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The Entire Core Protein of HCV JFH1 is Required for Efficient Formation of Infectious JFH1 Pseudoparticles

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Abstract

The vast majority of hepatitis C virus (HCV) strains cannot be grown in cell culture. Therefore, tests for neutralizing antibodies have relied heavily on retrovirus pseudoparticles displaying the envelope glycoproteins of HCV on their surface (HCVpp). Unfortunately, the envelope proteins of some strains, especially of JFH1, did not efficiently form functional HCVpp. We have manipulated the length and composition of the HCV core gene in the HCVpp expression vectors for 3 strains of HCV in an attempt to obtain more efficient production of pseudoparticles. The results demonstrated that the truncated core region included in the HCV expression plasmids of the classic pseudoparticle system was optimal for formation of strain H77pp, suboptimal for strain J6pp and insufficient for strain JFH1pp. Efficiency of JFH1pp formation increased 20-fold when the truncated core gene was replaced with the entire core gene. The full core from J6 and HK had modest effect on the production of infectious J6 and HK pp. The data suggested that pairs of HCV glycoproteins differ inherently in their ability to associate into functional heterodimers and that the core protein, provided *in cis* as the beginning of the polyprotein product, can in some cases facilitate this process, possibly by increasing the rate of proper folding of the glycoproteins.

INTRODUCTION

Hepatitis C is a serious disease noted for its extremely high rate of chronicity; currently over 170 million people worldwide have chronic hepatitis C. In contrast to HIV infections, which also exhibit a high rate of chronicity, the immune system remains functional in hepatitis C. In fact, HCV persists in the presence of high levels of antibodies to the two viral glycoproteins, E1 and E2 that are believed to comprise the viral receptor [Abrignani, 1997; Chang et al., 1997; Eckels et al., 1996]. For a long time, questions of why antibodies to the glycoproteins did not eliminate the virus were difficult to study since a cell culture system did not exist and the only animal model, the chimpanzee, was prohibitively expensive. This changed with the recent development of the HCVpp and a comparable cell culture (HCVcc) system which provided a practical means to identify and characterize antibodies that reacted with the E1 and E2 glycoproteins of HCV [Bartosch et al., 2003; Hsu et al., 2003; Zhong et al., 2005; Wakita et al., 2005; Lindenbach et al., 2005].

HCV is an enveloped RNA virus belonging to the genus *Hepacivirus* of the family *Flaviviridae* [Lindenbach & Rice, 2001]. Its positive-sense genome of 9.6 kb encodes a single polyprotein that is co- and posttranslationally processed by cellular and viral

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proteases to yield 3 structural and 7 nonstructural proteins [Penin et al., 2004]. The structural proteins are located at the N-terminus and are translated in the following order: capsid or core (c), E1 and then E2. Core consists of 191aa and its C-terminal 20aa serve as a signal sequence for E1 [Lo et al., 1995]. The HCVpp systems developed by Bartosch et al. and Hsu et al are based on pseudoparticles bearing E1 and E2 glycoproteins of HCV [Bartosch et al., 2003; Hsu et al., 2003]. In each case, pseudoparticles are generated by co-transfecting a plasmid encoding truncated core and full-length E1/E2 proteins with a plasmid or plasmids encoding a reporter gene [green fluorescent protein (GFP) or luciferase] and a retroviral packaging system. The assembled pseudoparticles released into the medium are able to infect cultured liver cells by virtue of their E1 and E2 components and the efficiency of infection can be quantified by the level of reporter gene expression. Although this system consists mainly of non-HCV components, its use as a tool to study neutralization of HCV was validated by the demonstration that antibodies that neutralized HCV in the chimpanzee model neutralized HCVpp [Meunier et al., 2005].

In both HCVpp systems, the sequence encoding the C-terminal 20 or 60aa of core were included to provide the signal sequence for E1: the C-terminus of E1, in turn, serves as the signal sequence for E2 [Cocquerel et al., 2000]. Therefore, the HCV portion of this system is relatively easy to manipulate since it contains less than 2kb of HCV genome and is located on a separate plasmid.

In the HCVcc system, autonomously replicating infectious virions are produced initially from a full-length recombinant HCV genome and then amplified by passage in hepatic cells [Zhong et al., 2005]. As far as is known, the viruses made in cell culture are structurally equivalent to those in hepatitis C patients and, therefore, would seem ideal for studying anti-HCV. However, the HCVcc system is much less versatile than HCVpp for studying anti-E1 or E2 because only a limited number of strains can be cultured currently. Initially, only the genotype 2a strain JFH1 replicated in cell culture [Wakita et al., 2005]. A limited number of chimeric viruses expressing the glycoproteins of other strains in the JFH1 backbone have been developed but each has had singular requirements for viability and serial passage has usually been required to select adaptive mutations [Lindenbach et al., 2005; Yi et al., 2006; Yi et al., 2007; Delgrange et al., 2007; Kaul et al., 2007; Yi et al., 2007; Zhong et al., 2006; Gottwein et al., 2009]. Thus generation of a diverse or expansive set of test viruses would be time consuming and labor intensive.

Much remains to be learned about the humoral response to HCV glycoproteins. Given the tremendous diversity of HCV arising from the 6 genotypes, hundreds of subtypes, and a plethora of quasispecies, it is clear that a broad spectrum of viruses is required for comprehensive assays of anti-E1 and E2. For this reason, the HCVpp system has obvious advantages over the HCVcc system because, in theory, virtually any E1E2 glycoprotein pair that functions *in vivo* would be expected to function in HCVpp. Although many natural glycoprotein pairs formed functional pseudoparticles, others such as the glycoprotein pair of JFH1, the prototype virus of the HCVcc system, did not efficiently assemble into functional HCVpp even though these same glycoproteins were incorporated into infectious virions. The availability of a greater diversity of functional HCV glycoprotein pairs could be extremely valuable for determining structure-function relationships or immune evasion mechanisms.

Therefore, in this report we have attempted to answer the perplexing question of why JFH1pp were so difficult to produce and whether the results were relevant for production of other pp and, therefore, might aid in increasing the spectrum of glycoproteins available for testing.

MATERIALS AND METHODS

Cell culture

HCVpp stocks were produced in HEK-293T cells (ATCC), a human embryonic kidney cell line and HCVpp infections were performed in Huh-7.5 cells, a human hepatoma cell line, (kind gift from C. Rice). All cells were propagated in complete Dulbecco's modified Eagle's medium with L-glutamine (DMEM; Invitrogen) supplemented with penicillin/streptomycin (Sigma) and 10% fetal bovine serum (BioWhittaker). Cells were grown in 100 mm culture dishes (Corning) at 37°C in the presence of 5% CO₂.

Plasmid Constructs

The three plasmids used to generate HCVpp were from the Bartosch system and included the CMV-Gag-pol murine leukemia virus (MLV) packaging construct, a plasmid encoding an MLV-based transfer vector containing a CMV-GFP internal transcriptional unit, and the phCMV-7a expression vector encoding 60aa from the C terminus of core and all of E1 and E2 from a genotype 1a strain (gift from F.L. Cosset). The phCMV-7a plasmid was modified by standard cloning methods to generate all other vectors used. All constructs were verified by restriction digestion and sequencing. Detailed description of the cloning strategy, plasmids and primer sequences are available upon request. The plasmid lacking E1 and E2 served as a negative control.

Generation of HCV pseudoparticles

Three million HEK-293T cells were seeded in 100mm flat culture dishes (Corning) and allowed to adhere overnight. Cells were transfected with Lipofectamine Plus reagents (Invitrogen Carlsbad, CA) as per manufacturer's protocol. A total of 2.5 µg of plasmid DNA including 0.75 µg of CMV-Gag-pol packaging construct, 0.75 µg of MLV-GFP plasmid, and 1.0 µg of HCV core E1E2 expression vector were diluted in 250 µl of serum-free DMEM. Eight microliters of Plus reagent was added to the DNA solution and incubated at room temperature for 15 min. The DNA mix was added drop wise to 250 µl of serum-free DMEM containing 12 µl of Lipofectamine reagent and incubated at room temperature for 15 min. Cells were washed twice and covered with 2 ml serum-free DMEM. The DNA/Lipofectamine mixture was added to the cells and placed at 37°C. Three hours later, the transfection mixture was replaced with complete DMEM and cells were cultured for two days at 37°C. Transfection efficiency was consistently 80-90% based on visual examination of GFP expression and FACS analysis of GFP positive HEK-293T cells. Culture supernatants were harvested and filtered through a 0.45 µm filter (Millipore) to remove cells and debris. Infection of Huh7.5 cells was performed on the same day to avoid freeze-thawing of the virus stocks, and remaining culture supernatants were stored at -80 °C for subsequent analysis. An E1E2 negative control and untransfected cells were also included in each experiment: since in all cases, less than 0.04% of the control cells scored positive, they are shown only for figure 1.

Infection of Huh 7.5 cells with HCV pseudoparticles

Huh-7.5 cells were seeded at a concentration of 40,000 cells per well in 24-well plates and cultured overnight. The next day, 200 µl of HCVpp-containing HEK-293T cell transfection supernatant was mixed with 50 µl of 5X Polybrene (Sigma) diluted in serum-free DMEM to give a final concentration of 4µg/ml. Cell supernatants were removed and the HCVpp/Polybrene mixture was added to the cells and incubated for 5 hours at 37°C with shaking every hour. The infection mixture was replaced with 1 ml complete DMEM. Four days later, culture supernatants were removed and the monolayer was washed once with 300 µl of 1X PBS (Invitrogen; pH 7.4), covered with 300µl Trypsin-Versene (Bio-Whittaker), and

incubated at 37°C for 15 min. Detached cells were transferred to 1.5 ml Eppendorf tubes, washed with 1 ml PBS, resuspended in 300µl of PBS and placed on ice. For FACS analysis 20,000 cells were analyzed for GFP expression using FACScan (BD) with the settings: FSC, E-1 linear, amp gain 4-59; SSC, 360 linear, amp gain 1; FL1, 340 log. FACS analysis of uninfected cells was performed as a negative control.

Purification of HCVpp

HCVpp were purified from the 293T culture supernatant using 20% sucrose. Briefly, 4 ml of culture supernatant was layered on 1 ml 20% sucrose and ultracentrifuged at 35,000 rpm for 1 h at 4 °C. Purified pps were resuspended in 30 µl TN buffer [50 mM Tris (pH 8.0), 10 mM NaCl] and used for immunoblotting analysis.

Immunoblotting analysis of HCVpp protein expression

Transfected HEK-293T cells were lysed at 48 hours posttransfection with 1X lysis buffer (Promega) as per manufacturer's protocol. Lysates and purified pp were separated on 4-12% Bis-Tris NuPage gels (Invitrogen) under reducing conditions. After electrophoresis, proteins were transferred to PVDF membranes (Invitrogen), and blots were processed using SNAP i.d. system (Millipore) as per manufacturer's protocol. Briefly, the membrane was blocked for 5 min at room temperature with 0.5% skim milk in 1X PBS-T (0.1% Tween-20). For detection of JFH1 core protein, a monoclonal antibody which recognizes the N-terminus of JFH1 core (mouse α -core; Anogen) was diluted 1:160 in PBS-T and incubated with the blot at room temperature for 10 min. For detection of JFH1 E2, a rabbit antibody to H77 HVR1 [LMF 87 (Farci et al., 1996)] was used at 1: 5000 dilution and incubated at room temperature for 10 min. After extensive washing with PBS-T, the blots were incubated for 10 min at room temperature with an HRP-conjugated α -mouse (1:16,000) and α -rabbit (1:30,000) secondary antibodies. After additional washing, blots were developed with SuperSignal® West Femto Chemiluminescent Substrate (Pierce).

RESULTS

Complete core gene enhances JFH1pp production

The E1E2 expression plasmids in the pseudoparticle assays developed by Bartosch et al. and Hsu et al. encode only the last 60 or 20 C-terminal amino acids, respectively, of the 191aa core protein [Bartosch et al., 2003; Hsu et al., 2003]. Although constructs expressing similar lengths of truncated core protein have been used successfully to produce pseudoparticles representing multiple strains and genotypes of HCV, a JFH1 E1E2 expression plasmid encoding the C-terminal 63 amino acids of core produced negligible quantities of pp (Fig. 1A). Since Merola et al. had reported that the core protein could enhance correct folding of E1 protein *in vitro* [Merola et al., 2001], vectors containing variable lengths of JFH1 core gene were constructed and tested to determine if they differed in efficiency of JFH1pp production. As a positive control, an expression plasmid encoding 60 amino acids of core and E1E2 of H77 strain was included since it consistently produced high yields of infectious pp.

Increasing the size of core to encode all 191aa, rather than just the last 63, increased the yields of JFH1 infectious pp over 20-fold (Fig. 1A). Shorter regions encoding the last 127 or 95 C-terminal aa had only a 3-fold or no effect, respectively (Fig. 1A). A C-terminal region of 159aa was approximately twice as efficient as that with 127aa but was still 4-fold less efficient than the entire 191aa region (data not shown). The results demonstrated the importance of core sequence, or at least the NH₂ terminal region, for JFH1 pp generation. However, note that the yield of H77 pp generated in the presence of partial core was still 4

times greater than that of JFH1 with complete core. As expected, Western blot analysis of pps did not detect any core (data not shown).

The stimulation of JFH1 particle production by core raised the question of whether H77 particle production could be increased by lengthening the core gene in the H77 expression plasmid. E1E2 expression vectors encoding all 191 amino acids or only the C-terminal 60 amino acids of H77 core were compared for their ability to generate infectious H77 pp (Fig. 1B). The two vectors produced similar results suggesting that the requirement for the core gene in pp formation differed between JFH1 and H77.

Core protein, rather than core gene RNA, enhances JFH1 particle production

The enhanced yield of infectious particles following incorporation of the entire core gene into the plasmid region directly preceding E1 could reflect a property of the core protein itself or, alternatively, it could be RNA-related and reflect greater mRNA stability or translatability. If the RNA itself was the critical variable, substitution of the JFH1 core nucleic acid sequence for that of the H77 core sequence in the H77 vector should result in decreased production of infectious H77pp since it should decrease the H77 mRNA stability or translatability to that of JFH1 mRNA. Since the 20 core aa preceding E1 in the HCV polyprotein are believed sufficient to provide the core/E1 cleavage site and the signal sequence for E1, H77 chimeric constructs containing either the full-length core gene of JFH1 or only the region encoding the C-terminal 20aa of JFH1 in-frame with the E1E2 gene of H77 were tested for pp formation. The results were quite informative. Regardless of the size or source of the core gene all three constructs produced similar yields of infectious H77 pp (Fig. 2): yield with 20 or 191aa of JFH1 core was as efficient as that with 60aa of H77 core. Therefore, the RNA sequence per se was not relevant. Furthermore, since the protein sequence of JFH1 and H77 core differ by 20% (4 of 20 aa in this region), the data suggested that the E1 and E2 glycoprotein of H77 have intrinsic ability to form functional complexes and that core protein is not required in this case.

Effects of heterologous core on JFH1 pp production

A plasmid encoding 60aa of core and E1 and E2 of strain J6 produces more infectious pp than does a similar construct of JFH1 (data not shown). Both are genotype 2a strains and differ from each other by 12/191aa in core. In contrast, H77 strain is a genotype 1a strain that differs from JFH1 in 20/191aa in core. Given the different efficiencies of pp production, it was of interest to determine if the core protein of either H77 or J6 could replace that of full-length JFH1 for JFH1 pp production.

Data comparing the yields of various pps in the same experiment confirmed that the homologous 60aa core/E1E2 construct of H77 was superior to any other construct tested (Fig. 3). Although the homologous 60aa core/E1E2 construct of J6 did produce a substantial quantity of pp, it was not as efficient as the construct encoding all 191aa of JFH1 core preceding E1 and E2 of JFH1 (Fig. 3). None of the three chimeric constructs encoding the E1E2 glycoproteins of JFH1 preceded by sequences encoding full or partial core of H77 or J6 was as efficient as the homologous JFH1 construct (Fig. 3). Of note, however, J6 full-length core was about twice as efficient as the more distantly related H77 full-length core and full-length H77 core was ten times more effective than the truncated H77 core.

Core protein is more effective when synthesized *in cis*

Previous results demonstrated that the last 20aa of core were sufficient to permit production (hence translation and processing) of H77 pp (Fig. 2). Therefore a plasmid encoding the last 20aa of JFH1 core followed by E1 and E2 of JFH1 was constructed and tested in the Bartosch system by itself or in combination with a second HCV plasmid encoding only full-

length JFH1 core. Synthesis of the core protein via a separate plasmid did not increase pp yield appreciably suggesting that core was most useful when translated as part of the polyprotein (Fig. 4A).

Western blot analysis of the cell lysates demonstrated that similar quantities of core protein were synthesized whether the full length core gene was provided *in cis* or *trans* (Fig. 4B).

Effects of longer incubation time

The previous experiments all tested pp harvested at 2 days posttransfection. When pp harvested at 3 days posttransfection were tested, the trends were similar but the differences were smaller. Homologous pp strain of H77 was produced most efficiently, full-length core of JFH1 but not of H77 was required for efficient production of homologous pp, and J6 core was more effective than H77 core for producing JFH1 pp (Fig. 5). However, whereas production of H77 pp at two days was routinely 3.0 to 4.2 fold higher than production of JFH1 pp, at three days this number decreased to about half that: also, the full length core of the two 2a genotype strains, JFH1 and J6, were equally effective in producing JFH1 pp.

Full core slightly enhances the generation of infectious J6 and HK pp

The envelope glycoproteins of some other HCV strains, for example J6 (genotype 2a) and HK (genotype 6a) are also inefficient in assembling into infectious pp. Since full core enhanced the generation of infectious JFH1 pp, it was possible that core might similarly affect the production of J6 and HK infectious pp. We, therefore, compared the E1E2 expression vectors encoding the C-terminal 60 amino acids or all 191 amino acids of J6 and HK core for their ability to produce infectious J6 and HK pp (Fig. 6). Similar to the results from JFH1, the full core from both the strains enhanced the generation of infectious J6 and HK pp, but this enhancement was only 2-fold and 3-fold, respectively, as compared to the truncated core.

H77 HVR1 can replace the HVR1 of other HCV strains

The enhancement by core protein of JFH1 pp production raised the question of whether core increased the expression of JFH1 glycoproteins. However, antibodies for the detection of JFH1 E1 or E2 by Western were not available to answer this question. HVR1 sequences vary tremendously without apparently affecting the function of E2; therefore, since the antibody LMF 87 reacts very well with HVR1 of H77, we substituted the JFH1 HVR1 with H77 HVR1 in the JFH1 E1E2 expression vector. Although this substitution decreased the infectivity of JFH1 pp slightly (Fig. 7A), this decrease was compensated for by the ability to now detect JFH1 E2 protein (Fig. 7B). Western blot analysis demonstrated that constructs encoding either truncated core or full core expressed similar amounts of JFH1 E2 (Fig. 7B) suggesting inclusion of full core did not increase expression of JFH1 glycoproteins. The viability also of J6 and HK pp was preserved following substitution of their HVR1 with that of H77 (Fig. 7A) suggesting this technique could be utilized to detect many of HCV E2 glycoproteins for which antibodies are not available.

DISCUSSION

Although the HCVcc much more closely resemble authentic HCV than do HCVpp, the relative ease with which HCVpp can be constructed makes them a more versatile and comprehensive system with which to study neutralization with a diverse collection of antibodies, viral strains and quasispecies. In the few cases compared thus far, HCVpp and HCVcc appear to be similarly neutralized [Meunier et al., 2008]. For this reason we felt it important to determine why the glycoproteins of some strains were so inefficient in forming infectious HCVpp, yet, at least for JFH1, they readily assembled into infectious HCVcc.

Our data suggest that there is an inherent difference in the ability of different sets of E1E2 glycoproteins to assemble into functional units. The H77 glycoproteins were able to assemble without any demonstrable reliance on core protein (except for signal sequence) whereas JFH1 glycoproteins were at the other end of the spectrum and required full-length core, synthesized *in cis*, for any significant assembly. Strain J6 appeared to have an intermediate requirement for core. Core sequence appeared to be important since homologous core protein facilitated functional complex formation more efficiently than the heterologous core did. Interestingly, a closely related core protein (i.e., 2a genotype J6 for 2a genotype JFH1) could partially substitute for the homologous one. Formation of infectious J6 and HK pseudoparticles, like JFH1, was also inefficient, but, unlike JFH1, was enhanced only slightly when truncated core gene was replaced with the entire core gene.

These results support the conclusion of Merola et al. that, in an *in vitro* translation system core protein aided E1 folding [Merola et al., 2001]. Although core has been reported to interact with E1 when provided *in trans* [Lo et al., 1996], core enhanced JFH1pp formation only when provided *in cis* (Fig. 2). The fact that differences among the strains diminished with longer incubation periods before harvest suggests that the core protein may be a chaperone that is acting to enhance the rate of proper folding and association of E1 and E2.

As the name hypervariable region implies, the HVR1 region in the E2 protein exhibits a tremendous degree of sequence variability in clinical specimens and has been proposed to function as a decoy to focus the immune response to non-critical regions. Indeed, Forns et al showed that the HVR1 is not required for infection of chimpanzees [Forns et al., 2000]. Therefore, it seemed possible that the HVR1 of one strain could be substituted for that of another strain while maintaining the structure-function relationships of E2. However, substitution of JFH1, J6 or HK HVR1 with that of H77 HVR1 in the respective E1E2 expression vector resulted in a small, but measurable decrease in the infectivity of the pp suggesting that HVR1 is not functionally inconsequential but acts in concert with the rest of E2 in the steps leading to infectious particle assembly or to cell entry and that sequence differences in HVR1 are not all random. Indeed, Bartosch et al, showed that nonconservative substitution of conserved amino acids in HVR1 decreases virus infectivity suggesting that the HVR1 plays an important role in virus infection [Bartosch et al., 2005]. This may relate to the observation that HVR1 is essential for E2 binding to the scavenger receptor class B type I (SR-BI) [Scarselli et al., 2002]. Although HVR1 switching is not without consequence, it should provide a more natural change than insertion of a foreign tag epitope and substitution of one HVR1 with another provides a minimally intrusive approach for antibody detection of functional E2 of various strains. Such HVR1 swaps may also provide insight into exactly how HVR1 functions. These findings emphasize how much remains to be discovered about the way in which HCV glycoproteins and their different domains interact and promote cell entry.

A number of implications follow from these data. First they suggest that production of HCVpp of perhaps many strains might benefit from incorporation of full-length core into the glycoprotein-expressing plasmid. Second, attempts to produce recombinant E1E2 complexes, whether for vaccine development or otherwise, might achieve better success if core is included. Third, generation of HCVcc chimeras might depend on the sequence of core in ways not appreciated to date. It seems reasonable to speculate that selection of mutations in core during adaptation to growth in cell culture might represent an RNA packaging effect but it could actually represent an advantage for glycoprotein function. Finally, these data suggest that studies of the interactions of HCV structural proteins might provide different results depending on which strain was analyzed.

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REFERENCES

- Abrignani S. Immune responses throughout hepatitis C virus (HCV) infection: HCV from the immune system point of view. *Springer Semin Immunopathol.* 1997; 19:47–55. [PubMed: 9266630]
- Bartosch BJ, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 protein complexes. *J Exp Med.* 2003; 197:633–642. [PubMed: 12615904]
- Bartosch BJ, Verney G, Dreux M, Donot P, Morice Y, Penin F, Pawlotsky JM, Lavillette D, Cosset FL. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. *J Virol.* 2005; 282:32357–32369.
- Chang KM, Rehmann B, Chisari FV. Immunopathology of hepatitis C. *Springer Semin Immunopathol.* 1997; 19:57–68. [PubMed: 9266631]
- Cocquerel L, Wychowski C, Minner F, Penin F, Dubuisson J. Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a major role in the processing, subcellular localization, and assembly of these envelope proteins. *J Virol.* 2000; 74:3623–3633. [PubMed: 10729138]
- Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouille Y, Dubuisson J, Wakita T, Duverlie G, Wychowski C. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. *J Gen Virol.* 2007; 88:2495–2503. [PubMed: 17698659]
- Eckels D, Flomenberg P, Gill JC. Hepatitis C virus: models of immunopathogenesis and prophylaxis. *Transfusion.* 1996; 36:836–844. [PubMed: 8823462]
- Farci P, Shimoda A, Wong D, Cabezon T, De Gioannis D, Strazzer A, Shimizu Y, Shapiro M, Alter HJ, Purcell RH. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci USA.* 1996; 93:15394–15399. [PubMed: 8986822]
- Forns X, Thimme R, Govindarajan S, Emerson SU, Purcell RH, Chisari FV, Bukh J. Hepatitis C virus lacking the hypervariable region 1 of the second envelope protein is infectious and causes acute resolving or persistent infection in chimpanzees. *Proc Natl Acad Sci USA.* 2000; 97:13318–13323. [PubMed: 11078521]
- Gottwein JM, Scheel TK, Jensen TB, Lademann JB, Prentoe JC, Knudsen ML, Hoegh AM, Bukh J. Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology.* 2009; 49:364–377. [PubMed: 19148942]
- Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci USA.* 2003; 100:7271–7276. [PubMed: 12761383]
- Kaul A, Woerz I, Meuleman P, Leroux-Roels G, Bartenschlager R. Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. *J Virol.* 2007; 81:13168–13179. [PubMed: 17881454]
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science.* 2005; 309:623–626. [PubMed: 15947137]
- Lindenbach BD.; Rice, CM. *Flaviviridae: the viruses and their replication.* In: Knipe, DM.; Howley, PM.; Griffin, DE.; Lamb, RA.; Martin, MA.; Roizman, B.; Straus, SE., editors. *Fields Virology.* 4th. Lippincott Williams & Wilkins; Philadelphia, PA: 2001. p. 991-1041.
- Lo S, Masiarz YF, Hwang SB, Lai MM, Ou JH. Differential subcellular localization of hepatitis C virus core gene products. *Virology.* 1995; 213:455–461. [PubMed: 7491770]

- Lo SY, Selby MJ, Ou JH. Interaction between hepatitis C virus core protein and E1 envelope protein. *J Virol.* 1996; 70:5177–5182. [PubMed: 8764026]
- Merola M, Brazzoli M, Cocchiarella F, Heile JM, Helenius A, Weiner AJ, Houghton M, Abrignani S. Folding of hepatitis C virus E1 glycoprotein in a cell-free system. *J Virol.* 2001; 75:11205–11217. [PubMed: 11602760]
- Meunier JC, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, Emerson SU, Cosset FL, Purcell RH, Bukh J. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *Proc Natl Acad Sci USA.* 2005; 102:4560–4565. [PubMed: 15767578]
- Meunier JC, Russell RS, Goossens V, Priem S, Walter ED, Union A, Faulk KN, Bukh J, Emerson SU, Purcell RH. Isolation and characterization of broadly neutralizing human monoclonal antibodies to E1 glycoprotein of Hepatitis C virus. *J Virol.* 2008; 82:966–973. [PubMed: 17977972]
- Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology.* 2004; 39:5–19. [PubMed: 14752815]
- Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* 2002; 21:5017–5025. [PubMed: 12356718]
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from cloned viral genome. *Nat Med.* 2005; 11:791–796. [PubMed: 15951748]
- Yi M, Ma Y, Yates J, Lemon SM. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. *J Virol.* 2007; 81:629–638. [PubMed: 17079282]
- Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci USA.* 2006; 103:2310–2315. [PubMed: 16461899]
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA.* 2005; 102:9294–9299. [PubMed: 15939869]
- Zhong J, Gastaminza P, Chung J, Stamataki Z, Isogawa M, Cheng G, McKeating JA, Chisari FV. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. *J Virol.* 2006; 80:11082–11093. [PubMed: 16956932]

Fig. 1.

(A). The length of JFH1 core but not of H77 core affects HCVpp generation. Plasmids encoding various N-terminal truncations of JFH1 core preceding the two envelope glycoproteins were used to generate HCVpp which were tested for their ability to infect Huh7.5 cells as quantified by FACS analysis for GFP expression. The positive control, an expression plasmid encoding 60 amino acids of core and E1E2 of H77 strain, consistently produced high yields of infectious HCVpp. Results represent three independent infections with the same inoculum. (aa) Core: number of C-terminal amino acids of indicated strain; E1E2: donor strains for E1E2 genes. Controls consisting of non-infected cells or cells infected with pp lacking glycoproteins were included in all experiments, and were consistently negative.

(B). E1E2 expression vectors encoding all 191 or just the 60 C-terminal aa of H77 core were compared for HCVpp production as in Fig 1A.

Fig. 2. JFH1 core RNA sequence does not decrease H77pp yield

The core gene region in the H77 E1E2 expression vector was replaced with the entire JFH1 core gene (191aa) or with that encoding the C-terminal 20aa. HCVpp were generated and tested as in Fig 1A. Solid bar: homologous core; hatched bar: heterologous core.

Fig. 3. J6 core produces an intermediate increase in JFH1 pp generation

The core region of the genotype 2a JFH1 expression vector was replaced with core sequence from genotype 1a H77 or genotype 2a J6 and production of infectious HCVpp was analyzed as in Fig. 1A. Solid bar: homologous core; hatched bar: heterologous core.

Fig. 4.

(A). JFH1 core enhances HCVpp generation *in cis* but not *in trans*. In the Bartosch system, a vector encoding only the JFH1 full core was co-transfected with a JFH1 E1E2 expression vector encoding the C-terminal 20aa of JFH1 core into HEK 293T cells. The HCVpp were quantified as in Fig. 1A. Solid black bar: Core provided *in cis*; solid grey bar: core provided *in trans*.

(B). Similar amounts of core were produced *in cis* and *in trans*. Transfected and untransfected HEK-293T cells were lysed 48 hours posttransfection, and analyzed by western blot with mouse α -core. Lanes M: protein marker; UT: untransfected; WT: cells transfected with JFH1 E1E2 expression vector encoding JFH1 full core; C+C20: cells co-transfected with JFH1 full length core expression vector and JFH1 E1E2 expression vector encoding C-terminal 20aa of JFH1 core.

HCVpp were harvested at 72 hr rather than the 48 hr posttransfection time used for Fig 1-4. Quantification of HCVpp was as for Fig 1A. Solid black bar: homologous core; hatched bar: heterologous core; solid grey bar: core provided *in trans*.

Fig. 6. J6 and HK full core slightly enhance the infectious pp production

E1E2 expression vectors encoding C-terminal 60 aa or all 191 of J6 and HK core were compared for HCVpp production as in Fig 1A.

Fig. 7.

(A). Substitution of JFH1, J6 and HK HVR1 with H77 HVR1. The HVR1 region of JFH1, J6 and HK in the E1E2 expression vector was substituted with that of H77. HCVpp were generated and tested as in Fig 1A. Solid bar: homologous HVR1; hatched bar: substituted HVR1.

(B). Full core does not effect E2 protein expression. Purified pps were analyzed by Western blot with rabbit α -HVR1. Lanes 1: cells transfected with JFH1 E1E2 expression vector encoding C-terminal 20 aa of JFH1 core and H77 HVR1; 2: cells transfected with JFH1 E1E2 expression vector encoding JFH1 full core and H77 HVR1; 3: cells transfected with JFH1 E1E2 expression vector encoding JFH1 full core and JFH1 HVR1.