effect on HCV translation initiation might be less important. Therefore, it is not excluded that other viral proteins such as nonstructural protein(s) might cooperate in regulation of the IRES function, thereby, facilitating initiation of replication and taking over from the core protein, which could then become available to exert some of its

multiple functions such as multimerization at the ER membrane for virus assembly, attachment to lipid droplets (60), and putative involvement in diverse cellular processes (25-27). A potential cooperative effect of another viral protein has already been investigated in two studies, although their data are at odds, one claiming an inhibitory effect of both NS4A and NS4B on HCV IRES (17), and the other a stimulatory effect of NS4B and NS5A (18). Such a discrepancy concerning NS proteins, in addition to the controversy mentioned for HCV-C protein, in their modulation of HCV IRES function, highlights the difficulty of ascertaining any biological relevance of a result aimed at elucidating translation initiation mechanisms, due to the absence of any *in vitro* infection system. To date, replicons, even though the most efficient systems, are not available to study the regulatory mechanisms of HCV IRES translation nor to distinguish them from replication. Rather, reporter systems have been designed as convenient tools for *in vitro* and *in vivo* evaluation of the HCV IRES activity on the expression of the gene located downstream of it.

In vivo systems are useful both to determine the relevance of a possible biological effect detected *in vitro*, and to approach mechanisms occurring in the cell. Nonetheless, in the absence of any available virus infection system, *in vivo* affordable systems can mimic, although not replicate, a putative biological mechanism *via* heterologous expression vectors. Of note, these surrogate tools do not allow the control of the actual concentration of the expressed protein due to (i) the well-known overexpression resulting from these systems, (ii) the possible variation in expression over time during the cellular cycle, and (iii) the potential multiplicity of subcellular localizations of the expressed protein. This is precisely the case for HCV core protein as mentioned before (25), and it may also account for discrepancies between published data, owing to the different experimental procedures used. In cell-based systems, unlike acellular assays, there is an important membranous network which affects core protein distribution, depending on cell type and maybe on its actual effect on HCV IRES. Moreover, data reflect the overall steady-state inside the cell, without distinguishing different phases of the cell cycle during which HCV IRES efficiency has been reported to vary (61). In an *in vitro* assay, the system is more constrained, and the observed phenomenon is thus expected to faithfully represent the spontaneous interaction

between HCV IRES and core protein.

Nonetheless, we are aware of the necessity of a subsequent *in vivo* study to confirm the data presented here. This work is in progress, using a mammalian expression system allowing modulatory production of core protein.

In summary, our results collectively support a specific modulatory effect of HCV core protein on the translational activity of viral IRES, although the biological relevance of these data

requires further careful examination. Due to conflicting data obtained in this field, it is likely that the HCV IRES-core protein interaction has evolved to regulate the HCV life cycle, gifting the virus with a strategy ensuring its amplification *via* modulation of the host cell translation apparatus. Further studies are needed to dissect these molecular aspects, and such an RNA-protein interaction might be considered as an excellent target for future therapy (62).

REFERENCES

1. Hoofnagle, J. H. (1997) *Hepatology* **26**, 15S-20S.
2. Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) *Science* **244**, 359-362
3. Reed, K. E., and Rice, C. M. (2000) *Curr. Top. Microbiol. Immunol.* **242**, 55-84.
4. Tsukiyamakohara, K., Iizuka, N., Kohara, M., and Nomoto, A. (1992) *J. Virol.* **66**, 1476-1483
5. Rijnbrand, R. C., and Lemon, S. M. (2000) *Curr. Top. Microbiol. Immunol.* **242**, 85-116
6. Reynolds, J. E., Kaminski, A., Kettinen, H. J., Grace, K., Clarke, B. E., Carroll, A. R., Rowlands, D. J., and Jackson, R. J. (1995) *EMBO J.* **14**, 6010-6020.
7. Honda, M., Brown, E. A., and Lemon, S. M. (1996) *RNA* **2**, 955-968.
8. Zhao, W. D., Lahser, F. C., and Wimmer, E. (2000) *J. Virol.* **74**, 6223-6226.
9. Malet, I., Wychowski, C., Huraux, J. M., Agut, H., and Cahour, A. (1998) *Biochem. Biophys. Res. Commun.* **253**, 257-264.
10. Laporte, J., Malet, I., Andrieu, T., Thibault, V., Toulme, J. J., Wychowski, C., Pawlowsky, J. M., Huraux, J. M., Agut, H., and Cahour, A. (2000) *J. Virol.* **74**, 10827-10833.
11. Fletcher, S. R., Ali, I. K., Kaminski, A., Digard, P., and Jackson, R. J. (2002) *RNA* **8**, 1558-1571.
12. Pestova, T. V., Shatsky, I. N., Fletcher, S. P., Jackson, R. J., and Hellen, C. U. (1998) *Genes & Development* **12**, 67-83
13. Kieft, J. S., Zhou, K., Jubin, R., and Doudna, J. A. (2001) *RNA* **7**, 194-206
14. Ji, H., Fraser, C. S., Yu, Y., Leary, J., and Doudna, J. A. (2004) *Proc. Natl. Acad. Sci. U S A* **101**, 16990-16995.
15. Shimoike, T., Mimori, S., Tani, H., Matsuura, Y., and Miyamura, T. (1999) *J. Virol.* **73**, 9718-9725.
16. Zhang, J., Yamada, O., Yoshida, H., Iwai, T., and Araki, H. (2002) *Virology* **293**, 141-150.
17. Kato, J., Kato, N., Yoshida, H., Ono-Nita, S. K., Shiratori, Y., and Omata, M. (2002) *J. Med. Virol.* **66**, 187-199
18. He, Y., Yan, W., Coito, C., Li, Y., Gale, M., Jr., and Katze, M. G. (2003) *J. Gen. Virol.* **84**, 535-543.
19. Ray, R. B., Lagging, L. M., Meyer, K., and Ray, R. (1996) *J. Virol.* **70**, 4438-4443.
20. Marusawa, H., Hijikata, M., Chiba, T., and Shimotohno, K. (1999) *J. Virol.* **73**, 4713-4720.
21. Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T., and Brechot, C. (1997) *Proc. Natl. Acad. Sci. U S A* **94**, 1200-1205.
22. Large, M. K., Kittlesen, D. J., and Hahn, Y. S. (1999) *J. Immunol.* **162**, 931-938.
23. Tellinghuisen, T. L., and Rice, C. M. (2002) *Curr. Opin. Microbiol.* **5**, 419-427.
24. Ray, R. B., Lagging, L. M., Meyer, K., Steele, R., and Ray, R. (1995) *Virus Res.* **37**, 209-220.
25. Lai, M. M., and Ware, C. F. (2000) *Curr. Top. Microbiol. Immunol.* **242**, 117-134.
26. McLauchlan, J. (2000) *J. Viral Hepat.* **7**, 2-14.
27. Ray, R. B., and Ray, R. (2001) *FEMS Microbiol. Lett.* **202**, 149-156.
28. Wang, T. H., Rijnbrand, R. C., and Lemon, S. M. (2000) *J. Virol.* **74**, 11347-11358
29. Kim, Y. K., Lee, S. H., Kim, C. S., Seol, S. K., and Jang, S. K. (2003) *RNA* **9**, 599-606.
30. Li, D., Takyar, S. T., Lott, W. B., and Gowans, E. J. (2003) *J. Gen. Virol.* **84**, 815-825.

31. Laporte, J., Bain, C., Maurel, P., Inchauspe, G., Agut, H., and Cahour, A. (2003) *Blood* **101**, 52-57.
32. Malet, I., Belnard, M., Agut, H., and Cahour, A. (2003) *J. Virol. Methods* **109**, 161-170.
33. Jang, S. K., Krausslich, H. G., Nicklin, M. J., Duke, G. M., Palmenberg, A. C., and Wimmer, E. (1988) *J. Virol.* **62**, 2636-2643.
34. Ruggli, N., Tratschin, J. D., Mittelholzer, C., and Hofmann, M. A. (1996) *J. Virol.* **70**, 3478-3487.
35. Macejak, D. G., and Sarnow, P. (1991) *Nature* **353**, 90-94.
36. Cortay, J. C., Negre, D., Scarabel, M., Ramseier, T. M., Vartak, N. B., Reizer, J., Saier, M. H., Jr., and Cozzone, A. J. (1994) *J. Biol. Chem.* **269**, 14885-14891.
37. Tanaka, T., Kato, N., Nakagawa, M., Ootsuyama, Y., Cho, M. J., Nakazawa, T., Hijikata, M., Ishimura, Y., and Shimotohno, K. (1992) *Virus Res.* **23**, 39-53.
38. Bukh, J., Apgar, C. L., and Yanagi, M. (1999) *Virology* **262**, 470-478.
39. Boulant, S., Becchi, M., Penin, F., and Lavergne, J. P. (2003) *J. Biol. Chem.* **278**, 45785-45792. Epub 42003 Sep 45782.
40. Reboud, A. M., Dubost, S., Buisson, M., and Reboud, J. P. (1980) *J. Biol. Chem.* **255**, 6954-6961.
41. Santolini, E., Migliaccio, G., and La Monica, N. (1994) *J. Virol.* **68**, 3631-3641.
42. Ray, P. S., and Das, S. (2004) *Nucleic Acids Res.* **32**, 1678-1687.
43. Matsumoto, M., Hwang, S. B., Jeng, K. S., Zhu, N., and Lai, M. M. (1996) *Virology* **218**, 43-51.
44. Nolandt, O., Kern, V., Muller, H., Pfaff, E., Theilmann, L., Welker, R., and Krausslich, H. G. (1997) *J. Gen. Virol.* **78**, 1331-1340.
45. Hunt, S. L., Kaminski, A., and Jackson, R. J. (1993) *Virology* **197**, 801-807.
46. Bergqvist, A., and Rice, C. M. (2001) *J. Virol.* **75**, 772-781.
47. Dumas, E., Staedel, C., Colombat, M., Reigadas, S., Chabas, S., Astier-Gin, T., Cahour, A., Litvak, S., and Ventura, M. (2003) *Nucleic Acids Res.* **31**, 1275-1281.
48. Scheper, G. C., Thomas, A. A., and Voorma, H. O. (1991) *Biochim. Biophys. Acta* **1089**, 220-226.
49. Brown, E. A., Zhang, H. C., Ping, L. H., and Lemon, S. M. (1992) *Nucleic Acids Res.* **20**, 5041-5045
50. Tanaka, Y., Shimoike, T., Ishii, K., Suzuki, R., Suzuki, T., Ushijima, H., Matsuura, Y., and Miyamura, T. (2000) *Virology* **270**, 229-236
51. Honda, M., Rijnbrand, R., Abell, G., Kim, D., and Lemon, S. M. (1999) *J. Virol.* **73**, 4941-4951
52. Honda, M., Beard, M. R., Ping, L. H., and Lemon, S. M. (1999) *J. Virol.* **73**, 1165-1174
53. Cristofari, G., Ivanyi-Nagy, R., Gabus, C., Boulant, S., Lavergne, J. P., Penin, F., and Darlix, J. L. (2004) *Nucleic Acids Res.* **32**, 2623-2631.
54. Fan, Z., Yang, Q. R., Twu, J. S., and Sherker, A. H. (1999) *J. Med. Virol.* **59**, 131-134.
55. Kieft, J. S., Zhou, K., Jubin, R., Murray, M. G., Lau, J. Y., and Doudna, J. A. (1999) *J. Mol. Biol.* **292**, 513-529.
56. Sansonno, D., Lauletta, G., and Dammacco, F. (2004) *J. Viral Hepat.* **11**, 27-32.
57. Chang, M., Williams, O., Mittler, J., Quintanilla, A., Carithers, R. L., Jr., Perkins, J., Corey, L., and Gretch, D. R. (2003) *Am. J. Pathol.* **163**, 433-444.
58. Hambidge, S. J., and Sarnow, P. (1992) *Proc. Natl. Acad. Sci. U S A* **89**, 10272-10276.
59. Gamarnik, A. V., and Andino, R. (1998) *Genes Dev.* **12**, 2293-2304.
60. McLauchlan, J., Lemberg, M. K., Hope, G., and Martoglio, B. (2002) *EMBO J.* **21**, 3980-3988.
61. Honda, M., Kaneko, S., Matsushita, E., Kobayashi, K., Abell, G. A., and Lemon, S. M. (2000) *Gastroenterology* **118**, 152-162.
62. Jubin, R. (2003) *Curr. Opin. Investig. Drugs* **4**, 162-167.

FOOTNOTES

The authors are grateful to Dr. Peter Sarnow for supplying the plasmid SV CAT BiP Fluc containing the BiP IRES sequence, and to Dr. Cezslaw Wychowski for supplying the plasmid pA/C-4 containing the CSFV IRES. They are indebted to Dr. Kunitada Shimotohno for providing us with the plasmid pCMV-980, Dr. John Bukh for the gift of plasmid pGBB, and Dr. Colette Jolivet-Reynaud for the monoclonal antibody 19D96D6. The authors wish to thank Sofia Lourenço and Frédéric Bertrand for their help. This work was supported in part by the Association pour la Recherche contre le Cancer (grant No. 4301 for J. P. L.), the Agence Nationale pour la Recherche sur le Sida (ANRS), the Centre National de la Recherche Scientifique (CNRS), and the Institut National de la Santé et de la Recherche Médicale (INSERM) (ATC Hépatite C). S. B. and S. B. are recipients of doctoral grants from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (MENRT).

FIGURE LEGENDS

Fig. 1. Schematic diagrams of different constructs used. The upper panel shows the bicistronic (*left*) and monocistronic (*right*) reporter vectors containing CMV and T7 promoters. In the bicistronic system, the upstream FLuc gene and the downstream RLuc gene are separated by different IRES sequences. The different IRES sequences used (*) are described in Materials and Methods, and indicated in the lower frame. *Left*, heterologous IRESes are as follows: two viral, EMCV and CSFV IRESes, and the cellular BiP IRES. *Right*, homologous HCV IRESes are based on the representation of Honda *et al.* (52) from genotype 1b. HCV IRES sequence comprises the complete 5' UTR and 30-nucleotide core-coding sequence (nt 1-371). IRES from genotype 1a differs from that of genotype 1b by only a few nt as specified in the text. IRES-1a mutants Q511, Q519, Q12 and Q22 described elsewhere are depicted. I, II, III, IV, four structural domains shaded; the initiator AUG codon in stem-loop IV is circled.

Fig. 2. Interference of HCV core protein with HCV IRES-40S ribosomal subunit primary complex. Reaction mixtures containing 40S ribosomal subunit, purified HCV core protein 1b and ³²P-labeled HCV IRES RNA according to different combinations were overlaid onto a 5-20% sucrose gradient, and centrifuged for 1h at 4°C. Gradient fractions were collected from the bottom as indicated, and components in each fraction were analyzed as described in Materials and Methods. Distributions (A) of 40S ribosomal subunit (optical density at 280 nm), (B) of labeled HCV IRES (radioactivity counts per minute: cpm) and (C) of HCV core protein (Western blotting) are shown.

Fig. 3. The stimulatory effect of exogenous addition of purified HCV core protein 1b on HCV IRES 1b translation efficiency. *In vitro* coupled transcription-translation (TNT) reactions were programmed with 200 ng of bicistronic reporter vector vb IRF of genotype 1b (*left panels*) and with 200 ng of each of monocistronic vectors vm FLuc plus vm IRLuc (*right panels*). Varying amounts of the core protein (10 to 1000 ng) were added to respective reporter translation reactions as depicted on the X-axis. (A) Luciferase activities are indicated in the presence of increasing amounts of HCV-C protein diluted in buffer containing DM. FLuc activity (cap-dependent) (*striped bars*); RLuc activity (HCV-IRES-dependent) (*shaded bars*). (B) Changes in HCV-IRES-directed RLuc activity relative to FLuc activities upon increasing concentrations of HCV-C protein diluted in buffer with DM (*solid bars*) and without DM (*open bars*). Relative HCV IRES efficiency was estimated from proportional luciferase activity (RLuc/FLuc) of *in vitro* translation products with that of the corresponding vector in the absence of core protein normalized to 1 (100% activity); *Left panel*, bicistronic context; *right panel*, monocistronic context. Each column represents the average IRES activity from triplicate translation samples, and error bars indicate the standard deviations.

Fig. 4. HCV protein acts on HCV IRES efficiency at the translational level. *In vitro* translation reactions of rabbit reticulocyte lysates (RRLs) were programmed with 200 ng of transcripts obtained *in vitro* from bicistronic (A) or monocistronic (B) plasmid reporters, and supplemented with increasing amounts of HCV-C protein diluted in buffer with (*solid bars*) or without (*open bars*) dodecyl-maltoside (DM) as indicated. Relative IRES efficiencies were determined as described in Fig. 3. Each column represents the average IRES activity from triplicate translation samples, and error bars indicate the standard deviations.

Fig. 5. Effect of heterologous core proteins on HCV IRES-dependent translation efficiency. RRL system reactions were programmed with bicistronic (A) and monocistronic (B) reporters respectively, and supplemented as depicted on the X-axis with various core proteins from HCV (solid bars), HIV (shaded bars) or GBV-B (open bars), diluted in DM at the indicated concentrations. Relative IRES efficiencies were determined as described in Fig. 3. The columns represent the means and the bars the standard deviations of the experiment performed in triplicate.

Fig. 6. Effect of HCV-C protein on heterologous IRESes. *In vitro* translation reactions of RRL were programmed with 200 ng of *in vitro* transcripts obtained from bicistronic (left panels) and monocistronic (right panels) reporter vectors containing the following heterologous EMCV (squares), CSFV (circles) and BiP (triangles) IRESes, and supplemented with increasing amounts of HCV-C protein as indicated. (A) Luciferase activities obtained for transcripts containing EMCV IRES in the presence of increasing amounts of HCV-C protein diluted in buffer with DM; left, bicistronic RNA; right, monocistronic RNA; FLuc activity (cap-dependent) (striped bars); RLuc activity (EMCV-IRES-dependent) (shaded bars). (B) Relative IRES efficiencies (RLuc/Fluc ratios) determined as described in Fig. 3, after programmed of translation reactions with respective transcripts and addition *in trans* of increasing amounts of HCV-C protein diluted in buffer with DM. (C) identical experiments were performed with HCV-C protein diluted without DM. Each value represents the average relative IRES activity from triplicate translation samples, and the error bars represent the standard deviation.

Fig. 7. Effect of HCV-C protein on HCV IRES 1b and different variants. *In vitro* translation reactions of RRL were programmed with 200 ng of *in vitro* transcripts obtained from bicistronic (left panels) and monocistronic (right panels) reporter plasmids containing the following HCV-1a IRES (squares) and different mutants Q12 (open triangles), Q22 (open squares), Q511 (triangles) and Q519 (circles) described in Materials and Methods, and supplemented with increasing amounts of HCV-C protein as indicated, diluted in buffer with DM (A) and without DM (B). Relative IRES efficiencies were determined as described in Fig. 3. Each value represents the average relative IRES activity from triplicate translation samples, and the error bars represent the standard deviation.

Table 1. Comparison of relative IRES efficiencies from IRESes used in that study, in absence of HCV core protein,

System used	bicistronic	monocistronic
HCV-1b ^a	1.2 ^b	4.9
HCV-1a	1.2	3.9
CSFV	1	6.4
EMCV	1.4	19.7
BiP	0.4	6

^a Different IRESes are described in Materials and Methods

^b Relative IRES efficiencies of different IRESes normalized to 1 (100% efficiency) before the addition of the HCV core protein in either bi- or monocistronic system.

Table 2. Comparison between HCV-C protein to HCV IRES proportions (core protein/IRES) observed within the infected cell and those calculated in our *in vitro* assay.

Observed values	within one cell ^a			<i>in vitro</i>			
HCV-core protein							
pg	7	- 56	(25) ^b	10×10^3	100×10^3	1000×10^3	5000×10^3
molecules ($\times 10^8$)	2.2	- 17.8	(7.9)	3 000	30 000	300 000	1 500 000
HCV IRES (RNA) ^c							
pg	15×10^{-7} - 134×10^{-7} (69×10^{-7})			57×10^3			
molecules	7	- 64	(33)	3×10^{11}			
stoichiometry							
core / IRES	24×10^6			1	10	100	500
fold excess in the cell							
to <i>in vitro</i> ($\times 10^4$)	NA			2 000	200	20	4

^a The values given per infected hepatocyte for HCV core protein (48) and for RNA genomes (49).

^b Range values; the average value in parentheses.

^c The values for HCV IRES were calculated on the basis of 371 nt for transcript from monocistronic plasmid vm-IR 1b (1300 nt).

Figure 1

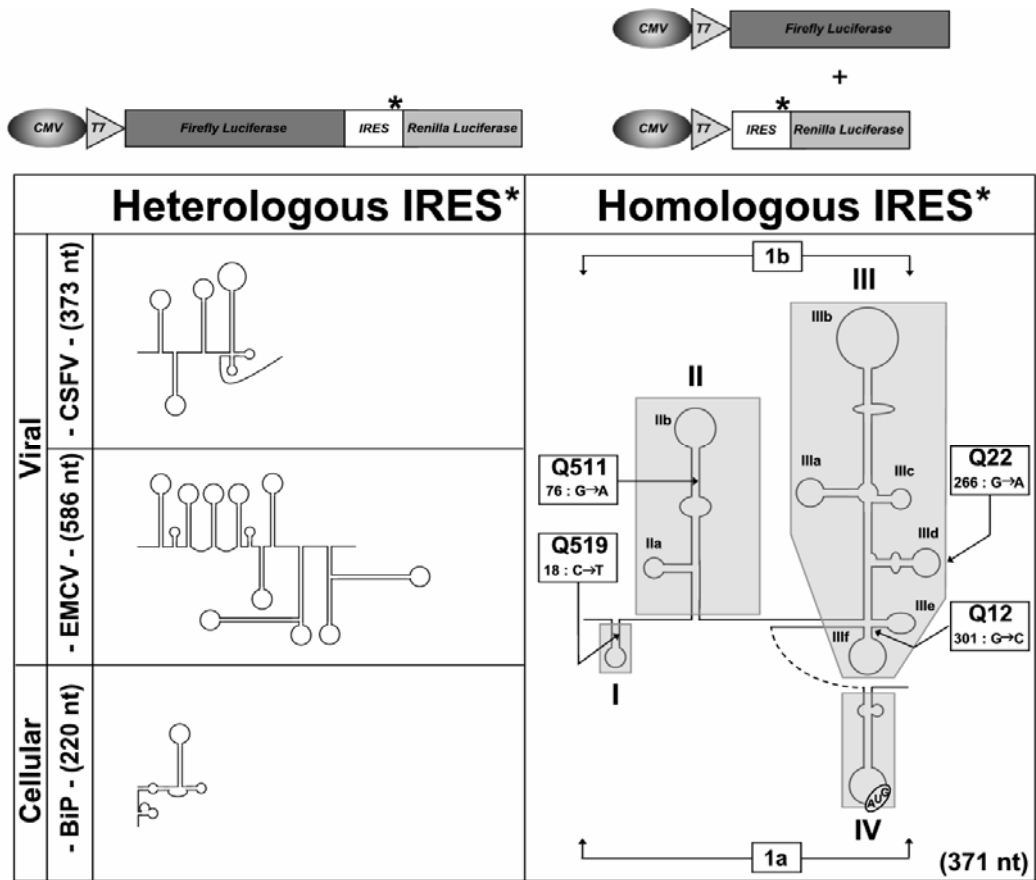


Figure 2

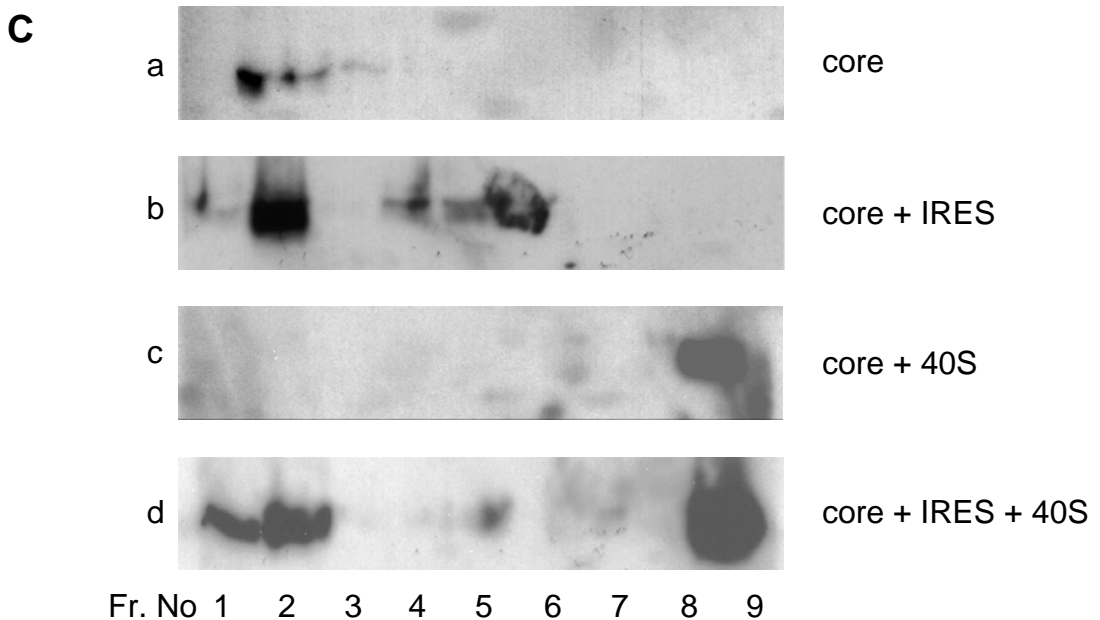
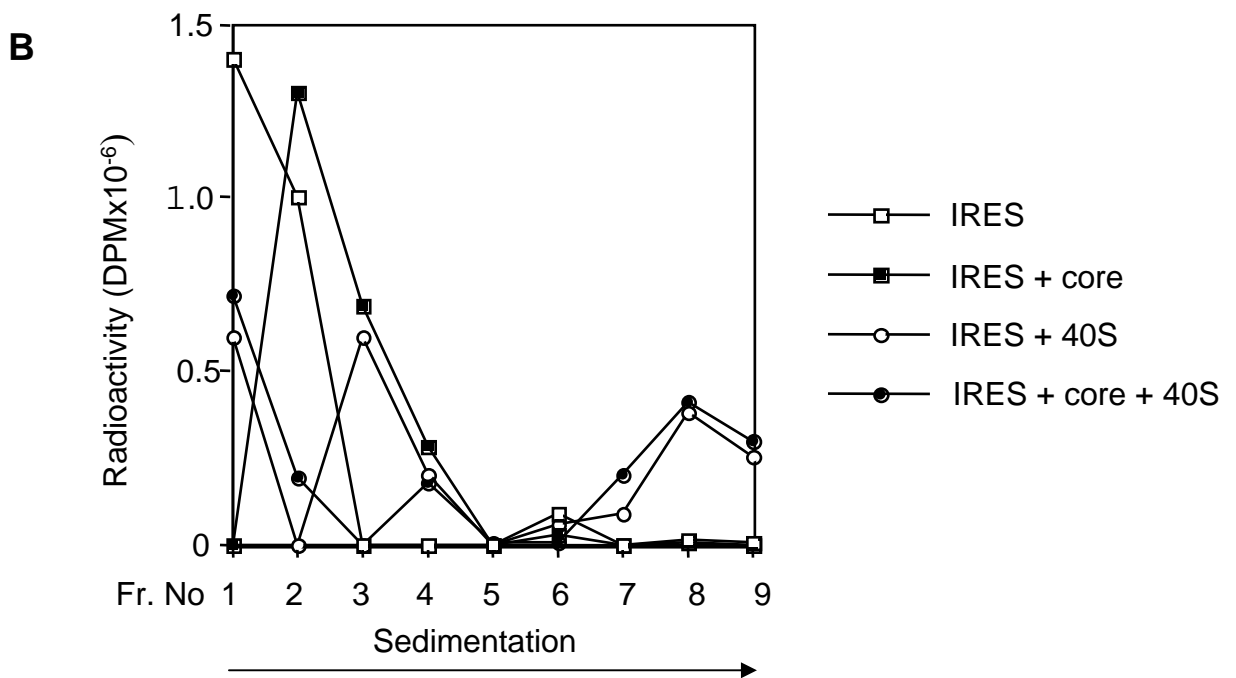
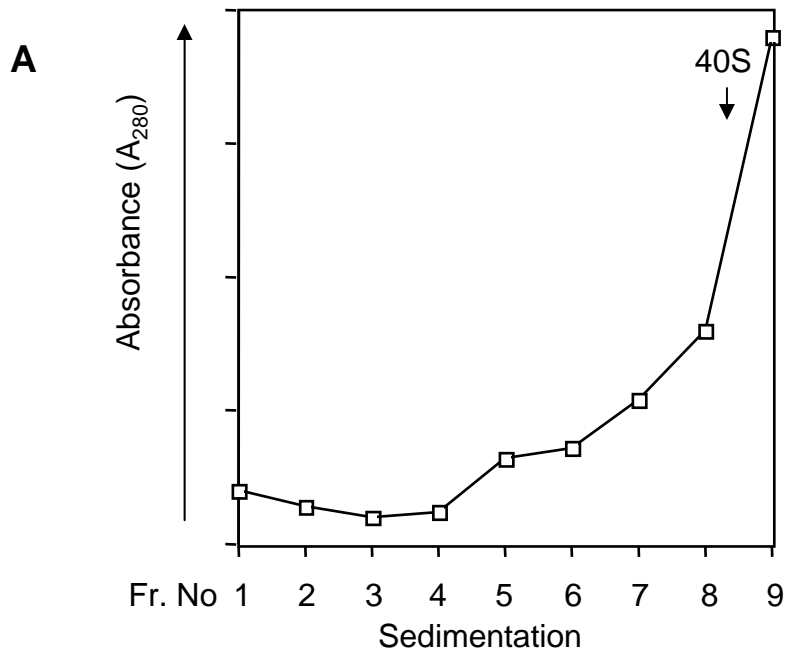
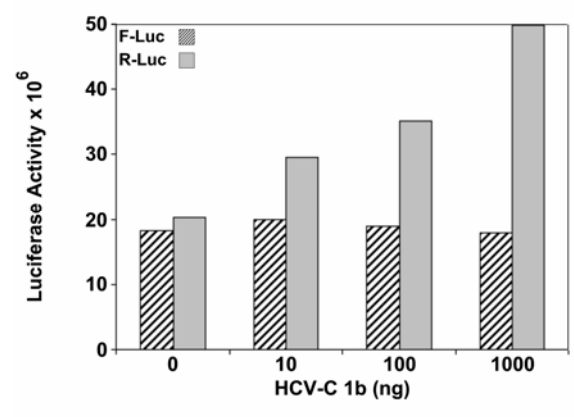
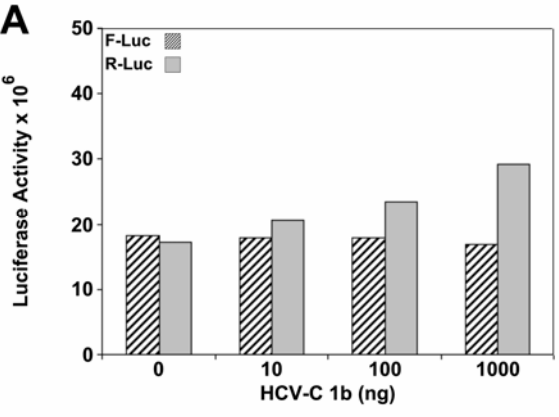


Figure 3

Bicistronic

Monocistronic

A



B

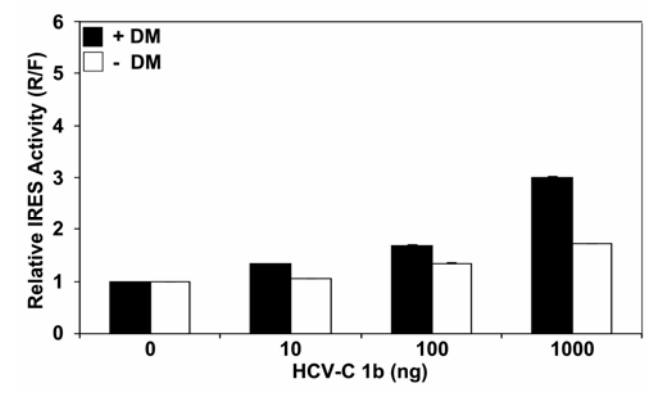
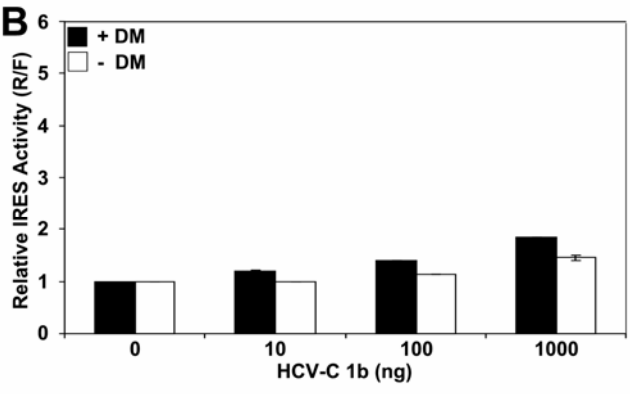


Figure 4

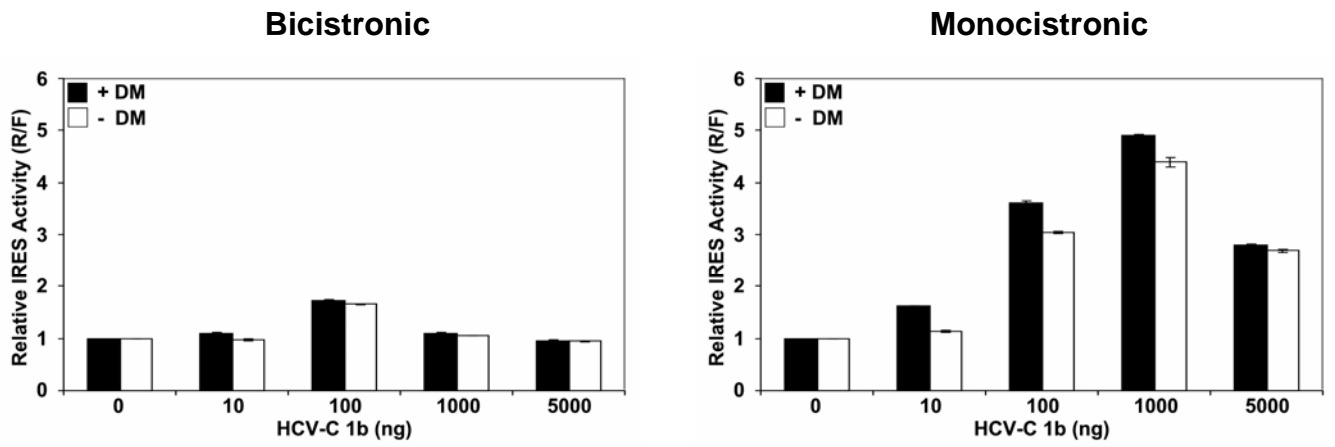


Figure 5

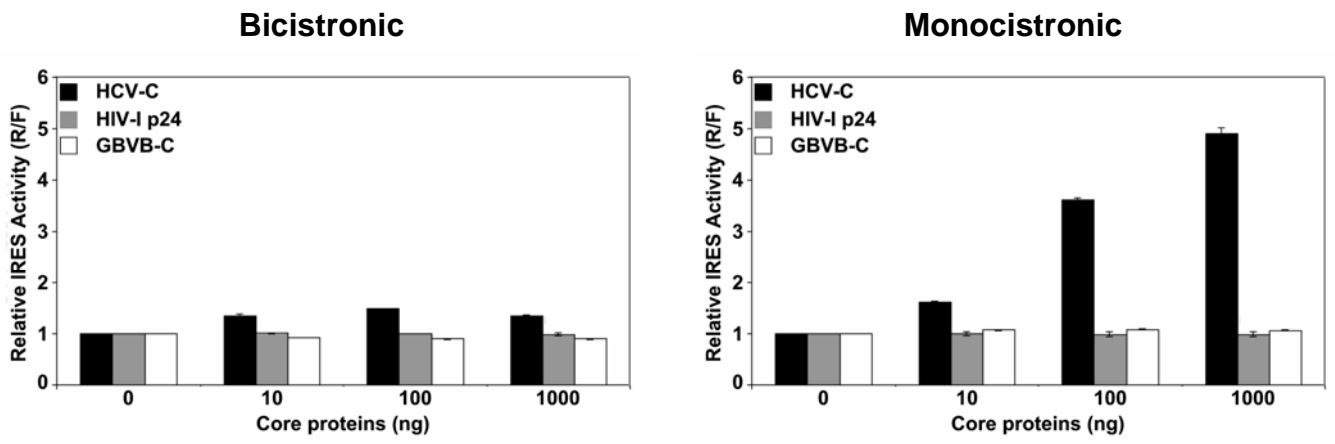
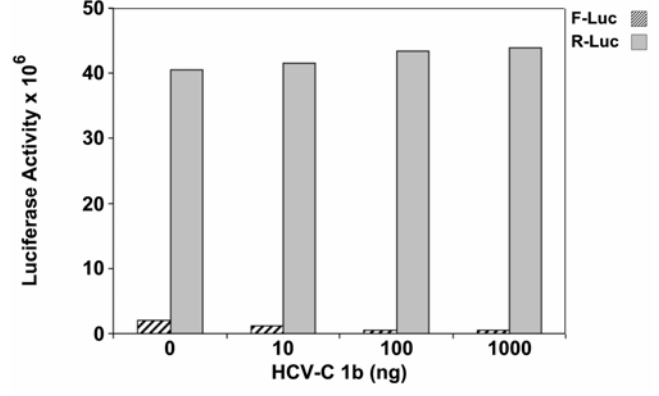
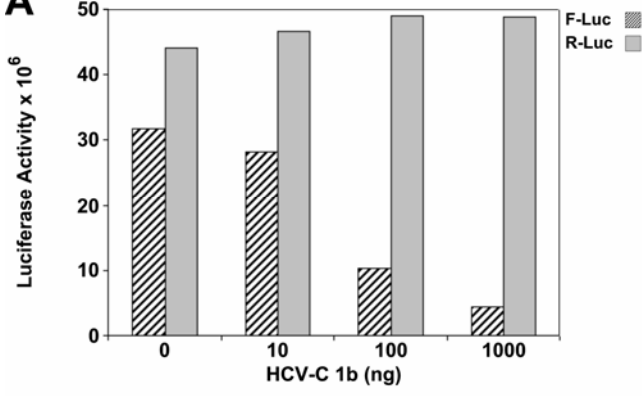


Figure 6

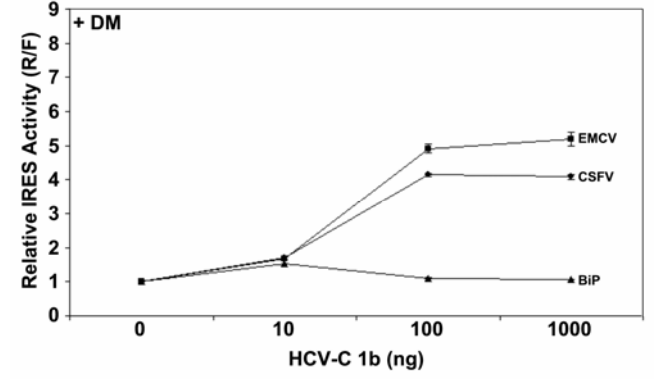
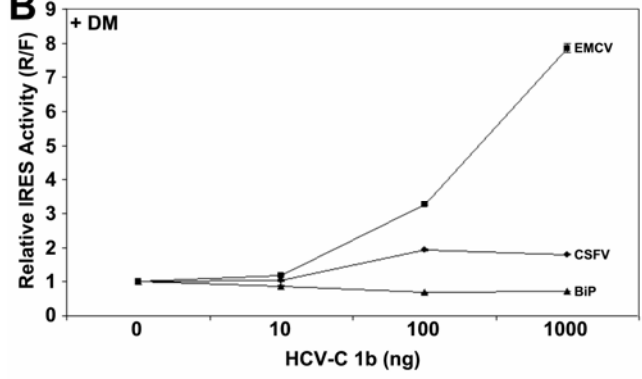
Bicistronic

Monocistronic

A



B



C

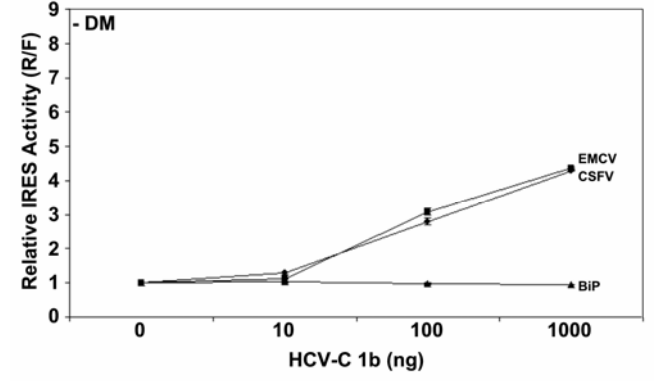
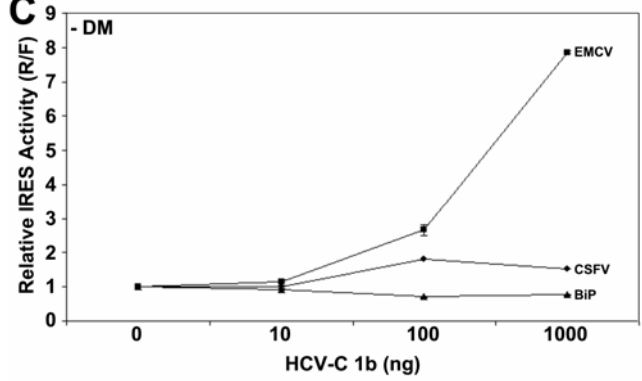
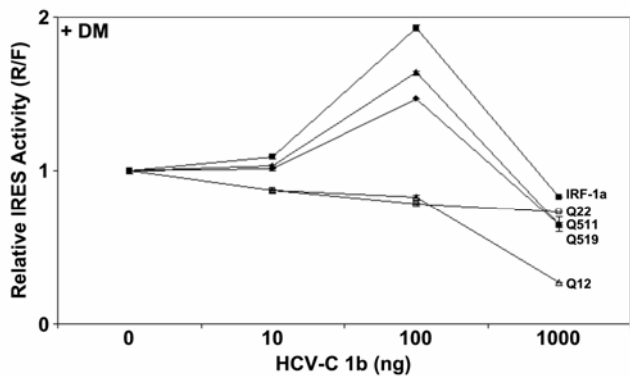


Figure 7

Bicistronic



Monocistronic

