ELSEVIER

A 2a/1b full-length p7 inter-genotypic chimeric genome of hepatitis C virus is infectious *in vitro*

G. Haqshenas [a,*], X. Dong [a,b], G. Ewart [c], S. Bowden [d], E.J. Gowans [a,b]

[a] *The Macfarlane Burnet Institute, GPO Box 2284, Melbourne, VIC 3001, Australia*
[b] *Department of Microbiology, Monash University, Clayton, VIC 3800, Australia*
[c] *Biotron Ltd., Canberra, Australia*
[d] *Victorian Infectious Diseases Reference Laboratory, North Melbourne, VIC 3051, Australia*

Abstract

The p7 protein of hepatitis C virus (HCV) functions as an ion channel in planar lipid bilayers, and its function is vital for the virus life cycle. In this study, we replaced either the entire or partial p7 of genotype 2a (strain JFH1), an HCV strain that replicates and produces virus progeny *in vitro*, with the corresponding regions of the p7 protein from genotype 1b (Australian isolate, HCV-A). Compared to wild type, the chimeric viruses reached their peak of infectivity with a delay but they produced a comparable titer to the wild type virus and the progeny viruses were able to infect naive permissive cells. Amantadine treatment of wild type and chimeric viruses reduced the virus titers by about 50% and 45%, respectively. Therefore, in this study, for the first time, we demonstrated that genotype 2a (JFH1 strain) genome encoding a full-length genotype 1b p7 gene produces infectious particles *in vitro*. These chimeric viruses are valuable instruments for comparative studies of the p7 proteins.
© 2006 Elsevier Inc. All rights reserved.

Keywords: p7 protein; JFH1 strain; Chimeric virus; Hepatitis C virus; Amantadine

Introduction

Hepatitis C virus (HCV) is a major etiological agent of chronic hepatitis, which can lead to hepatocellular carcinoma and is currently the leading single indicator for liver transplantation in the developed world. The threat posed to the health systems in developed and developing countries by HCV infection continues to grow at an alarming rate. In the most recent report of WHO on HCV prevalence, it has been estimated that 123 million individuals are infected with the virus worldwide.

HCV is classified in the family *Flaviviridae*, genus *Hepacivirus* and is a small enveloped virus with a positive-sense single stranded RNA genome. The genome encodes a polyprotein that is co- and post-translationally cleaved into at least 10 structural and nonstructural (NS) viral proteins (Fields et al., 1996). One of these, the p7 protein, is a small, membrane-associated protein of 63 amino acids that is located at the junction of the structural and NS proteins (Lin et al., 1994). Thus far, it has not been determined if the p7 protein is a structural or an NS protein. The protein is comprised of two tails located in the lumen of the endoplasmic reticulum (ER), two hydrophobic transmembrane α-helices, TM1 and TM2, and an intervening basic loop located in the cytoplasm (Carrere-Kremer et al., 2002). Using plasmid expression vectors, p7 has been shown to be mainly localized in the endoplasmic reticulum (Carrere-Kremer et al., 2002; Griffin et al., 2005) and possibly in mitochondrial membranes while a small percentage of the over-expressed p7 protein was detected in the plasma membrane (Griffin et al., 2004). However, we have recently shown that p7 is localized to the ER, not mitochondria, in cells transfected with a replication-competent recombinant genome of the JFH1 strain of HCV (Haqshenas et al., in press).

It has recently been shown that the p7 protein forms ion channels in planar lipid bilayers (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2004). p7 belongs to a family of proteins known as viroporins, which homo-oligomerize to form ion channels in cellular membranes (Gonzalez and Carrasco, 2003). Similar to that which has been shown for other viral ion channel forming proteins like the influenza virus proteins, M2

* Corresponding author. Fax: +61 3 92822100.
E-mail address: haqshenas@burnet.edu.au (G. Haqshenas).

(Duff and Ashley, 1992; Duff et al., 1994; Pinto et al., 1992), BM2 (Mould et al., 2003), and NB (Sunstrom et al., 1996), HIV proteins Vpu (Ewart et al., 1996) and Vpr (Piller et al., 1996), alphavirus 6K protein (Melton et al., 2002), Severe Acute Respiratory Syndrome (SARS) coronavirus (SARS-CoV) E protein (Wilson et al., 2004), GBV-B p7 (Premkumar et al., 2006) and Dengue virus M protein (Premkumar et al., 2005), the p7 protein seems to have an essential role in the HCV life cycle. In an *in vivo* study, p7 was shown to be essential for HCV replication as viral RNA with the p7 region deleted or mutated was non-infectious in chimpanzees (Sakai et al., 2003). However, p7 is dispensable for viral RNA replication as replicons which lack the p7 gene replicate efficiently (Blight et al., 2000; Lohmann et al., 1999). Therefore, it is concluded that p7 is not required for efficient HCV genome replication, but is essential for the production of infectious virions (Sakai et al., 2003).

Recently, the H77 (Yi and Lemon, 2004) and JFH1 strains of HCV genotypes of 1a and 2a, respectively, were demonstrated to replicate and produce progeny virus in cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Yi et al., 2006; Zhong et al., 2005). Very recently, chimeric JFH1 viruses encoding the genotypes 1a, 1b and 3a structural proteins, p7 and partial NS2 were also constructed (Koutsoudakis et al., 2006; Pietschmann et al., 2006). Although these cell culture systems will play a significant role to increase our understanding of the molecular biology of HCV and drug discovery, there remains a need for a cell culture system to discover ion channel inhibitors and investigate their specific effects on the p7 protein.

Currently, there are no specific targeted antiviral agents effective against HCV. The current treatment for HCV is a combination of pegylated interferon-alfa and ribavirin, which is only effective in about 50% of patients. This therapy produces considerable side effects and can lead to the emergence of resistant virus strains (Fargion et al., 2004). The p7 protein, as an ion channel forming protein, is a potential target for ion channel inhibitors. p7 ion channel activity in planar lipid bilayers is inhibited by hexamethylene amiloride (Premkumar et al., 2004), long-alkyl-chain iminosugar derivatives (Pavlovic et al., 2003) and amantadine, which also inhibited p7 activity in an hemadsorption assay (Griffin et al., 2003; Griffin et al., 2004). In clinical trials, amantadine has been shown to significantly increase the number of HCV-infected patients who showed a sustained virological response (Berg et al., 2003; Deltenre et al., 2004; Mangia et al., 2004) while other data have not supported these findings (Thuluvath et al., 2004). These contrasting findings may be a reflection of amantadine resistance of the ion channel function of p7 found in some HCV strains (Steinmann et al., 2005).

Collectively, there is an urgent demand for authentic cell culture systems to examine ion channel inhibitors on the p7 protein of different genotypes. Therefore, in the current study, we investigated if the JFH1 strain could be used as a backbone to generate chimeric viruses encoding the p7 protein of genotype 1b of HCV. Such chimeric viruses would be valuable tools to investigate the specific effects of ion channel inhibitors on the function of the p7 protein.

Results

p7 protein of HCV-A of genotype 1b has amantadine-resistant ion channel activity

Before the chimeric viruses were synthesized, we examined the ability of HCV-A.p7 to form functional ion channels. As shown in Fig. 1B, following the addition of the full-length p7 peptide to the CIS chamber of the planar lipid planar rig, ion currents were detected after brief periods of stirring. This indicates that the p7 has incorporated and formed ion channels in the lipid bilayer separating the CIS and TRANS chambers. Similarly, it was found that the N-terminal 38 residues comprised of the lumenal tail, TM1, and the cytosolic loop could form ion channels (data not shown). These data are in agreement with the computer modeling showing that only TM1 is involved in the channel formation (Patargias et al., 2006). As can be seen in Fig. 1C, amantadine at a final concentration of 100 μM did not block the ion channel activity of the protein.

The recombinant genomes are replication-competent

Before the generation of the chimeric viruses, we compared the deduced amino acid sequences of the p7 of genotype 2a (JFH1) and 1b (HCV-A). The sequences shared about 60% identity in a pairwise alignment (Fig. 2). Two chimeric JFH1 cDNA constructs were generated, one encoding the partial and the other one encoding the entire p7 gene of genotype 1b (Fig. 3), in response to a previous report that the amino acid sequences of the lumenal tails of p7 protein were crucial for E2-p7 and p7-NS2 cleavage (Sakai et al., 2003).

In order to demonstrate that the chimeric genomes were replication-competent, Huh7 cells were transfected with wild
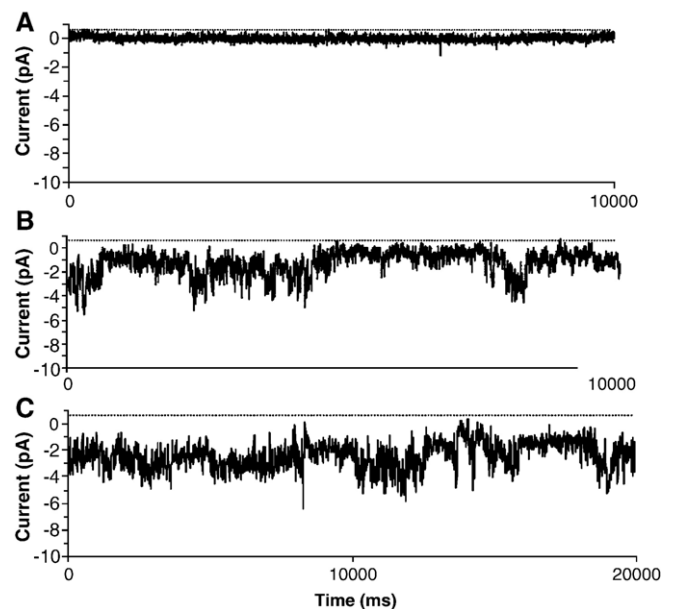

Fig. 1. Ion channel activity of the HCV-A.p7 in planar lipid bilayers. (A) The current across the lipid bilayer in the absence of protein. (B) Ion channel activity of the full-length HCV-A.p7. (C) Ion channel activity of the HCV-A.p7 protein in the presence of 100 μM amantadine.

JFH1 (gen. 2a)   ALEKLVVLHAAS AANCHGLLYFAIFFVAAWHIRGRVVPLTTYCLTGLWPF
                  ||| •|• .|..||::|.|:||.||||::||:||...•|.::.:||.
HCV-A (gen. 1b)   ALENLVVLNAASVAGAHGILSFLVFFCAAWYIKGRLVPGAAYAIYGVWPL

JFH1 (gen. 2a)   CLLLMALPRQAYA
                  .|||:||| •|||•|
HCV-A(gen. 1b)   LLLLLALPPRAYA

Fig. 2. Comparison analysis of the deduced amino acid sequences of p7 proteins of JFH1 and HCV-A isolates of HCV. The lumenal tails are shown in boxes.

type and recombinant JFH1 genomes and examined 2 to 3 days post-transfection by IF with anti-core antibody. The lysates of the infected cells were also subjected to immunoblot analysis. As shown in Fig. 4A, cells transfected with the recombinant genomes showed an intense signal by IF. The positive cells showed a core-specific punctuated cytoplasmic staining pattern as described previously (Hope et al., 2002). In Western blot analysis, a band with an approximate size of 20 kDa, consistent with the molecular weight of the core protein, was observed in the cells transfected with the wild type and chimeric genomes (data not shown). As the control GNDJFH1 mutants failed to generate any signal in either IF or Western blot, these data indicate that the recombinant genomes were replication-competent. Since the results generated from GNDJFH1 mutant were identical to those from G-Fp7 and G-Pp7, in the next steps, we only used GNDJFH1 as a control.

The chimeric genomes produce infectious particles

After transfection, the Huh7 cells were passaged to determine if they were persistently infected. After each passage, the cells were examined for core protein by IF. At the first passage, the proportion of core antigen-positive cells in the culture transfected with the wild type RNA slightly increased (from 40–50% to about 60–70%) while the proportion of positive cells in the culture transfected with the chimeric genomes decreased slightly (from 40–50% to about 30–40%, data not shown). High numbers of non-adherent cells were noted continuously in the culture transfected with the wild type RNA, while the Pp7 and Fp7-transfected cells failed to show a CPE until passage 3. This is consistent with the IF data showing that the percentage of core antigen-positive cells in the wild type-transfected culture remained at 90–100% until passage 5 and then declined sharply to 10–20%. The cells became totally negative by passage 6. To investigate if a virus-resistant cell subset dominated during the course of infection, we re-exposed the cells to the virus and showed that the cells were successfully re-infected (data not shown). In the Pp7- and Fp7-transfected cells, the proportion of

positive cells declined to about 30% in passage 2 but increased significantly in passage 3 (about 70–80% positive, data not shown). About 30–50% of the cells were still positive for core antigen at passage 7 when the experiment was concluded. To determine if the supernatants of the cells were infectious, naive Huh7 cells were infected with the supernatant fluid containing the highest viral titers. Three days post-infection, the cells were examined by anti-core-antigen IF test and shown to be positive (Fig. 4A). The lysates of the infected cells were also subjected to Western blot analysis and showed a band with an approximate size of 20 kDa in the cells transfected with the wild type and chimeric genomes (Fig. 4B).

We also examined the viral RNA titer in the cell culture supernatant of each passage. As shown in Fig. 5A, the titer of the wild type viral RNA in the supernatant increased at passage one and remained high during the next passages before it gradually decreased to undetectable levels at passage 7. In the case of the chimeric genomes, the virus titer declined slightly in passage 2 but increased in passage 3–4 and remained at this level until the end of the experiment (Fig. 5A). The chimeric viruses did not reach the peak titer of wild type at any time point showing that the chimera replicated with slower kinetics than the wild type. The maximum titers of the wild type JFH1, P-p7 and F-p7 that were achieved in this study were 7.4×10^7, 3.6×10^7 and 3.5×10^7 viral RNA copies/ml, respectively. The specific infectivity of the wild type and F-p7 was calculated as 2900 and 1600 RNA copies per FFU, respectively. We also determined the infectivity titers of the wild type and the chimeric viruses of the supernatants up to passage 6 post-transfection. Consistent with the results from the viral RNA titers, the wild type virus reached its peak in early passages while Pp7 and Fp7 chimeras reached their highest infectivity titers at passage 3 and 5, respectively (Fig. 5B). Immunoblot analysis of the cells transfected with wild type and chimeric genomes, and the cells infected with the wild type and chimeric viruses did not reveal a major difference in the pattern of proteins that reacted with the monoclonal antibody AP33 (Fig. 6). This suggests that the cleavage of E2 from its precursor protein was not affected by the insertion and did not change during passaging the transfected cells.

The heterologous p7 gene in the chimeric viruses is stable

RNA viruses commonly introduce compensatory mutations in the heterologous genes to increase viral fitness. To investigate if any mutation occurred in the HCV-A.p7 genes in the backbone of JFH1, we examined the nucleotide sequences of
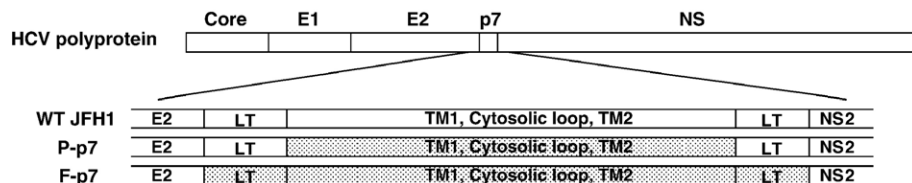

Fig. 3. Schematic representation of the wild type and chimeric JFH1 polyproteins. The p7 protein is comprised of two luminal tails (LT) and two transmembrane domains (TM1 and TM2) and an intervening cytosolic loop. Hatched boxes indicate the JFH1 p7 sequences that were replaced by the corresponding regions in HCV-A.p7 to generate the partial (P-p7) and complete (F-p7) JFH1/HCV-A.p7 chimeras.
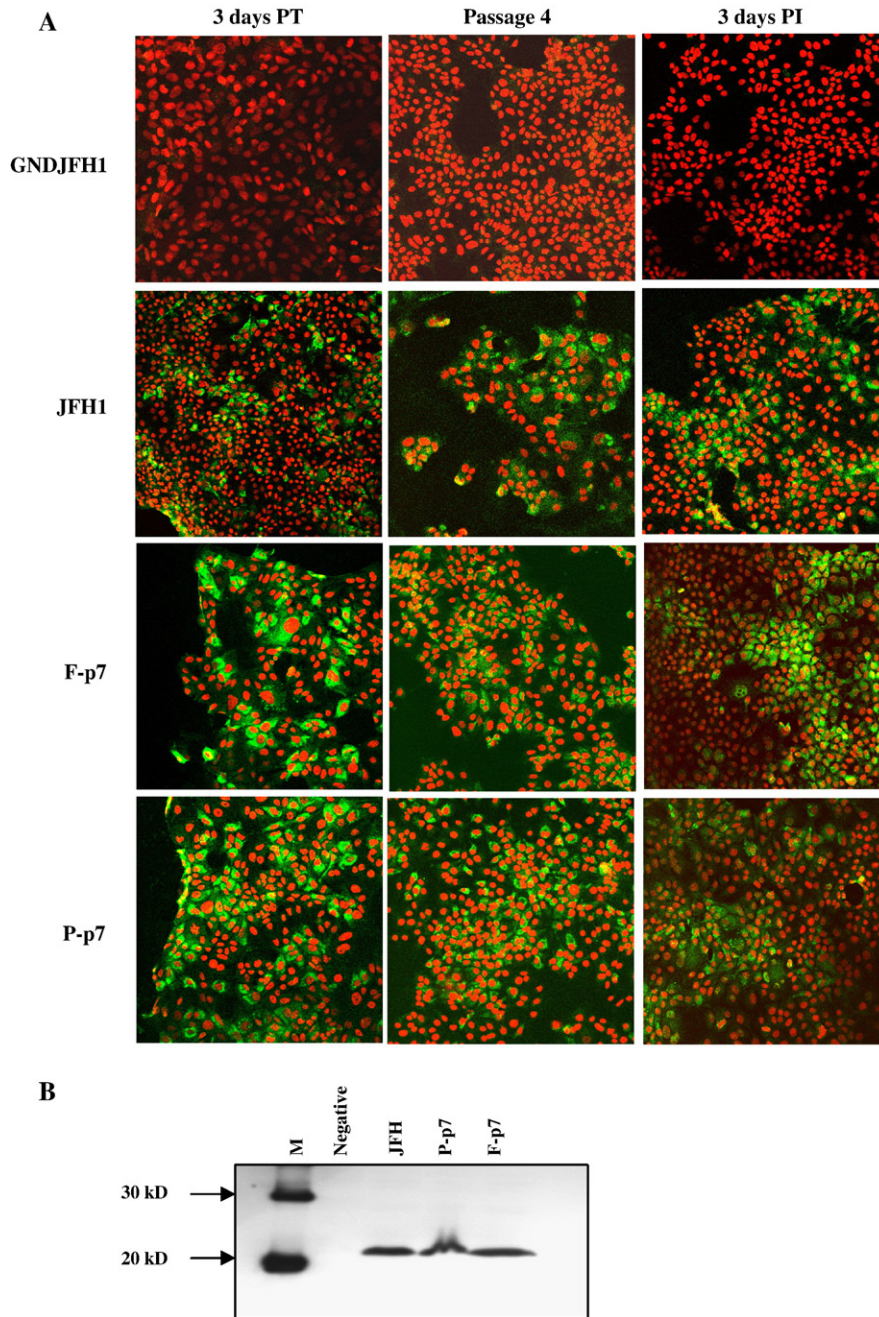
Fig. 4. Wild type and chimeric genomes were replication-competent in transfected cells and produced infectious progeny. (A) Huh7 cells transfected with the *in vitro* synthesized transcripts of GNDJFH1 (top panels), wild type JFH1 (second from top), mutant JFH1 encoding the entire HCV-A.p7 (F-p7, third from top) and mutant JFH1 encoding the partial HCV-A.p7 (P-p7, bottom). Three days post-transfection, the cells were stained with anti-core antibody and counterstained with propidium iodide. (B) Detection of HCV core protein by immunoblot analysis. The Huh7 cells were infected with the wild type and chimeric viruses. At day 3 post-infection, the mock-infected and infected cells were directly lysed in 2× Laemmli buffer and subsequently subjected to SDS-PAGE in 12% gel. Negative: mock infected cells, JFH1: wild type virus, P-p7: JFH1 encoding the partial HCV-A.p7, F-p7: JFH1 encoding the entire HCV-A.p7, PT: post-transfection, PI: post-infection, M: MagicMark protein size marker (Invitrogen).

the nascent recombinant viruses. The viral RNA was extracted from the cell culture supernatant of the passage 5 culture and was subsequently used in a one step RT-PCR with genome-specific primers, FE2 and RNS2 (Table 1). Agarose gel electrophoresis of the PCR products from the chimeras showed one distinct band with an electrophoretic mobility similar to that of the product from the wild type virus (Fig. 7A). Nucleotide sequencing of the products did not reveal any mutations in the

sequence encoding p7 and flanking regions in the chimeric viruses (Figs. 7B, C).

Amantadine reduced the wild type and chimeric virus titer in the supernatant

It has been reported that the strain J4 of genotype 1b is sensitive to amantadine (Griffin et al., 2003) while the JFH1
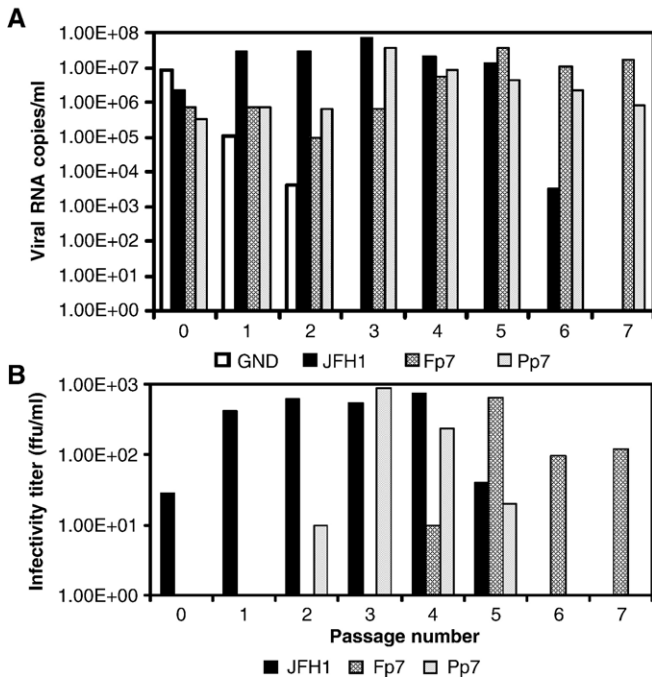
Fig. 5. Production of infectious HCV after transfection of the full-length RNA transcripts of wild type and chimeric JFH1 into Huh7 cells RNA. Using DMRIE-C (Invitrogen), 4 μg of the transcripts were transfected into Huh7 cells. The supernatants were harvested at the indicated time points post-transfection. (A) The virus titers in the supernatants were determined by a third-generation HCV bDNA 3.0 assay (Bayer Diagnostics) as described in Materials and methods. (B) The infectivity of the supernatants corresponding to the top figure was determined, and the values were presented as focus forming unit (FFU) per ml. GND: replication defective construct of JFH1, JFH1: JFH1 wild type, P-p7: chimeric JFH1 genome encoding the partial HCV-A.p7, F-p7: chimeric JFH1 genome encoding the entire HCV-A.p7.

strain of genotype 2a is resistant to this drug (Steinmann et al., 2005). We investigated whether the full-length p7 chimera (F-p7) with an identical TM1 to strain J4 is sensitive to amantadine. As shown in Fig. 8, 30 μM amantadine reduced the wild type and chimeric virus titer in the supernatant at day 3 post-infection by about 0.3 and 0.25 log, respectively. This effect was not seen at day 1 post-infection and was not due to amantadine-induced cytotoxicity, as determined by the MTT assay (data not shown).

Discussion

This study describes the generation of two novel chimeric viruses in which the partial or the entire p7 protein gene of the JFH1 strain of HCV was substituted with the corresponding gene from a genotype 1b virus (HCV-A). These novel chimeric viruses are valuable tools for comparative studies of the function of p7 in an authentic cell culture system and for the examination of the effects of potential ion channel inhibitors in vitro.

p7 protein is a small protein of HCV that is highly membrane associated. The protein is comprised of two lumenal tails, two TM domains with an intervening cytosolic loop. The lumenal tails at the N- and C-termini of the protein were previously reported to contain genotype-specific sequences since the

chimeric 1a/2a virus was infectious only when the lumenal tail sequences of the backbone genome of genotype 1a were left unaltered in the chimeric genome (Sakai et al., 2003). Differences in the amino acids of the N-terminal lumenal tails of genotype 1a (H77C) and genotype 2a (J6) p7 proteins are restricted to positions 4 and 9. In H77C, there are two polar amino acids of asparagines at positions 4 and 9 while in J6 there are two charged amino acids of lysine and histidine at the corresponding positions (Sakai et al., 2003). The J4 p7, like the HCV-A.p7, contains two asparagines at positions 4 and 9 while JFH-1 p7, like J6 p7, contains a lysine and a histidine residue at the corresponding positions, respectively. Moreover, the differences between JFH1 and HCV-A in the C-terminal lumenal tail are greater than the differences between J6 and H77C (Sakai et al., 2003). Nevertheless, in the present study, despite these differences, the full-length JFH1/HCV-A.p7 chimera was infectious and produced infectious particles. This might reflect the difference between an in vivo and an in vitro system to examine the chimeric HCV genomes. To further examine the possibility of the existence of genotype-specific sequences in the lumenal tails, the chimeric 1a/2a genomes can now be examined in an in vitro assay since a cell culture system is now available to grow genotype 1a and its chimeric viruses (Yi et al., 2006). In this study, although the chimeric genomes produced infectious viruses with comparable titers to wild type, compared to the wild type, the chimeric viruses reached their peak of infectivity with a delay. Sequencing analysis of the insert regions did not reveal any compensatory mutation in the p7 and flanking regions. Moreover, the immunoblot analysis did not demonstrate a major difference in the processing of the E2-p7-NS2 precursor proteins of the wild type and chimeras following either transfection or infection. However, it is likely that a compensatory mutation occurred in the viral genome, outside of the p7 and flanking regions, which made the chimeric viruses more replication-competent.

Until recently, HCV studies were hindered by the lack of a robust cell culture system to grow the virus in vitro. Recently, Wakita et al. (2005) reported a productive infection of Huh7 cells with the JFH1 isolate of genotype 2a. Since then, Lindenbach et al. (2005) generated a JFH1/J6 chimeric virus, containing structural genes of J6 and NS genes of JFH1, and demonstrated that the cells were productively infected with the chimera. Very recently, Yi et al., (2006) demonstrated a complete replication cycle of genotype 1a of HCV in cell culture. More recently,
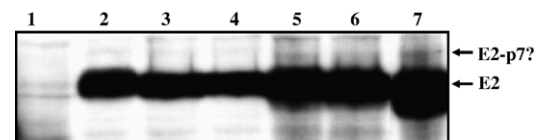

Fig. 6. Immunoblot analysis of the cells transfected with the wild type and chimeric genomes or infected with the wild type and chimeric viruses. The same number of cells was used in the transfection and infection experiments. 150 μl of Laemmli buffer was added to each well of a 6-well plate, and 12 μl of each sample was analyzed onto a 10% SDS-PAGE gel. The monoclonal antibody AP33 was used to detect the E2 protein, 1: mock-transfected cells, 2: JFH1-transfected cells, 3: Pp7-transfected cells, 4: Fp7-transfected cells, 5: JFH1 infected cells, 6: Pp7 infected cells, 7: Fp7 infected cells.

Table 1

Name	Position [a]	Nucleotide sequence
FE2	2257–2276	GGTTGAGCACAGGCTCACGG
RNS2	2979–2998	CGGGCAGAATATAGTGACGG
F-FAus.p7		**ACCACCACGGGCATACGCC**TATGACGCACCTGTGCACGG
R-FAUS.p7		**GGACCACCAGGTTCTCTAAGGC**TGCTTCGGCCTGGCCCAAC
F-pAUS.p7		**CTGCTCCTGCTGGCGCTACCA**CGGCAGGCTTATGCCTATGAC
R-pAUS.p7		**AATGCCATGTGCTCCGGCCAC**ACTCGCAGCGTGCAAGACG
M13 rev. primer		CAGGAAACAGCTATGAC

HCV-A.p7 related sequences are in bold.
[a] Nucleotide positions are referred to full-length JFH1 genome (accession number: AB047639).

during the preparation of this manuscript, chimeric JFH1 viruses encoding the genotype 1a, 1b and 3a structural proteins, p7 and partial NS2 were also constructed (Koutsoudakis et al., 2006; Pietschmann et al., 2006). Despite the importance of these reports, the lack of a cell culture system to support the replication of all the HCV genotypes remains a major obstacle to study different HCV genotypes and develop antiviral drugs that are effective against all genotypes. Moreover, in the current cell culture systems, it is very difficult to justify the specific effects of any ion channel inhibitors on the function of p7 protein. Therefore, in the present study, based on the JFH1 strain, we generated two novel chimeric viruses that encode only the genotype 1b p7 gene to facilitate the comparative study of the ion channel activity of the genotype 1 and 2 p7 proteins since genotype 1 is responsible for a high proportion of HCV-infected patients in most geographic areas. In most Asian countries, genotype 1b is predominant while in the United States and Europe genotype 1a and 1b are predominant (Bukh et al., 1995; Lau et al., 1996). The ion channel activity of genotype 1b p7 protein (Yanagi et al., 1998) has been shown to be sensitive to amantadine (Griffin et al., 2004). The amino acid sequence of the

J4 isolate only differs in two amino acid positions to the HCV-A p7: one in the cytosolic loop and one in TM2 (Fig. 9). Using computer modeling and electron microscopy, it has been proposed that only TM1, but not TM2, is involved in ion channel formation (Carrere-Kremer et al., 2002; Griffin et al., 2003; Patargias et al., 2006). We have also examined the N-terminal subunit of p7 for ion channel activity and confirmed the previous reports by showing that the N-terminal subunit of p7 *per se*, independent from the C-terminal subunit, forms ion channel in an artificial lipid bilayer (unpublished data). Furthermore, J4 p7 has been shown to be sensitive to amantadine in an artificial lipid bilayer and also in an *in vitro* system (Griffin et al., 2003; Griffin et al., 2004). Computer modeling of the p7 protein of J4 strain demonstrated that amantadine resides within the pore, formed by oligomerization of TM1, in the vicinity of His-17 and interacts with Ser-21 via hydrogen bonding (Patargias et al., 2006). Therefore, we examined the sensitivity of the full-length chimeric virus, containing an identical N-terminal subunit to the J4 strain p7 protein, for sensitivity to amantadine. This study demonstrated that amantadine has a similar effect on the life cycles of the chimeric (encoding the full-
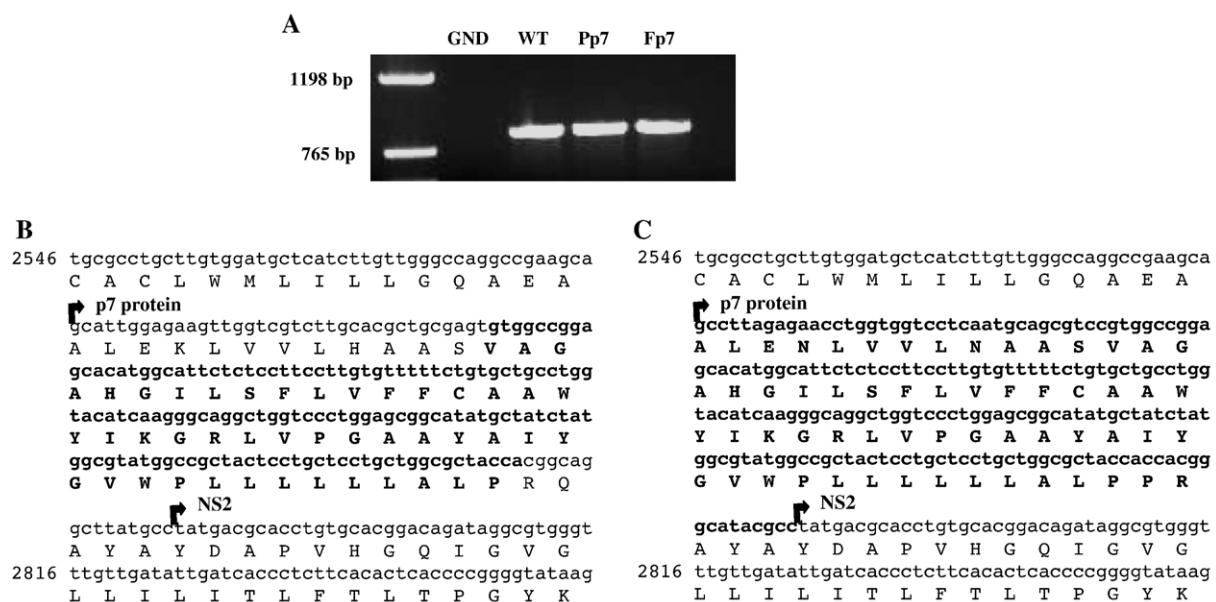

Fig. 7. The p7 proteins in the chimeric viruses are stable. (A) The RNA was extracted from passage 4 of the wild type and the chimeric viruses containing partial p7 (Pp7) and the entire p7 (Fp7) proteins and subjected to RT-PCR amplification. The PCR products were visualized in a 1% agarose gel. (B and C) The PCR products were then sequenced, and the depicted amino acid sequences have been shown.
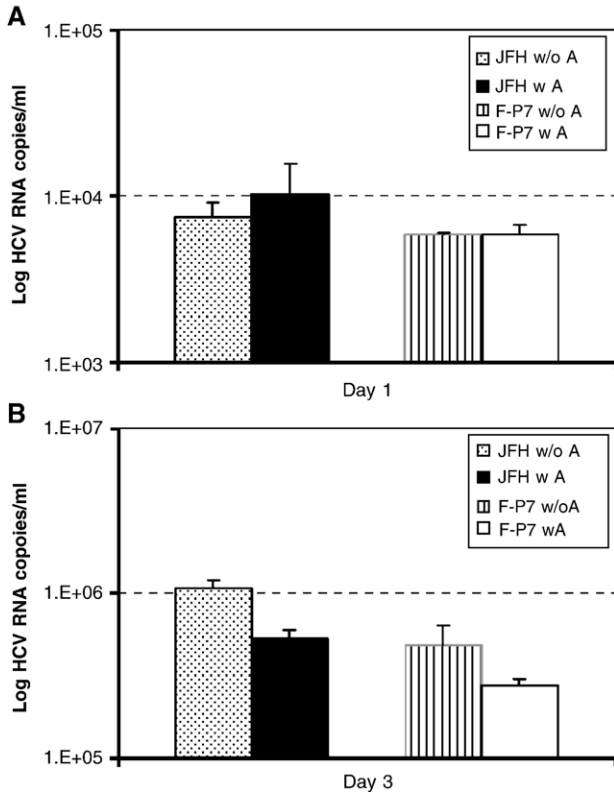
Fig. 8. The effects of amantadine on the replication of wild type and chimeric JFH1 (F-p7) viruses. Amantadine at a final concentration of 30 µM was added to the cell culture medium of Huh7 cells 1 h prior to and during the infection. Supernatant samples were collected at day 1 and 3 post-infection as described in Materials and methods. The virus titer was determined by a third-generation HCV bDNA 3.0 assay (Bayer Diagnostics) as described in Materials and methods. W-A: with amantadine, Wo-A: without amantadine.

length HCV-A.p7) and wild type viruses as determined by the titer of the virus secreted into the supernatant. However, a much larger reduction in the titer of the chimeric virus was expected, due to the previous published data (see above). We also demonstrated that the ion channel activity of the HCV-A (genotype 1b) p7 protein was resistant to amantadine in artificial lipid bilayers. The discrepancy between our results on HCV-A (genotype 1b) and the published data on J4 p7 (genotype 1b) may suggest a role for the cytosolic loop and TM2 in the response to amantadine. To examine this, we are currently in the process of changing the two amino acids to convert HCV-A.p7 to J4 p7. Although the results of the amantadine effect on JFH1 wild type are in contrast to previously published data (Steinmann et al., 2005), they are in agreement with the more recently

published data showing that amantadine reduced the number of JFH1 foci by about 70% in cell culture (Boume et al., 2005). The mechanism of the effect of amantadine on the HCV life cycle is not clear yet. However, the results presented in this manuscript suggest that this mechanism is far more complex than simply blocking ion channel pores. It has been documented that the infection of cells with porcine circovirus type 2 that does not contain any ion channel forming protein is inhibited by amantadine through inhibition of clathrin-mediated endocytosis (Nauwynck et al., 1999). HCV, like porcine circovirus type 2, enters the cells via clathrin-mediated endocytosis (Blanchard et al., 2006) and, therefore, the effect of amantadine on the HCV life cycle might partially be through the blocking of this function. Collectively, because the only difference between the wild type and the chimera is the p7 protein and irrespective of the sensitivity of p7 to amantadine, the drug has almost the same effect on the viral life cycle, it is possible that this effect is not through the inhibition of p7 protein.

In conclusion, we believe that the novel chimeric viruses described in this manuscript are valuable tools for the study of the function of the p7 protein and also to specifically examine the effects of potential ion channel inhibitors in a genuine cell culture system.

Materials and methods

Cells and plasmids

Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (P.A. Biologicals Co., Melbourne, Australia), 100 IU of penicillin/ml and 100 µg of streptomycin/ml. The wild type and replication-defective mutant full-length JFH1 cDNA constructs were kindly provided by Dr. Wakita (Wakita et al., 2005). The full-length Australian HCV cDNA (HCV-A) was constructed in our laboratory (Trowbridge and Gowans, 1998). E. coli strain JM109 (Promega, Madison, Wis) was used in all transformation experiments.

Examination of HCV-A.p7 for ion channel activity and amantadine sensitivity

A synthetic p7 peptide, based on the sequence of HCV-A, was synthesized at GenScript Corporation (NJ, USA) and tested in an planar lipid bilayers, as described previously (Premkumar et al., 2004), to determine if it has ion channel activity. Similarly, a peptide was synthesized corresponding to the N-terminal 38
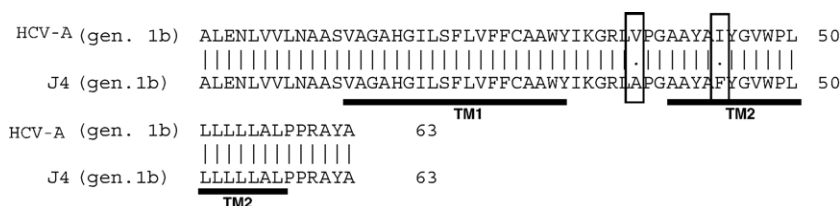

Fig. 9. Comparison analysis of the deduced amino acid sequences of p7 proteins of J4 and HCV-A isolates of HCV. TM1 and TM2 are underlined (Sakai et al., 2003).

residues (ALENLVVLNAASVAGAHGILSFLVFFCAAW-YIKGRLVP) and was also examined for ion channel activity. Amantadine sensitivity of the p7 ion channel was determined by using 100 μM amantadine as described before (Premkumar et al., 2004).

Construction of the recombinant cDNA

The full-length wild type and defective GND mutant of JFH1 were digested with XbaI and NotI restriction enzymes, blunt ended and re-circularized using standard protocols (Sambrook et al., 1989). The recombinant plasmids were called wtJFH-N and GJFH-N, respectively. Two chimeric JFH1/HCV-A.p7 fragments were then synthesized by fusion PCR, using the primers shown in Table 1. In the first chimera, the entire p7 gene of JFH1 was substituted by the HCV-A.p7. In the second, the partial p7 protein (TM1, TM2 and cytosolic loop) of JFH1 was replaced by the corresponding region of HCV-A to ensure that the lumenal tails of the p7 protein of JFH1 remained unchanged. The chimeric fragments were then digested with SphI and cloned into the wtJFH-N and GJFH-N plasmids digested with the corresponding enzyme to create 5′JFH-Fp7 and 5′JFH-Pp7 (containing the full-length and partial HCV-A.p7 genes), respectively. These constructs were then digested with EcoRI and KpnI, and the released fragments containing IRES, core, E1, E2, partial or full-length HCV-A.p7 and NS2 were cloned into wtJFH1 and mutant GNDJFH1 (digested with the corresponding enzymes). The resultant recombinant JFH1 constructs were named J-Fp7 (JFH1 encoding full-length HCV-A.p7), G-Fp7 (GNDJFH1 encoding full-length HCV-A.p7), J-Pp7 (JFH1 encoding partial HCV-A.p7) and G-Pp7 (GNDJFH1 encoding partial HCV-A.p7). The nucleotide sequences of the fragments were confirmed by using FE2 and RNS2 primers (Table 1) in an automated cycle sequencing reaction.

In vitro RNA transcription and transfection

RNA transcripts were synthesized as described (Huang et al., 2005). To examine the replication competency of the recombinant genomes, 4 μg of in vitro generated RNA transcripts was transfected into cells in 6-well plates (Nalge Nunc, Rochester, NY) using 10 μl of DMRIE-C (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The transfection mixture was removed 5 h later, the cells washed twice with DMEM and complete medium added. The cells were trypsinized and passaged every 3–4 days for 7 passages as described previously (Zhong et al., 2005).

Immunofluorescence (IF) and immunoblot analysis

The transfected and infected cells were fixed with cold acetone for IF analysis then stained with goat anti-HCV core antibody (ViroStat, Portland, ME) followed by Alexa Fluor 488-conjugated donkey anti-goat (Molecular Probes). The cells were then counterstained with propidium iodide and examined with a BioRad confocal microscope.

For immunoblot analysis, the cells were directly lysed in 2× Laemmli buffer (BioRad, Hercules, CA). Goat anti-HCV core antibody and the monoclonal antibody AP33 (Owsianka et al., 2005) were used to detect the HCV core and E2 proteins, respectively. ECL Western blotting detection reagents (Amersham Pharmacia Biosciences) were used according to the manufacturer's instructions to detect the immunocomplexes.

Quantification of HCV RNA

The supernatant of each passage was filtered through a 0.45 μM filter and stored at −70 °C. From each passage, 75 μl of the supernatant fluids was collected and 50 μl of aliquots used for quantification. HCV viral loads were determined using the Versant HCV RNA 3.0 bDNA assay (Bayer HealthCare, Tarrytown, NY) according to the manufacturer's instructions.

Amantadine treatment of wild type and chimeric viruses infected cells

Overnight cultures of Huh7 cells were treated with amantadine (Sigma) at final concentrations of 30 μM for 1 h that was not toxic for the cells in an MTT-based cytotoxicity assay (data not shown). The cells were then infected with the wild type and chimeric JFH1 samples containing 3.5×10^6 and 2.5×10^6 RNA copies/ml, respectively, in the presence of amantadine for 5 h. The cells were washed twice with DMEM, and fresh medium containing 30 μM amantadine was added. To determine whether amantadine has any effect on the progeny virus production, the cells were grown for 3 days and 200 μl samples collected on day 1 and 3 post-infection for quantification of HCV RNA. After each sampling, a same volume of fresh medium containing amantadine was added to each culture.

References

Berg, T., Kronenberger, B., Hinrichsen, H., Gerlach, T., Buggisch, P., Herrmann, E., Spengler, U., Goeser, T., Nasser, S., Wursthorn, K., Pape, G.R., Hopf, U., Zeuzem, S., 2003. Triple therapy with amantadine in treatment-naive patients with chronic hepatitis C: a placebo-controlled trial. Hepatology 37 (6), 1359–1367.

Blanchard, E., Belouzard, S., Goueslain, L., Wakita, T., Dubuisson, J., Wychowski, C., Rouillé, Y., 2006. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J. Virol. 80 (14), 6964–6972.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290 (5498), 1972–1974.

Boume, N., Veselenak, R.L., Pyles, R.B., Yi, M.K., 2005. Amantadine reduces infectious hepatitis C virus (HCV) focus formation but not replication of RNA from different HCV genotypes in cell-based assays. Global Antiv. J. 1, 11–15.

Bukh, J., Miller, R.H., Purcell, R.H., 1995. Genetic heterogeneity of the hepatitis C virus. Princess Takamatsu Symp. 25, 75–91.

Carrere-Kremer, S., Montpellier-Pala, C., Cocquerel, L., Wychowski, C., Penin, F., Dubuisson, J., 2002. Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. J. Virol. 76 (8), 3720–3730.

Deltenre, P., Henrion, J., Canva, V., Dharancy, S., Texier, F., Louvet, A., De Maeght, S., Paris, J.C., Mathurin, P., 2004. Evaluation of amantadine in chronic hepatitis C: a meta-analysis. J. Hepatol. 41 (3), 462–473.

Duff, K.C., Ashley, R.H., 1992. The transmembrane domain of influenza A M2 protein forms amantadine-sensitive proton channels in planar lipid bilayers. Virology 190 (1), 485–489.

Duff, K.C., Gilchrist, P.J., Saxena, A.M., Bradshaw, J.P., 1994. Neutron diffraction reveals the site of amantadine blockade in the influenza A M2 ion channel. Virology 202 (1), 287–293.

Ewart, G.D., Sutherland, T., Gage, P.W., Cox, G.B., 1996. The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. J. Virol. 70 (10), 7108–7115.

Fargion, S., Fracanzani, A.L., Valenti, L., 2004. Treatment choices for people infected with HCV. J. Antimicrob. Chemother. 53 (5), 708–712.

Fields, B.N., Knipe, D.M., Howley, P.M., 1996. Fields Virology. Lippincott-Raven Publishers, Philadelphia, PA. (Eds.).

Gonzalez, M.E., Carrasco, L., 2003. Viroporins. FEBS Lett. 552 (1), 28–34.

Griffin, S.D., Beales, L.P., Clarke, D.S., Worsfold, O., Evans, S.D., Jaeger, J., Harris, M.P., Rowlands, D.J., 2003. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, amantadine. FEBS Lett. 535 (1–3), 34–38.

Griffin, S.D., Harvey, R., Clarke, D.S., Barclay, W.S., Harris, M., Rowlands, D.J., 2004. A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria. J. Gen. Virol. 85 (2), 451–461.

Griffin, S., Clarke, D., McCormick, C., Rowlands, D., Harris, M., 2005. Signal peptide cleavage and internal targeting signals direct the hepatitis C virus p7 protein to distinct intracellular membranes. J. Virol. 79 (24), 15525–15536.

Haqshenas, G., Mackenzie, J.M., Dong, X., and Gowans E.J., in press. The HCV p7 protein is localised in the endoplasmic reticulum when it is encoded by a replication competent genome. J. Gen. Virol.

Hope, R.G., Murphy, D.J., McLauchlan, J., 2002. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. J. Biol. Chem. 277 (6), 4261–4270.

Huang, Y.W., Haqshenas, G., Kasorndorkbua, C., Halbur, P.G., Emerson, S.U., Meng, X.J., 2005. Capped RNA transcripts of full-length cDNA clones of swine hepatitis E virus are replication competent when transfected into Huh7 cells and infectious when intrahepatically inoculated into pigs. J. Virol. 79 (3), 1552–1558.

Koutsoudakis, G., Kaul, A., Steinmann, E., Kallis, S., Lohmann, V., Pietschmann, T., 2006. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J. Virol. 80 (11), 5308–5320.

Lau, J.Y., Davis, G.L., Prescott, L.E., Maertens, G., Lindsay, K.L., Qian, K., Mizokami, M., Simmonds, P., 1996. Distribution of hepatitis C virus genotypes determined by line probe assay in patients with chronic hepatitis C seen at tertiary referral centers in the United States. Hepatitis Interventional Therapy Group. Ann. Intern. Med. 124 (10), 868–876.

Lin, C., Lindenbach, B.D., Pragai, B.M., McCourt, D.W., Rice, C.M., 1994. Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. J. Virol. 68 (8), 5063–5073.

Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309 (5734), 623–626.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285 (5424), 110–113.

Mangia, A., Leandro, G., Helbling, B., Renner, E.L., Tabone, M., Sidoli, L., Caronia, S., Foster, G.R., Zeuzem, S., Berg, T., Di Marco, V., Cino, N., Andriulli, A., 2004. Combination therapy with amantadine and interferon in naive patients with chronic hepatitis C: meta-analysis of individual patient data from six clinical trials. J. Hepatol. 40 (3), 478–483.

Melton, J.V., Ewart, G.D., Weir, R.C., Board, P.G., Lee, E., Gage, P.W., 2002. Alphavirus 6K proteins form ion channels. J. Biol. Chem. 277 (49), 46923–46931.

Mould, J.A., Paterson, R.G., Takeda, M., Ohigashi, Y., Venkataraman, P., Lamb, R.A., Pinto, L.H., 2003. Influenza B virus BM2 protein has ion channel activity that conducts protons across membranes. Dev. Cell 5 (1), 175–184.

Nauwynck, H.J., Duan, X., Favoreel, H.W., Van Oostveldt, P., Pensaert, M.B., 1999. Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis. J. Gen. Virol. 80 (2), 297–305.

Owsianka, A., Tarr, A.W., Juttla, V.S., Lavillette, D., Bartosch, B., Cosset, F.L., Ball, J.K., Patel, A.H., 2005. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J. Virol. 79 (17), 11095–11104.

Patargias, G., Zitzmann, N., Dwek, R., Fischer, W.B., 2006. Protein–protein interactions: modeling the hepatitis C virus ion channel p7. J. Med. Chem. 49 (2), 648–655.

Pavlovic, D., Neville, D.C., Argaud, O., Blumberg, B., Dwek, R.A., Fischer, W.B., Zitzmann, N., 2003. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc. Natl. Acad Sci. U.S.A. 100 (10), 6104–6108.

Pietschmann, T., Kaul, A., Koutsoudakis, G., Shavinskaya, A., Kallis, S., Steinmann, E., Abid, K., Negro, F., Dreux, M., Cosset, F.L., Bartenschlager, R., 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Natl. Acad Sci. U.S.A. 103, 7408–7413.

Piller, S.C., Ewart, G.D., Premkumar, A., Cox, G.B., Gage, P.W., 1996. Vpr protein of human immunodeficiency virus type 1 forms cation-selective channels in planar lipid bilayers. Proc. Natl. Acad Sci. U.S.A. 93 (1), 111–115.

Pinto, L.H., Holsinger, L.J., Lamb, R.A., 1992. Influenza virus M2 protein has ion channel activity. Cell 69 (3), 517–528.

Premkumar, A., Wilson, L., Ewart, G.D., Gage, P.W., 2004. Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. FEBS Lett. 557 (1–3), 99–103.

Premkumar, A., Horan, C.R., Gage, P.W., 2005. Dengue virus M protein C-terminal peptide (DVM-C) forms ion channels. J. Membr. Biol. 204 (1), 33–38.

Premkumar, A., Dong, X., Haqshenas, G., Gage, P.W., 2006. Amantadine inhibits the function of an ion channel encoded by GB virus-B but fails to inhibit virus replication. Antiv. Ther. 11 (3), 289–295.

Sakai, A., Claire, M.S., Faulk, K., Govindarajan, S., Emerson, S.U., Purcell, R.H., Bukh, J., 2003. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. Proc. Natl. Acad Sci. U.S.A. 100 (20), 11646–11651.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual 2nd ed.

Steinmann, E., Koutsoudakis, G., Kallis, S., Zitzmann, N., Saint, N., Penin, F., Pieschmann, T., Bartenschlager, R., 2005. Analysis of amantadine and iminosugar derivates as potential inhibitors of the HCV life cycle. Abstract P-292. 12th International Symposium on HCV and Related Viruses. Montreal, Canada.

Sunstrom, N.A., Premkumar, L.S., Premkumar, A., Ewart, G., Cox, G.B., Gage, P.W., 1996. Ion channels formed by NB, an influenza B virus protein. J. Membr. Biol. 150 (2), 127–132.

Thuluvath, P.J., Maheshwari, A., Mehdi, J., Fairbanks, K.D., Wu, L.L., Gelrud, L.G., Ryan, M.J., Anania, F.A., Lobis, I.F., Black, M., 2004. Randomised, double blind, placebo controlled trial of interferon, ribavirin, and amantadine versus interferon, ribavirin, and placebo in treatment naive patients with chronic hepatitis C. Gut 53 (1), 130–135.

Trowbridge, R., Gowans, E.J., 1998. Molecular cloning of an Australian isolate of hepatitis C virus. Arch. Virol. 143 (3), 501–511.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

Wilson, L., McKinlay, C., Gage, P., Ewart, G., 2004. SARS coronavirus E protein forms cation-selective ion channels. Virology 330 (1), 322–331.

Yanagi, M., St Claire, M., Shapiro, M., Emerson, S.U., Purcell, R.H., Bukh, J., 1998. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. Virology 244 (1), 161–172.

Yi, M., Lemon, S.M., 2004. Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells. J. Virol. 78 (15), 7904–7915.

Yi, M., Villanueva, R.A., Thomas, D.L., Wakita, T., Lemon, S.M., 2006. From the cover: production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad Sci. U.S.A. 103 (7), 2310–2315.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad Sci. U.S.A. 102, 9294–9299.