Amino acids 1–20 of the hepatitis C virus (HCV) core protein specifically inhibit HCV IRES-dependent translation in HepG2 cells, and inhibit both HCV IRES- and cap-dependent translation in HuH7 and CV-1 cells

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A self-modulating mechanism by the hepatitis C virus (HCV) core protein has been suggested to influence the level of HCV replication, but current data on this subject are contradictory. We examined the effect of wild-type and mutated core protein on HCV IRES- and cap-dependent translation. The wild-type core protein was shown to inhibit both IRES- and cap-dependent translation in an in vitro system. This effect was duplicated in a dose-dependent manner with a synthetic peptide representing amino acids 1–20 of the HCV core protein. This peptide was able to bind to the HCV IRES as shown by a mobility shift assay. In contrast, a peptide derived from the hepatitis B virus (HBV) core protein that contained a similar proportion of basic residues was unable to inhibit translation or bind the HCV IRES. A recombinant vaccinia–HCV core virus was used to examine the effect of the HCV core protein on HCV IRES-dependent translation in cells and this was compared with the effects of an HBV core-recombinant vaccinia virus. In CV-1 and HuH7 cells, the HCV core protein inhibited translation directed by the IRES elements of HCV, encephalomyocarditis virus and classical swine fever virus as well as cap-dependent translation, whereas in HepG2 cells, only HCV IRES-dependent translation was affected. Thus, the ability of the HCV core protein to selectively inhibit HCV IRES-dependent translation is cell-specific. N-terminal truncated (aa 1–20) HCV core protein that was expressed from a novel recombinant vaccinia virus in cells abrogated the inhibitory phenotype of the core protein in vivo, consistent with the above in vitro data.

INTRODUCTION

It is estimated that about 3 % of the worldwide population is infected with hepatitis C virus (HCV) and more than 80 % of infected individuals develop persistent infection (World Health Organisation, 1999). It is unclear why such a high proportion of individuals fails to resolve the infection. However, several studies suggest that multiple factors might be involved, including the high genetic variability of the genome (Ray et al., 1999; Simmonds, 1995), extrahepatic replication (Laskus et al., 1998; Muller et al., 1993) and the lack of an effective early immune response (Minton et al., 1998; Zibert et al., 1997). No consensus has emerged. Similar to many other viruses, it is most likely that HCV has developed a strategy to evade the host defence to ensure survival.

HCV is a member of the Flaviviridae, along with the pestiviruses and flaviviruses (Robertson et al., 1998). The genome is a single-strand positive-sense RNA molecule of approximately 9600 nucleotides that contains a single long open reading frame (ORF) which is flanked by untranslated regions (UTR) at the 5' and 3' ends. The 5' UTR is a highly conserved region that contains an internal ribosome entry site (IRES), which initiates translation by a cap-independent mechanism (Rijnbrak & Lemon, 2000). Translation yields a polyprotein, which is cleaved into three structural (core, E1, E2/p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B).
The core protein, which is thought to form the viral capsid, is located in the most N-terminal portion of the polyprotein. Evidence has accumulated to suggest that the core protein may inhibit the host response to virus infection through multiple mechanisms (Lai & Ware, 1999; McLauchlan, 2000). In addition, the core protein has been shown to bind heterogeneous nuclear ribonucleoprotein K (hnRNPK), which is involved in cellular pre-mRNA splicing and nuclear RNA transport, and modulate cellular RNA transcription (Ray et al., 1995, 1997; Shrivastava et al., 1998).

It has also been suggested that HCV has a self-modulating mechanism to maintain a low level of replication and expression that may promote virus persistence. To account for this, it was speculated that stem–loop IV of the HCV IRES might be stabilized by interaction with a viral protein, to result in inhibition of translation (Honda et al., 1996). The core protein was later shown to bind positive- but not negative-strand HCV RNA, an interaction that resulted in suppression of translation (Shimoike et al., 1999). Amino acids (aa) 1–75 of the core protein were previously reported to be responsible for the interaction with the viral RNA (Santolini et al., 1994). In addition to binding the viral RNA, the core protein can interact with itself and with the E1 and E2 proteins (Lo et al., 1996). It was also reported that the HCV core protein reduced the efficiency of HCV translation by binding to the IRES (Shimoike et al., 1999) and aa 33–44 were recently shown to interact with the IRES and contribute to the inhibition of translation (Zhang et al., 2002). In contrast, a previous study suggested that the core protein did not appear to have any specific effect on HCV IRES-directed translation, and instead, it was reported that suppression of IRES-directed translation resulted from an RNA–RNA interaction (Wang et al., 2000).

The potential role of the HCV core protein in the efficiency of HCV IRES-directed translation is a key issue in understanding the replication and expression of HCV. Consequently, the aim of this study was to clarify the nature of the HCV core–IRES interaction. We examined the effect of the wild-type and mutated core protein on the expression of reporter genes in an in vitro system and the effect of the protein expressed from a recombinant vaccinia virus on HCV IRES-directed translation in different cell lines.

**METHODS**

**Cells.** Monkey kidney (CV-1) cells and the human hepatocellular carcinoma cells, HuH7 and HepG2, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% or 10% foetal calf serum (FCS) with penicillin and streptomycin.

**Plasmids.** The plasmids pCore and pA20, which encode aa 1–167 and aa 21–167 of the HCV core protein respectively, were synthesized by insertion of the appropriate PCR-generated fragment from p5’UTR-A2 (Trowbridge & Gowans, 1998) into pcDNA3 (Invitrogen). pA20 was engineered to encode a methionine at the start of the ORF and a stop codon at the end. Plasmid pA1, in which the start codon of the core protein was deleted from pCore, was made using the Quickchange Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer’s instructions. A series of C-terminal truncated core proteins was generated by appropriate restriction enzyme digestion of pCore. The reporter plasmid pIRES-CAT (Lott et al., 2001), which contains the HCV 5’ UTR sequence and 27 nt of the downstream core protein-coding sequence ligated in-frame with the chloramphenicol acetyltransferase (CAT) gene, was constructed in pGEM-T (Promega). pCAP-LUC and pIRES-CAT were constructed by inserting the firefly luciferase gene into pGEM-T. Transcription from these plasmids is controlled by the T7 promoter and expression controlled by the HCV IRES and a cap-dependent mechanism respectively.

A bicistronic reporter pcAT, from which expression of firefly luciferase and CAT were controlled by cap- and IRES-dependent mechanisms respectively, was constructed by ligating pCAP-LUC and pIRES-CAT in pcDNA3. Three additional bicistronic constructs, which contained the IRES elements from HCV, encephalomyocarditis virus (EMCV) and classical swine fever virus (CSFV) (referred to as HIRES, EIRES and CIRES) inserted between the Renilla luciferase (R-LUC) and CAT genes (Lott et al., 2001), were also used as reporter molecules. Thus, the expression of R-LUC and CAT was directed by cap- and IRES-dependent mechanisms respectively.

**In vitro transcription of RNA.** The plasmids were linearized by the appropriate restriction enzyme and then purified by phenol/chloroform extraction followed by isopropanol precipitation or by the BRESAclean DNA purification system (Bresatec). RNA was synthesized from each plasmid by T7 RNA polymerase with or without the addition of RNA capping analogue (Gibco-BRL) as appropriate. The RNAs were purified by phenol/chloroform extraction and isopropanol precipitation followed by two washes with 70% ethanol. The RNA pellets were dissolved in RNase-free water. The quality and quantity of the RNAs were checked by agarose gel electrophoresis and the concentration was determined by optical density measurement.

**In vitro translation.** Wild-type core protein and the pA1 and pA20 protein products were expressed in a 25 µl rabbit reticulocyte lysate translation reaction (RRL; Promega) loaded with 500 ng of the respective RNA. This reaction will be referred to as RRL1. The products were analysed by SDS-PAGE and immunoblot as previously described (Wang et al., 1997). Radiolabelled products were visualized by SDS-PAGE followed by PhosphorImager analysis (Molecular Dynamics) and by immunoblot to confirm the authenticity of the synthesized proteins. To examine the effect of the expressed protein on IRES- or cap-dependent translation, an aliquot of the above RRL1 was then added to a second RRL containing 500 ng of pIRES-CAT RNA and/or 100 ng of pCAP-LUC RNA. This reaction will be referred to as the reporter translation reaction (RRL2). All translation reactions were carried out at 30°C for 90 min unless noted otherwise. The products of RRL2 were analysed as described above and all experiments were carried out in triplicate.

In some experiments, synthetic peptides were added to the RRL2. The peptides were synthesized by Mimotopes (Australia) to > 90% purity. The sequences of the peptides are: (1) HCV core aa 1-20-MSYNPKPQRKTRKNTNRPRQ; (2) HCV E2 HVR1 aa 384-419-DTHHTTGGVAQRTDRTGFLFFGFSPGQK; (3) HBV core aa141-160-STLPETTVRGRKRRPRRT; (4) HbsAg aa 202-213-IPQSLDSWWTSL.

**RNA–protein binding assay.** The RNA–protein binding assay was carried out essentially as described (Furuya & Lai, 1993) with slight modifications. Briefly, 32P-labelled RNA was mixed with peptide in 10 µl of binding buffer (10 mM HEPES, 2.5 mM MgCl2, 40 mM HCl, 5% glycerol, 2.5 mM DTT, 20 U RNase inhibitor). The binding reaction was incubated at 30°C for 10 min and then mixed with 5× loading buffer (50% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol, 2× TBE). The samples were analysed by electrophoresis on a 4% native polyacrylamide gel. The gel was pre-electrophoresed at
80 V for 1 h prior to loading the samples in 0.5× TBE and electrophoresis was performed at 80 V for 1.5 h. Labelled RNA was detected by PhosphorImager analysis of the dried gel.

**Recombinant vaccinia viruses.** Two recombinant vaccinia viruses (RecVV), RecVV-HCC and RecVV-HBC, were used to express either the full-length HCV core protein (aa 1–191) or the full-length HBV core protein (aa 1–183), respectively. These were synthesized and supplied by J. Hammond and B. Couper (Australian Animal Health Laboratory, Geelong, Australia). Expression of the core proteins from the recombinant viruses was controlled by the VVP7.5 promoter in the thymidine kinase (TK) gene locus of Western Reserve (WR). The virus stocks were prepared and titrated as previously described (Boyle et al., 1985).

To construct the N-terminal truncated version of RecVV-HCC (RecVV-A20), the PCR product, encoding aa 21–191 of the HCV core protein, was inserted into the pBCB06 transfer vector (Boyle et al., 1985). The recombinant plasmid was transfected into CV1 cells, previously infected with WR, then followed by two cycles of TK phenotype selection in 143B ë− cells. The DNA sequence of the recombinant transfer vector was confirmed and the authenticity of the recombinant virus was further confirmed by PCR amplification of the inserted fragment.

**Infection and transfection.** Confluent monolayers of CV-1, HuH7 and HepG2 cells were prepared in 6-well plates (TPP) and infected with trypsinized VV at an m.o.i. of 10 in 0.5 ml of DMEM + 1% FCS). The inoculum was replaced with fresh culture medium after incubation for 1 h in a humidified CO2 incubator at 37°C and the cells were subsequently incubated overnight (18–24 h). Before transfection, the cells were washed twice with 1 ml OptiMEM (Gibco-BRL). 1 µg each of pRES-CAT and pCAP-LUC RNA, or 2 µg of each bicistronic reporter RNA, were prepared with Lipofectin reagent (Gibco-BRL) per well according to the manufacturer’s protocol.

**CAT and luciferase assays.** The cells were washed once with ice-cold PBS, and then lysed with 0–3 ml of buffer provided with the dual luciferase assay (DLA; Promega) for 10 min. The cell debris was removed by centrifugation and the supernatant transferred to a fresh tube. CAT activity was determined by ELISA (Roche) and LUC and WT activities were measured by the DLA, following the instructions of the manufacturer.

**Relative translational efficiency (RTE).** The RTE was calculated by normalizing the activities of the reporter molecules in the RRL2 containing the RNA(−) Control and in RecVV-HBC-infected cells to 100%.

**RESULTS**

**Core protein inhibits both IRES- and cap-directed translation in a dose-dependent manner in an in vitro system**

To investigate the effect of the HCV core protein on HCV IRES- and cap-directed translation, the core protein was pre-synthesized and then added to a reporter translation reaction (RRL2). In this experiment, we used aa 1–167 of the protein, which is similar in size to the mature core protein (Santolini et al., 1994). The protein was produced in an RRL1 reaction by translation of pCore RNA and an aliquot of this reaction was then transferred to the RRL2. An aliquot of RRL1 containing pA1 RNA and one without the addition of any RNA [RNA(−) control] represented controls. Core protein expressed in this manner did not require purification, as the protein was contained in the same medium as the reporter translation reaction. The pCore translation product was detected as an 18 kDa polypeptide by immunoblot and the product expressed from pA1 appeared as a 6 kDa protein when it was labelled with [35S]methionine (data not shown). This product is consistent with initiation at the methionine in the −1 ORF (nt position 257) of an ORF which extends to nt position 431 (i.e. 59 aa) of the Australian HCV isolate (Trowbridge & Gowans, 1998). Five µl of the respective RRL1 was added to the pRES-CAT and/or pCAP-LUC RNA programmed RRL2 and the effect on translation measured by quantification of the CAT and luciferase products. The levels of CAT and luciferase expression in the RNA(−) control reaction were normalized to 100%. The addition of the core protein to the reporter translation reaction resulted in an inhibition of CAT and luciferase expression (Fig. 1A) that was found to be 60% and 56% respectively by PhosphorImager analysis (Fig. 1B). In contrast, the pA1 control only showed a slight inhibition of translation (10% and 5% respectively). Translation inhibition by the pCore product

![Fig. 1](http://vir.sgmjournals.org)
was dose-dependent (data not shown). These results indicate that translation was inhibited by the pCore translation product and not by RNA including the pΔ1RNA, which is identical to pCore RNA (except for the removal of the initiation codon), or any component of the RRL. Furthermore, in order to generate similar levels of protein, it was necessary to add 5-fold more IRES RNA compared with cap-dependent RNA (see Materials and Methods) because cap-dependent translation is more efficient in our hands; consequently, the inhibitory effect of the core protein is actually 5-fold more specific for the HCV IRES.

**The N-terminal 21 aa of core protein are sufficient to produce a similar inhibition to core 1–167 in the *in vitro* system**

To map the major region within the core protein responsible for the inhibitory effect, a series of C-terminal truncated core proteins was synthesized (Fig. 2A). The molarity of the different RNA molecules was equalized in the RRL1, and it was assumed that the efficiency of synthesis of the different proteins was similar. Therefore, 5 μl of the respective RRL1 was added to RRL2. A similar level of inhibition on IRES- and cap-directed translations (~50–60%) was noted in all reactions that contained core protein including the 24 and 21 aa forms, but not in the reaction containing the pΔ1 product (Fig. 2B). Thus a major portion of the core antigen inhibition activity against IRES- and cap-dependent translation is located in the N terminus of the protein.

**Amino acids 1–20 of the HCV core contribute to translation inhibition in *cis* and removal of these residues results in loss of translation inhibition in the *in vitro* system**

To further examine the role of aa 1–20 in translation inhibition, pΔ20, in which the codons for aa 1–20 were removed, was synthesized. The addition of equal amounts of pΔ20 and pCore RNA to the respective RRL1 resulted in a 5-fold increase in the expression of core aa 21–167 over that...
of core aa 1–167. This increase in translation efficiency of pΔ20 shows that aa 1–20 can also inhibit translation in cis (Fig. 3A). The volume of the respective RRL1 was adjusted to ensure that equal amounts of pCore and pΔ20 proteins were then added to the respective RRL2 reactions. The addition of the pΔ20 protein did not affect pIRES-CAT and pCAP-LUC translation relative to that of the RNA(−) control (Fig. 3), whereas the addition of pCore reduced pIRES-CAT translation by 62 % (±12 %) and pCAP-LUC translation by 81 % (±15 %), consistent with the results described above. Thus, removal of aa 1–20 results in loss of the inhibitory phenotype; these data confirmed that aa 1–20 of the core protein contain the major region responsible for translation inhibition in cis and in trans.

Dose-dependent inhibition of translation by a synthetic peptide

The above data were generated with wild-type and mutant core proteins that were synthesized in one RRL and then added to a second (reporter) reaction. Thus, although this experimental design made it unnecessary to purify the core protein, it was necessary to duplicate the data using a protein or peptide that was synthesized independently. This would confirm that the inhibitory activity was due to expression of the core protein. Consequently, a synthetic 20-mer peptide representing aa 1–20 of the core protein was added to the pIRES-CAT and pCAP-LUC reporter translation reactions. Peptides derived from (i) the HBV core protein that contained the same proportion of basic residues as the HCV core 1–20 peptide, (ii) the HCV envelope protein 2 (E2) HVR1 and (iii) hepatitis B surface antigen (HBsAg) represented controls. Varying amounts of the peptide were added to achieve molar ratios of RNA : peptide that ranged from 1 : 10 to 1 : 500. The HCV core peptide inhibited both HCV IRES- and cap-dependent translation in a dose-dependent manner (Fig. 4). In contrast, the control peptides showed no translation inhibition even at high concentrations (Fig. 4). The lowest concentration of core peptide (ratio 1 : 10) showed only a slight inhibition of translation, while the highest concentration (1 : 500) inhibited both IRES- and cap-dependent translation by 80 %.

HCV core peptide 1–20 binds to the HCV IRES RNA

To determine the possible mechanism of this translation inhibition by the HCV core peptide, an in vitro RNA-binding assay was performed and the products were analysed in a native polyacrylamide gel. The HCV core peptide was able to bind to the HCV IRES, leading to a shift in the
electrophoretic mobility of the labelled RNA at a ratio of 50:1 or greater, whereas the HBV core peptide showed no such activity, even at high concentrations (Fig. 5A). In this native polyacrylamide gel, the HCV IRES RNA always appeared as two bands, whereas in a denaturing gel, a single band with the predicted electrophoretic mobility was detected (data not shown). The mobility shift induced by the peptide was subtle (note the slight decrease in the electrophoretic mobility of the RNA in tracks 50 and 100) and is consistent with a 20-mer peptide with a molecular mass of 2554 binding to the 368 nt RNA with a predicted molecular mass of 121 000. Peptide ratios of 200 induced aggregation which resulted in retention of the RNA at the top of the gel. This can be viewed as an extreme form of RNA binding.

The binding efficiency of the HCV core peptide was dose-dependent as was the translation inhibition in vitro. However, if the ratio of RNA:peptide was < 1:50, no shift was noted. To address this point further, we set up four groups of RNA-binding reactions in which four different concentrations of core peptide (2.5, 5, 10 and 20 μM) were added to variable amounts of RNA to achieve RNA:peptide ratios of 1:10, 1:100 and 1:200. The results of this experiment show that if the concentration of peptide was 5 μM, no electrophoretic shift was detected even at a RNA:peptide molar ratio of 1:200 (Fig. 5B, C). However, when the concentration of peptide was increased to ≥10 μM, RNA binding was evident in the reactions in which the peptide was ≥100-fold excess over the RNA (Fig. 5). These results show that a peptide concentration of 10 μM is required, and

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**Fig. 5.** The core peptide binds the HCV IRES in a dose dependent manner and depends on a threshold level of the core peptide. (A) Varying concentrations of the HCV core peptide (MSTNPQKQRKTKRNTNRRPQD) or the HBV core peptide (STLPETTVVRRGRSPRRRT) were incubated with radiolabelled HCV IRES RNA in binding buffer for 10 min at 30°C. The complex was analysed by a native polyacrylamide gel. (B) Four groups of IRES RNA binding reactions were set up, in which the concentration of the core peptide was 2.5, 5, 10 and 20 μM respectively, and then a variable concentration of HCV IRES RNA was added to achieve a ratio of RNA:peptide of 1:10, 1:100 and 1:200 in each group. The position of the labelled RNA was measured by PhosphorImager analysis. (C) The proportion of labelled RNA which was retained on the top of the gel (boxed area) was calculated and compared in each reaction shown in panel B.
provided that this minimum concentration is achieved, then the core peptide can bind to the HCV IRES.

**HCV core protein inhibits HCV IRES-directed translation in vivo in a cell-specific manner**

To examine the effect of the HCV core protein on HCV IRES-directed translation in vivo, the HCV core protein was expressed from a recombinant vaccinia virus (RecVV-HCC), and an HBV core recombinant vaccinia virus (RecVV-HBC) represented a control. We have shown that the level of expression from pIRES-CAT and pCAP-LUC in RecVV-HCC-infected cells was similar to that in cells infected with the WR strain of vaccinia virus (data not shown). The effect of the expression of the core protein was examined in three different cell lines, CV-1, HuH7 and HepG2. The cells were infected with the respective viruses at an m.o.i. of 10, transfected with the different RNA reporter molecules after overnight incubation and the expression of the reporter proteins was measured 5 h later.

Initially, we co-transfected the RecVV-infected cells with pIRES-CAT and pCAP-LUC RNA. The levels of CAT and luciferase expressed in the RecVV-HBC-infected cells were normalized to 100 % (Fig. 6A). By comparison, the expression of CAT in all three cell lines infected with RecVV-HCC was reduced by 45–70%. The expression of luciferase in RecVV-HCC-infected CV-1 and HuH7 cells showed a similar reduction, but no such reduction was noted in the RecVV-HCC-infected HepG2 cells. Thus, expression of the HCV core protein appeared to inhibit both HCV IRES- and cap-dependent translation in the CV-1 and HuH7 cells, but was specific for the HCV IRES in HepG2 cells.

We then tested the effect of the HCV core protein on CAT and luciferase expression from a bicistronic reporter molecule, pcCAT, in which the luciferase and CAT genes were separated by the HCV IRES. The results obtained using this bicistronic RNA duplicated those obtained after transfection with the monocistronic RNAs (data not shown).

The above data were derived using the HCV IRES from a genotype 1b virus (Trowbridge & Gowans, 1998). We then extended the study to examine the effect of expression of the HCV core protein (1b) on an IRES element derived from a genotype 1a virus and compared this against IRES elements derived from EMCV and CSFV. These bicistronic vectors have been described previously (Lott et al., 2001). As described above, RecVV-HBC was used as a negative control. In CV-1 and HuH7 cells, the HCV core protein appeared to inhibit translation from all three IRES elements and cap-dependent translation to similar degrees (Fig. 7). In contrast, in HepG2 cells, the HCV core protein only inhibited translation from the HCV IRES and had no effect on the EMCV and CSFV IRES elements, or on cap-dependent translation.

**Amino acids 1–20 of the HCV core protein is important for translation inhibition in vivo**

Since in vitro translation inhibition was lost when aa 1–20 of the HCV core protein was removed, a RecVV construct was synthesized that encoded aa 21–191, to evaluate this observation in vivo. This virus was used to infect HuH7 and HepG2 cells and the cells were then transfected with the bicistronic pcCAT RNA. CAT and luciferase activities were assayed as described above. Consistent with the above results, infection of both cell types with RecVV-HCC resulted in a 60 % inhibition of CAT activity (Fig. 8). However, in cells infected with RecVV-A20, CAT expression was only reduced by ~15 % in both cell lines. The activity of luciferase in cells infected with RecVV-HCC or RecVV-A20 was similar to the above results (Fig. 6A), i.e. HCV core protein showed no inhibitory effect on cap-directed translation in HepG2 cells but inhibited cap-directed translation by 60 % in HuH7 cells. Similarly, and consistent with the IRES-CAT data, deletion of aa 1–20 from the core protein virtually ablated the inhibitory effect on cap-directed expression in the HuH7 cells (Fig. 8, left panel). The results clearly demonstrate that the bulk of the activity associated with the HCV core protein-related inhibition of IRES- or cap-directed translation resides within aa 1–20 of the protein.

**DISCUSSION**

Although previous studies (Shimoike et al., 1999; Wang et al., 2000) agreed that translation from the HCV IRES could be...
inhibited by expression of the HCV core protein, the data on
the mechanism were conflicting. On the one hand, it was
suggested that the core protein itself was responsible
(Shimoike et al., 1999) and on the other, it was suggested
that an interaction between the core RNA and the IRES was
responsible (Wang et al., 2000). We have re-examined this
phenomenon by in vitro analysis and analysis in cultured
cells, and present four major conclusions from our work.
First, the HCV core protein can inhibit IRES-directed
translation; aa 1–20 at the N-terminus of the core protein
are responsible for HCV IRES-binding and the translation
inhibition phenotype. Second, the HCV core (aa 1–20)
peptide-IRES interaction (and presumably inhibition of
translation in vivo) depends not only on a minimum,
threshold level of the peptide but also on a high ratio of
peptide : RNA. Third, translation inhibition by the HCV core
protein is specific for the HCV IRES in HepG2 cells but
inhibits both HCV IRES- and cap-dependent translation in
HuH7 and CV-1 cells. Four, core protein can also inhibit
translation from the EMCV and CSFV IRES elements in
HuH7 and CV-1 cells, but not in HepG2 cells.

Our finding, that a synthetic peptide representing aa 1–20 of
the HCV core protein, can inhibit cap- and IRES-translation
is novel. Indeed, we have recently demonstrated (data not
shown) that the addition of this peptide to a cell monolayer
resulted in a reduction in cellular RNA synthesis. In contrast,
a 20 aa synthetic peptide derived from the HBV core protein,
which encapsidates HBV RNA during replication and which
can also bind RNA in a non-specific manner (Cohen &
Richmond, 1982), was unable to inhibit translation. This
peptide contained the same proportion of basic residues as
the HCV core peptide. This suggests that a basic residue-rich
domain is necessary, but not sufficient, for the inhibitory
phenotype shown by the HCV core peptide. The peptide
sequence itself is important.

The full-length HCV core protein was able to inhibit trans-
lation in cell cultures in a reproducible manner, whereas the
protein which lacked aa 1–20 was unable to do so. In con-
trast, the full-length HBV core protein was unable to inhibit
cap- or HCV IRES-dependent translation in cells. Thus the
in vitro data can be related to events in vivo. The specific effect

Fig. 7. The HCV core protein inhibits HCV, EMCV and CSFV IRES-directed CAT- and cap-directed luciferase-expression in
CV-1 and HuH7 cells, but only inhibits HCV IRES-directed expression in HepG2 cells. RecVV-HBC- (diagonal stripes) and
RecVV-HCC- (cross-hatching) infected CV1, HuH7 and HepG2 cells were transfected with bicistronic vectors in which the
HCV, EMCV and CSFV IRES elements respectively separated an upstream luciferase gene and a downstream CAT gene to
result in cap-directed luciferase expression and IRES-directed CAT expression. The expression of the reporter proteins is
shown as RTE compared with the levels in RecVV-HBC-infected cells that were normalized to 100 %.
of the HCV core protein in HepG2 cells as opposed to HuH7 and CV-1 cells may be related to differences in the intracellular distribution of the protein (Wang et al., 2000), although we were unable to demonstrate any difference in the localization patterns in the three cell lines used in our study (data not shown). If the HCV core protein inhibits translation and/or transcription (Ray et al., 1995, 1997; Shrivastava et al., 1998) in human hepatocytes, then this may influence cell viability. A recent study (Bantel et al., 2001) reported that a high proportion of hepatocytes in HCV-infected livers showed evidence of apoptosis, although it is not known if this is related to the expression of core or other HCV proteins. It has been suggested that the HCV core protein can sensitize the cell to apoptosis, although this may not necessarily be linked to reduced translation efficiency (Lai & Ware, 1999).

In HepG2 cells, the HCV core protein specifically inhibited translation from the HCV IRES, irrespective of whether the IRES element was derived from a 1a or 1b genotype, but had no effect on translation from the EMCV and CFSV IRES elements. These results are consistent with previous data in which the translation from the HCV IRES was specifically inhibited by the HCV core protein in HepG2 and HepT cells (Shimoike et al., 1999; Zhang et al., 2002). Our data also highlight yet another difference between HepG2 and HuH7 cells, as it has previously been demonstrated that the HuH7 cells but not the HepG2 cells support replication of the HCV replicon (Lohmann et al., 1999; Blight et al., 2000). We have sought an explanation for our results which differ to those showing that the effect is mediated by RNA (Wang et al., 2000). In our study, a ratio of 1:100 of RNA:peptide was necessary for binding, while 1:500 was necessary for complete inhibition of translation in vitro. In contrast, the concentration of HCV core used in vitro in the Wang study was much lower (Wang et al., 2000). There is an additional important difference between the two studies; in the Wang study, the bicistronic reporter plasmids were transfected 24 h prior to infection of the cells with recombinant baculovirus which encoded the HCV core protein, whereas we transfected our reporter plasmids after overnight infection of the cells with a recombinant vaccinia virus. Thus, it is possible that the level of expression of the HCV core protein in the Wang study was insufficient to inhibit HCV IRES function. Indeed, our study shows that a high concentration of core protein and a high ratio of protein:RNA (Fig. 5) are necessary for an interaction between the HCV core and the HCV IRES. These features are consistent with our understanding of HCV replication (Bartenschlager & Lohmann, 2000). Our in vitro data showed that a similar concentration of the core peptide was required for translation inhibition and RNA binding (Figs 4 and 5 respectively). Furthermore, this concentration is similar to that necessary for HCV core protein to induce assembly of an HCV nucleocapsid-like particle in vitro (Kunkel et al., 2001). Extrapolation of these data suggests that a high threshold concentration of the core protein, as might be found in the typical punctate staining patterns (Gowans, 2000), is required for RNA binding in vivo. This may either inhibit translation per se or reduce translation by removal of the RNA template through packaging. The latter mechanism may account for the difficulties in detecting HCV products in naturally infected liver samples (Gowans, 2000). In this respect, HCV resembles bovine viral diarrhoea virus because the bulk of the virus is rapidly secreted and very little remains cell-associated (Gong et al., 1996).

Other circumstantial evidence supports our conclusion that the HCV core protein inhibits HCV IRES activity; first, the efficiency of colony formation by the full-length replicon which encodes the structural proteins was reported to be 3–4 logs lower than that of the subgenomic replicons (Ikeda et al., 2002). Second, the level of replication of the full-length replicon in the selected cell lines is around 5-fold lower than that of the subgenomic replicon (Pietschmann et al., 2002). Together, these data suggest that one or more of the structural proteins regulates HCV replication. As E1/E2 have been shown to have no effect on HCV IRES function (Shimoike et al., 1999), it can be concluded that the core protein itself inhibits HCV IRES-directed translation. Finally, we have recently expressed the HCV core, E1 and E2 proteins from a Semliki forest virus replicon (Greive

Fig. 8. Removal of aa 1–20 from the HCV core protein results in loss of the translation inhibition phenotype in vivo. HuH7 and HepG2 cells were infected with RecVV-HBC, RecVV-HCC or RecVV-Δ20, and then transfected with the bicistronic pcCAT reporter RNA. The RTE was determined as described in the legend of figure 6.
et al., 2002). In similar studies, the level of expression of the HCV structural proteins was reduced by >100-fold when the expression was controlled by the HCV IRES (Greive, 2001), again suggesting that one or more of the HCV structural proteins inhibited HCV IRES-related translation. It is likely that the HCV IRES–core interaction has evolved to reduce the level of HCV replication consistent with persistent infection.

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**REFERENCES**


