

Figure 6. HCV triggers a transient inhibition of protein translation. Huh7.25.CD81 cells were transfected with 400 ng of CAT-IRES^{HCV}-LUC (A and B) or 50 ng of CAT-IRES^{EMCV}-LUC (C and D), together with the pRL-TK-RLUC plasmid (40 ng) and the HA-TRIM25 expressing plasmid (100 ng). 24 hrs post-transfection, cells were infected with JFH1 at an m.o.i of 0.2. **A and B:** At the indicated times, total cellular RNA was extracted and F-luc and GAPDH RNA were quantified by qRT-PCR. The graphs represent the number of copies of the IRES^{HCV}-luc (A) or of the IRES^{EMCV}-luc (C) RNA normalized to the number of copies of GAPDH RNA. Error bars represent the mean \pm S.D. for triplicates. **B and D:** At the indicated times, cell lysates were prepared and the IRES^{HCV}-luc or IRES^{EMCV}-luc activity was analysed by a reporter assay. For normalization, the levels of firefly luciferase activity were divided in each case by the ratio R-luc RNA/GAPDH RNA that was calculated after measurement of the R-luc RNA by RTqPCR using the total cellular RNA extracted for A and C. Error bars represent the mean \pm S.D. for triplicates. doi:10.1371/journal.pone.0010575.g006

induction. Huh7.25/CD81 cells were transfected with control siRNAs or siRNA directed against PKR, in conditions that allow complete depletion of PKR (Figure 7B). They were then transfected with TRIM25 in the presence of the IFN β -luciferase reporter plasmid. Cells that express normal endogenous levels of PKR presented an initial increase in IFN β -luciferase induction up to 12–15 hrs in response to infection with JFH1 followed by a sudden decline (Figure 7A), similarly to the results described in Figure 3. In contrast, induction of IFN β -luciferase continued to increase past 15 hrs post-infection in cells in which PKR expression was silenced. In both cases, a decrease of IFN expression at 24 hrs was correlated with strong NS3 expression in the cytosol (Figure 7A). Concomitant analysis of the activity of luciferase placed under a TK promoter revealed the same pattern of inhibition of expression during HCV infection, which was alleviated when PKR was silenced. This confirmed that HCV-mediated inhibition at the general level occurs through PKR

(Figure S3). Therefore, these results demonstrate the role of PKR activation in controlling IFN induction in HCV infection prior to MAVS cleavage by the HCV NS3/4A.

PKR positively controls HCV yields through the inhibition of the IFN induction pathway

We next examined whether there was a correlation between the control of IFN induction by PKR, and HCV yields. Huh7.25/CD81 cells were transfected with control siRNA or siRNA directed against PKR as in Figure 7, and infected with JFH1, in conditions where the IFN induction pathway was either active after transfection with TRIM25, or not (Figure 8). The virus yields were measured by RT-qPCR at different times post-infection. First, ectopic expression of TRIM25 triggered a significant inhibition of virus yields at 24 hrs post-infection (40%; Figure 8), as expected from its ability to restore IFN induction in Huh7.25/CD81 cells as shown in previous figures. Inhibition of virus yields

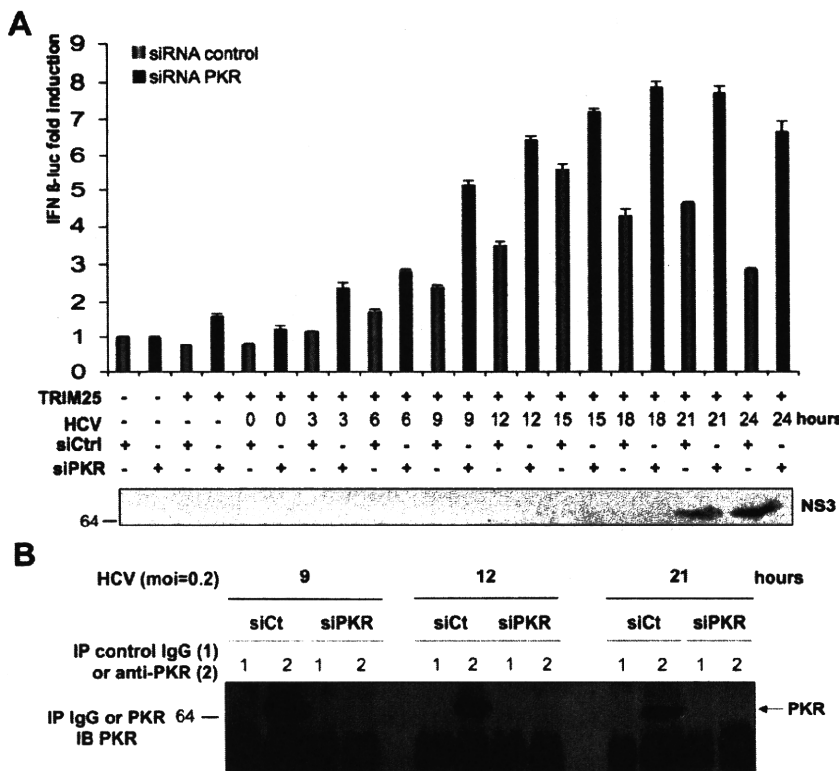


Figure 7. Depletion of PKR increases IFN induction in HCV-infected cells. Huh7.25.CD81 cells were first transfected with 25 nM of siRNA directed against PKR or with 25 nM of control siRNA and then transfected 24 hrs later with the pGL2-IFNβ-FLUC/pRL-TK-RLUC reporter plasmids and the TRIM25 expressing plasmid. 24 hrs post-transfection the cells were infected with JFH1 at an m.o.i. of 0.2. **A:** At the indicated times, one set of cells was treated for RNA extraction and the other for reporter assay as described in the legend to Figure 3. IFN expression was expressed as fold-induction over control cells that were simply transfected with pGL2-IFNβ-FLUC/pRL-TK-RLUC and either control siRNA or siPKR. The graph represents the levels of firefly luciferase activity normalized to the ratio R-luc RNA/GAPDH RNA. Error bars represent the mean ± S.D. for triplicates. In addition, cell lysates were pooled and analysed for the presence of NS3 as a marker of HCV infection. **B:** Huh7.25.CD81 cells were treated as in A, except for transfection with the reporter plasmids, to control the efficiency of PKR silencing. At the indicated times post-infection, cell extracts (1.7 mg) were incubated with normal mouse IgG or Mab 71/10 anti-PKR. The immunoprecipitated complexes were run on SDS-12.5% PAGE gels and blotted using Mab 71/10 (PKR). doi:10.1371/journal.pone.0010575.g007

did not increase later in infection (36% at 48 hrs post-infection; Figure 8), in agreement with the block on IFN induction downstream of TRIM25 imposed by the NS3/4A-mediated cleavage of MAVS. Depletion of PKR from Huh7.25/CD81 cells also triggered inhibition of virus yields. Interestingly, whereas this inhibition was limited in the absence of TRIM25 (13% and 9% inhibition at 24 and 48 hrs post-infection, respectively), it was more pronounced when the cells were expressing TRIM25, with a 28% and 31% further increase in inhibition after 24 and 48 hrs of infection, respectively (Figure 8). Altogether, the combined effect of TRIM25 expression and PKR depletion resulted in 58% of inhibition of HCV virus yields at 24 hrs and 56% at 48 hrs post-infection. These data demonstrate that HCV infection is particularly sensitive to the IFN induction pathway, and that the efficiency of infection benefits from an inhibition of this pathway at the translational level by PKR.

Pharmacological inhibitors of PKR increase IFN induction and inhibit HCV infection

Although best known for its antiviral proprieties, PKR is now also recognized for its negative effect on neurodegenerative disorders such as Huntington’s, Parkinson’s and Alzheimer’s

diseases [16]. For these reasons, pharmacological inhibitors of PKR have been developed. Here, we have used two PKR inhibitors, the oxindole-imidazole C16 compound [17] and the cell penetrating PRI peptide, that corresponds to one PKR motif (DRBM) involved in binding to its activators. This latter compound represents a more specific PKR inhibitor than C16 [18]. We examined the effects of these inhibitors on IFN induction and HCV replication during infection of Huh7.25/CD81 cells with JFH1 (Figure 9). The cells were transfected with TRIM25 as described in Figure 7, and incubated with either C16 or PRI after 11 hrs of infection. The results clearly show that both PKR inhibitors increased IFNβ promoter induction (Figure 9A), as well as restoring general level of protein expression (Figure S4). This increase was more pronounced at 15–18 hrs post-infection (Figure 9A). This highlights the importance of PKR activation during this time-frame to control IFN induction. Next, the effect of the PKR inhibitors on HCV replication was assayed. Huh7.25/CD81 cells were transfected with TRIM25, or not, to have a non-functional or functional RIG-I/MAVS pathway. Similarly to the experiment shown in Figure 8, PKR inhibitors were able to inhibit HCV yields only in conditions where the RIG-I/MAVS pathway was activated (Figure 9B).

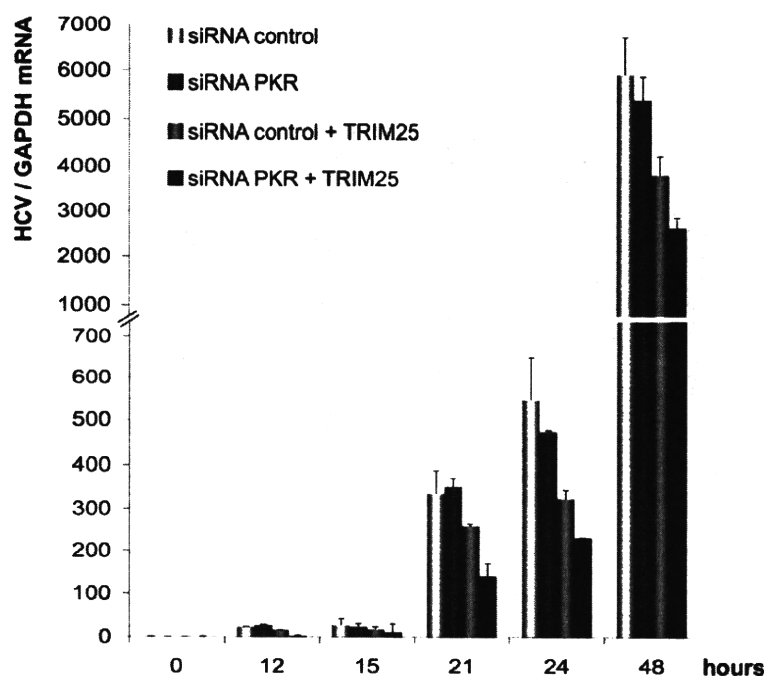


Figure 8. PKR positively controls HCV yield through inhibition of the IFN induction pathway. Huh7.25.CD81 cells were first transfected with 25 nM of siRNA directed against PKR or 25 nM of control siRNA and then transfected 24 hrs later with either empty vector or the TRIM25 expressing plasmid. 24 hrs post-transfection, the cells were infected with JFH1 at an m.o.i. of 0.2. At the indicated times, cells were processed for RNA extraction and HCV RNAs were quantified by qRT-PCR and normalized against RNA from GAPDH. Error bars represent the mean \pm S.D. for triplicates. doi:10.1371/journal.pone.0010575.g008

Discussion

Here we report the use of HCV permissive Huh7.25.CD81 cells to analyse the effect of HCV on the RIG-I/MAVS-mediated IFN induction pathway during the early hours after infection, before the HCV NS3/4A protease can cleave MAVS and abrogate this pathway. We first observed that the IFN induction pathway was deficient in these cells, but that it could be conveniently restored upon ectopic expression of the E3 ubiquitin ligase TRIM25. This protein has been shown to bind the threonine 55 residue of RIG-I through its C terminal SPRY domain, and uses the E3 ligase activity present in its N terminal RING domain to promote a Lys63-type ubiquitination of RIG-I in its CARD domain at lysine 172. This modification is important if RIG-I is to engage in a correct interaction with its downstream adapter MAVS [3]. The nature of the TRIM25 defect in Huh7.25.CD81 cells and the relation to activation of RIG-I is now under characterization. It is interesting to note here that this defect emphasizes the importance of the RIG-I/TRIM25 pathway in the control of HCV expression since both Huh7.25.CD81 and Huh7.5 cells, which were originally selected for their ability to replicate HCV subgenomic replicons, present a defect in this pathway at the level of RIG-I for Huh7.5 cells and of TRIM25 for Huh7.25.CD81 cells. Importantly, Huh7.25.CD81 cells can be considered as a more physiological model than Huh7.5 cells to study the interaction between the IFN induction pathway and HCV infection, since Huh7.5 cells have a point mutation at the threonine 55 residue of RIG-I [5] and therefore require ectopic expression of RIG-I to restore their IFN induction pathway. In contrast, ectopic expression of TRIM25 restores the IFN induction pathway downstream of RIG-I and therefore does not perturb the initial

interaction between RIG-I and the incoming HCV dsRNA structures.

Using Huh7.25.CD81 cells that ectopically express TRIM25, we observed that HCV triggers IFN β induction as early as 6 hrs after infection, demonstrating that the entry of the virus into the cell rapidly generates enough viral structured RNAs to activate RIG-I. NS3/4A expression was detected around 18 hrs post-infection, and MAVS cleavage after 24 hrs post-infection. We thus expected to observe an increase in IFN β induction at least until 18 hrs after infection. This was indeed found to be the case when we analysed IFN β induction at the RNA level. However, when we used a luciferase reporter gene placed under the IFN β promoter as a surrogate marker for production of the IFN β protein, it was intriguing to observe an arrest of IFN induction as rapidly as 12 hrs after infection, followed by a considerable decline in its expression afterwards. These results indicated a new control of IFN induction at the translational level during HCV infection in addition to –and earlier than– the NS3/4A-mediated MAVS cleavage. We have demonstrated that this inhibition is the result of a strong activation of the protein kinase dsRNA-dependent kinase PKR at 12 and 15 hrs after HCV infection and of the concomitant phosphorylation of its substrate eIF2 α .

Since this manuscript was submitted, work performed by U.Garaigorta & F. Chisari showed that HCV also activates PKR and eIF2 α phosphorylation to negatively control the antiviral action of IFN[19]. Both their and our data concur to point out that HCV diverts PKR away from its known antiviral role to use it to attenuate both the IFN-mediated antiviral response [19] and IFN induction itself (our data). Importantly, both studies show that PKR silencing has no effect on HCV production, unless cells are

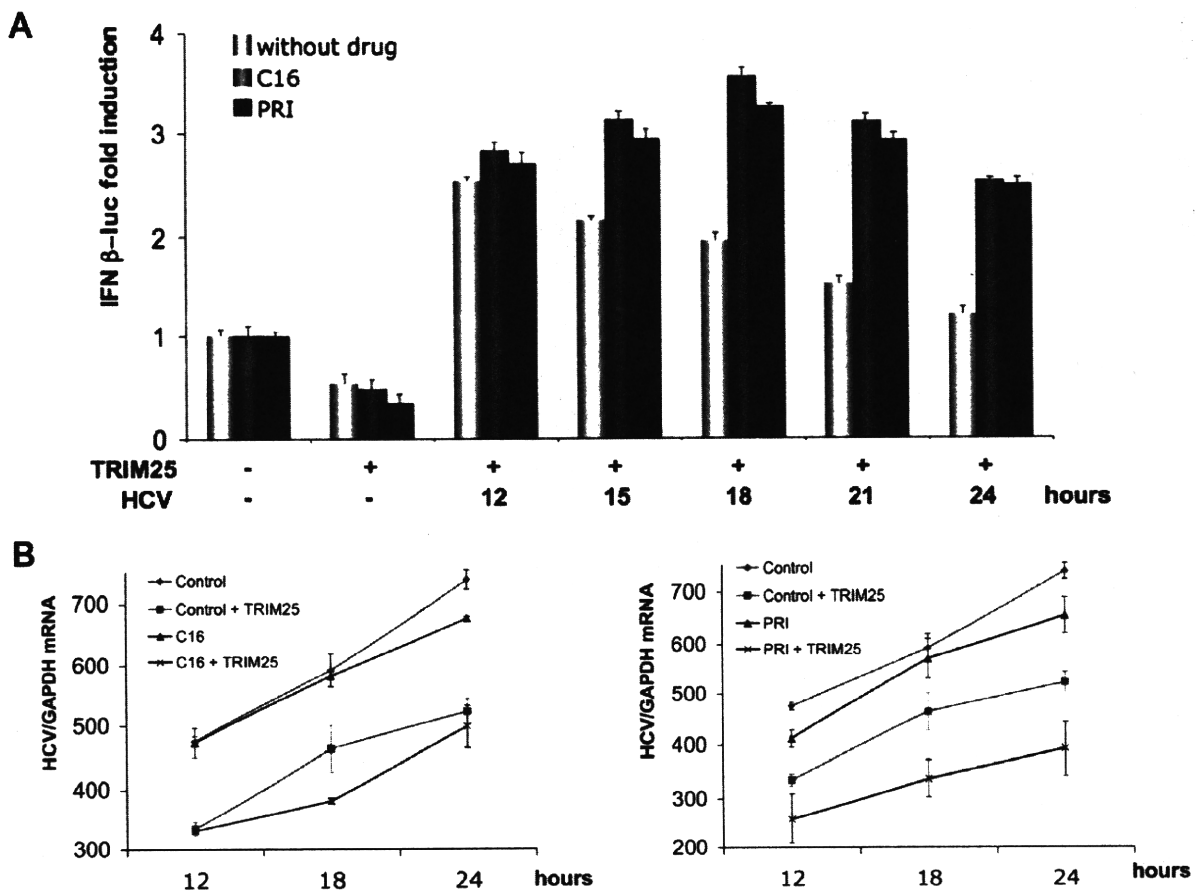


Figure 9. Pharmacological inhibitors of PKR increase IFN induction and inhibit HCV infection. **A:** Huh7.25.CD81 cells were first transfected with the pGL2-IFN β -FLUC/pRL-TK-RLUC reporter plasmids and the TRIM25 expressing plasmid. 24 hrs post-transfection, the cells were infected with JFH1 at an m.o.i of 0.2. At 11 hrs post-infection, cells were exposed to 200 μ M of C16 or 30 μ M of the PRI peptide as described in Materials and Methods. At the indicated times, one set of cells was treated for RNA extraction and the other for reporter assay as described in the legend to Figure 3. IFN expression was expressed as fold-induction over control cells that were simply transfected with pGL2-IFN β -FLUC/pRL-TK-RLUC. The graph represents the level of firefly luciferase activity normalized to the ratio R-luc RNA/GAPDH RNA. Error bars represent the mean \pm S.D. for triplicates. **B:** Huh7.25.CD81 cells were first transfected with the TRIM25 expressing plasmid or an empty plasmid. 24 hrs post-transfection, the cells were infected with JFH1 at an m.o.i of 0.2. At 11 hrs post-infection, cells were exposed to 200 μ M of C16 or 30 μ M of the PRI peptide as described in Materials and Methods. At the indicated times, cell lysates were processed for RNA extraction and HCV RNAs were quantified by qRT-PCR and normalized against RNA from GAPDH. Error bars represent the mean \pm S.D. for duplicates. doi:10.1371/journal.pone.0010575.g009

allowed to induce IFN upon ectopic expression of TRIM25 (our study) or are directly treated with IFN [19].

PKR is a 551 aa serine/threonine protein kinase with its catalytic domain at the C-terminus and regulating domains at the N-terminus. This N-terminus domain contains two tandem copies of a 70-amino acid dsRNA binding motif or DRBM (positions 6–79 and 96–169) that affiliate PKR to the large family of dsRNA binding proteins, which includes TRBP, PACT, DICER, ADAR, RNase III and NFAR's [20]. Binding of PKR to dsRNA induces its dimerization and a change of its conformation that frees access to its autophosphorylation site. Structural analysis has revealed that PKR dimers are arranged back to back, which prevents trans-phosphorylation of one monomer by the other and favours a model of autophosphorylation in cis or through the action of a PKR dimer on other PKR dimers or monomers [21]. Once autophosphorylated on its threonine T446, PKR is stabilized as a kinase and can phosphorylate its substrates, such as the α subunit

of the initiation factor eIF2. PKR is preferentially activated upon binding to long stretches of dsRNA (>33 bp) [22] but was recently shown also to be activated upon binding to RNA structures similar to those that activate RIG-I, such as short imperfect 16 bp stem-loop RNAs with 5' triphosphorylated ends. It is now proposed that perfect dsRNAs can activate PKR with no need for a 5'ppp, whereas if they are to activate dsRNA with minimal secondary structures are dependent on 5'ppp [23]. In addition, PKR activity can be regulated positively and negatively through interaction with the cellular proteins PACT and TRBP, two members of the dsRNA binding protein family. This type of interaction, which is independent of the presence of dsRNA, is complex. The TRBP/PACT interaction can modulate the activation of PKR by PACT and a cellular stress reverses it [24].

Our results point out the ability of HCV to trigger both induction of IFN and activation of PKR in the early hours of infection. The induction of IFN is initiated by the binding of RIG-

I to viral 5'ppp structured RNA, the 3' end poly-U/UC motif and/or replicative RNA duplexes [5,25]. In vitro experiments have shown that PKR can be activated upon binding to the HCV IRES [26] but it is currently not known exactly how HCV activates PKR in vivo. The use of antibodies directed against phosphorylated PKR or against phosphorylated eIF2 α allowed in vivo activation of PKR to be demonstrated, either upon HCV subgenomic replicon replication [27] or more recently after electroporation of Huh7 cells with JFH1 RNA [28]. Surprisingly, the latter study showed that silencing of PKR resulted in 10-fold higher viral yields compared to our study (Figures 8 and 9) and that of Garaigorta & Chisari [19], where no effect on viral yields could be observed unless the IFN induction pathway is triggered or IFN is added to the cells. Although it is difficult to compare different experimental systems, the discrepancy observed may be due to the different procedures used to express JFH1 in the cells: electroporation of its RNA in Kang's study, compared to infection with JFH1 viral stocks in our study and that of Garaigorta & Chisari. Interestingly, the differences might indicate that the mode and/or efficiency of interaction between the HCV structures required to activate PKR are crucial to determine how the virus will benefit or suffer from PKR action. PKR has also been shown to be activated through an interaction between its N terminal domain and the first 58 amino acids of HCV core protein [29]. Core is the first HCV protein to be processed upon translation of the viral polyprotein and may interact with endogenous PKR soon after its own apparition in the cytosol. The possibility evoked here that core is involved in PKR activation requires further investigation.

Our results show that the IFN induction pathway can be stimulated during HCV infection up to 12 hrs post-infection, followed by a decline concomitantly with activation of PKR and of its substrate eIF2 α . Importantly, depletion of the endogenous PKR by silencing, or inhibition of its function through the use of pharmacological inhibitors, prevents this decline. This indicates that HCV infection triggers an inhibition of general protein synthesis through PKR, whereas its own translation is not affected, as shown by the appearance of the viral proteins and an increase in expression of a reporter gene placed under the control of an IRES^{HCV}. These data are in agreement with a recent study which showed, using in vitro experiments, that PKR can inhibit both cap-mediated and IRES^{EMCV}-mediated translation, but not IRES^{HCV}-mediated translation [26]. Shimoike et al proposed that HCV uses alternate initiation factors that allow a mechanism of translation initiation distinct from other IRES and from the cellular machinery of general translation that is not inhibited by eIF2 α phosphorylation. The resistance of IRES^{HCV} to control of translation may be related to a positive effect *in trans* from the virus through some of its structures, such as the polyadenylated 3' UTR [30]. Alternatively, translation from the HCV IRES may occur in autophagy-like structures, together with translation from the incoming HCV RNA, where it may escape PKR action [31]. By both escaping and down regulating host cap-dependent translation, HCV may gain a further selective advantage for its own replication and propagation.

Interestingly, we found that the depletion of PKR from Huh7.25.CD81 cells, or incubation with the PKR inhibitors C16 and PRI, leads to inhibition of HCV replication, but only under conditions where the IFN induction pathway has been restored by the expression of TRIM25. Our results thus demonstrate that, among all the cap-dependent proteins the expression of which are sensitive to PKR, only the translation of proteins resulting from the activation of the IFN induction pathway represent a real constraint for HCV replication and

propagation. Early activation of PKR upon HCV infection (12–15 hrs post-infection) allows this virus to limit the expression of IFN and pro-inflammatory cytokines as soon as possible, before enough NS3/4A is translated from the HCV IRES to block this signaling pathway through MAVS cleavage (18 to 24 hrs post-infection) (see model in Figure 10). Through detailed kinetic experiments, we demonstrated that phosphorylation of PKR and of its substrate eIF2 α are restricted to a 3 hour-period between 12 and 15 hrs post-infection. Accordingly, the PKR inhibitors C16 and PRI efficiently restored IFN induction during this time. The use of both PKR silencing and the addition of pharmacological inhibitors of PKR demonstrates the importance of PKR in the control of IFN induction early after infection with HCV. These results highlight that the 3-hour period between 12 and 15 hrs post-infection is crucial for HCV to activate PKR to restrain IFN expression, once it has launched the IFN induction pathway through interaction of its replicative dsRNA forms with RIG-I. Its ability to activate PKR at the same time allows this induction to be moderated at the level of the translation of this cytokine before sufficient NS3/4A accumulates and abrogates IFN induction at the transcriptional level through MAVS cleavage.

It was intriguing to observe that PKR and eIF2 α phosphorylation decrease at 15 hrs post-infection with HCV, to reappear progressively and increase from 24 hrs post-infection until the 72 hr end-point of the experiment (Figures 4 and 5). The reason for this remains to be investigated and may indicate that PKR could be sensitive to the action of phosphatase(s) at certain time points of the infection.

PKR is well-recognized as an important effector of the antiviral response through its ability to arrest protein synthesis and its importance is highlighted by the number of viral and cellular products that are able to abrogate or modulate its action [13,32]. In the case of HCV, some viral proteins such as NS5A and a cytosolic soluble form of E2 were reported to interact with PKR, and were proposed to be viral inhibitors of the antiviral action of PKR [33,34,35].

Here, we show another aspect of the interaction of HCV with PKR, that reveals how HCV uses the ability of PKR to inhibit cellular cap-dependent translation, while avoiding its effect through its own translation driven by the HCV IRES. PKR silencing or PKR inhibitors have less effect on HCV production when the RIG-I/MAVS pathway is non-operative. This demonstrates that it is only IFN (and presumably also the pro-inflammatory cytokines resulting from the activation of the RIG-I/MAVS pathway) that represents a threat for viral propagation. The situation we describe here might correspond to primo-infection during the natural course of HCV infection in an IFN-free environment, allowing unabated propagation of the virus due to very limited production of IFN. In established chronic infections, whatever IFN has been produced can then trigger induction of a large number of ISGs (IFN-stimulated genes), such as RIG-I and TRIM25, which can sustain the IFN induction pathway, and ISG56 or viperin, which are involved in the inhibition of HCV translation and replication [36,37]. Although PKR is also recognized in several viral infections for its antiviral properties, it may be more suitable to control its action in the case of HCV infection. Therefore, we propose that a carefully-controlled use of PKR inhibitors, in conjunction with IFN/ribavirin, would be beneficial for the treatment of chronically HCV-infected patients, since it would lead to a boost in the induction of innate immunity and a sustained inhibition of the viral propagation.

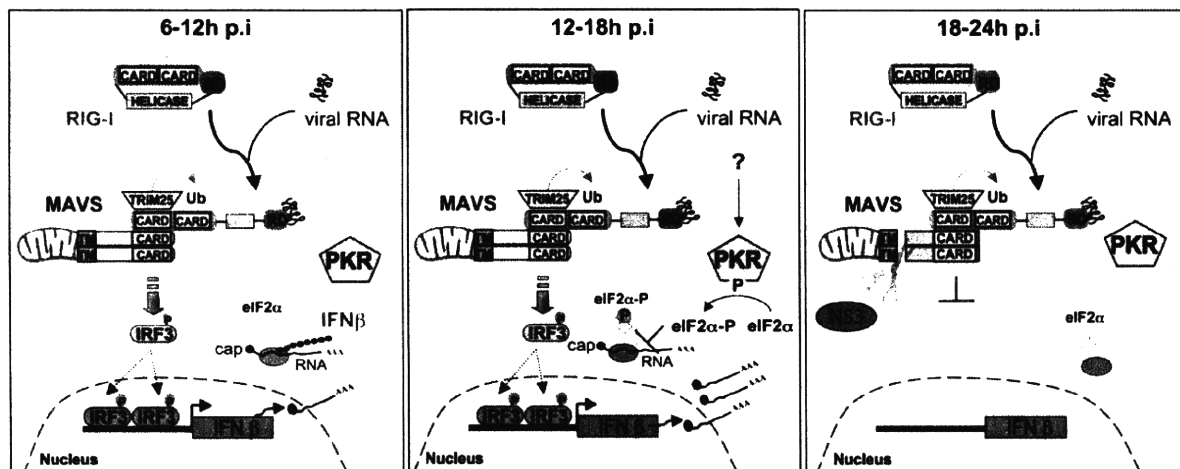


Figure 10. Model of regulation of IFN β induction at the very early steps of HCV infection. Left panel: 6–12 hrs after infection. After cell entry, the HCV genomic RNA is liberated, and thus dsRNA structures are accessible in the cytosol and can associate with the RIG-I RNA helicase. This triggers activation of RIG-I followed by its ubiquitination by the E3-ligase TRIM25, and interaction with the mitochondria-bound MAVS adapter. MAVS, in turn, activates downstream signalling kinases leading to IRF3 phosphorylation and induction of IFN β . Detection of IFN β expression starts at 6 hrs p.i. and increases until 12 hrs post-infection. Induced IFN β mRNA moves to the cytosol and is translated into IFN β protein through the cap-dependent process of cellular translation. Middle panel: 12–18 hrs after infection. HCV infection triggers the activation of PKR through a still unknown mechanism (either through its dsRNA or through a viral protein, such as core, or through activation of a cellular protein, such as PACT). Once activated, PKR phosphorylates its substrate, the α subunit of the eIF2 initiation complex and arrests the cap-dependent protein synthesis. As a result, translation of IFN β protein stops. Right panel: 18–24 hrs after infection. HCV proteins have been translated through a cap-independent mechanism and begin to accumulate in the cytosol. The viral NS3 protease cleaves MAVS at residue 508. This results in complete inactivation of MAVS, arrest in the recruitment of its downstream kinases and arrest of IFN induction. Note that after 15 hrs, PKR is no longer phosphorylated, hence no longer activated. The mechanism of this regulation is not yet known. RIG-I: Retinoic acid Inducible Gene 1; CARD: Caspase Recognition Domain; RD: Repressor Domain, Ub: Ubiquitin; MAVS: Mitochondria Adaptor Virus Signalling; TM: Transmembrane domain; PKR: Protein Kinase RNA-dependent; IRF3: Interferon Regulatory Factor 3; eIF2 α : α subunit of eukaryotic Initiation Factor; AAA: polyadenylated tail. P: phosphate group. doi:10.1371/journal.pone.0010575.g010

Materials and Methods

Cell culture

Huh7 and Huh7.5 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids (Gibco BRL) and 1% penicillin/streptomycin (Invitrogen). Huh7.25/CD81 cells were cultured in the same medium supplemented with 0.4 mg/ml geneticin (G418; PAA Laboratories).

Viruses

Sendai virus stocks were grown in the allantoic cavities of 9-day-old embryonated chicken eggs that were provided by D. Garcin, as described [38]. For the preparation of HCV stocks, Huh7.5 cells plated in 150 cm² flasks were inoculated with JFH1 at an m.o.i of 0.05, in serum-free DMEM. After 2 hrs at 37°C, the medium was removed and replaced with complete medium. Three days after infection, the cells were dissociated by trypsin treatment, and replated in 3 or 4 150 cm² flasks. After 3 more days, the JFH1-containing cell supernatants were collected, centrifuged at 1200 rpm for 10 min at 4°C, cleared of debris by passing through 0.45- μ m-pore-size filters, and stored at -80°C. The cells were trypsinated once again, passaged in 1 or 2 150 cm² flasks and viral supernatants were collected again after 24 hrs and stored at -80°C. For titration, Huh7.5 cells (10⁵ cells/well in a 24-well plate) were incubated in the presence of serial dilutions of the viral preparations. After three days, cells were washed with PBS, fixed for 20 min with 4% paraformaldehyde-PBS (PFA; Electron Microscopy Sciences), permeabilized by incubation for 1 hour

with PBS that contained 1% gelatin (Sigma) and 0.1% Tween-20 (Sigma), and incubated for 1 hour with 150 μ l of PBS-gelatin that contained serum from a patient chronically infected with HCV (genotype 1b) at a 1/500 dilution. Cells were washed and incubated with peroxidase-conjugated anti-human antibody (1/2500 dilution; Dako), and the presence of the virus was detected using the Vector NovaRED substrate kit for peroxidase (VECTOR). Infectious foci were counted under a microscope and the titer was calculated as focus forming units per ml. Titers of 6.10⁴ FFU/mL were obtained on a routine basis.

Plasmids, siRNAs and antibodies

The pcDNA3 expressing 5'HA tagged-TRIM25 was provided by D. Garcin. The CAT-IRES^{HCV}-LUC and CAT-IRES^{EMCV}-LUC constructs have been described [39]. The IFN β -firefly luciferase (pGL2-IFN β) and pEF-BOS FLAG-RIG-I plasmids were described previously [40]. The pcDNA3.1 cMyc-MAVS has been described [41]. Control (scrambled) siRNA and siRNA to PKR (GCAGGGAGUAGUACUUAUUUU) were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The siRNAs (final concentration 25 nM) were transfected for 24 h using FuGENE HD (ROCHE) before the transfection with other plasmids, or infection. Normal mouse IgG were from Santa Cruz Biotechnology. Monoclonal anti-PKR 71/10 antibody was obtained from A.Hovanessian [42]. Anti-actin antibody was from Sigma. Rabbit polyclonal antibodies were used to detect eIF-2 α phosphorylated at Serine 51 (Cell Signalling Technology), PKR phosphorylated at Thr451 (AbCAM), and MAVS (Alexis Biochemical Inc.). Mouse monoclonal antibodies were used to detect HCV NS3 and the HA tag (Chemicon and 12CA5; Roche, respectively).

Luciferase Assays

Huh7, Huh7.5 and Huh7.25.CD81 cells (80,000 cells/well; 24-well plates) were transfected with 40 ng of pRL-TK Renilla-luciferase (R-luc) reporter (Promega); 150 ng of pGL2-IFN β -Firefly luciferase (F-luc) reporter, 400 ng of CAT-IRES^{HCV}-Luc or 50 ng of CAT-IRES^{EMCV}-Luc reporter; and pEF-BOS FLAG-RIG-I, pcDNA3 HA-TRIM25 or pcDNA3 cMyc-MAVS as indicated. Transfections were performed in the presence of the FuGENE HD reagent (ROCHE) according to the manufacturer's instructions. Cells were then incubated for 24 hrs before being mock-treated or infected with either SeV or JFH1. At different times post-infection, cells were processed for a reporter assay. For most of our experiments concerning activation of PKR and inhibition of general translation, normalization of the data was performed using Renilla luciferase RNA rather than its activity. All assays were performed in triplicate in two different sets of cells, plated, transfected and infected in the same conditions. For the reporter assay, the medium was removed and 100 μ l of lysis buffer (Passive lysis buffer; Promega) was added to each well, and incubation was at room temperature for 15 minutes with gentle agitation. Luciferase activity was measured using the dual-luciferase reporter assay system, according to the manufacturer's instructions (Promega). RT-qPCR analysis was performed on Renilla luciferase RNA and GAPDH RNA after extraction of total cellular RNA. The measured amount of Renilla luciferase RNA was normalized to the amount of GAPDH RNA and this ratio was used to normalize the Firefly luciferase activity.

To estimate the efficacy of transfection, Huh7.25.CD81 cells (80,000 cells/well; 24-well plates) were separately transfected with 1 μ g of pcDNA3 Lac-Z in presence of FuGENE HD reagent. 24 hrs post-transfection, cells were fixed in 4% PFA and incubated for a few minutes with 200 μ l of X-Gal stain (PBS, pH 7.2; 2 mM MgCl₂; 4 mM potassium ferricyanide; 4 mM potassium ferrocyanide and 1 mg of X-Gal (Sigma) previously dissolved in DMSO). X-Gal-stained cells were counted in different sections of the plate and the cell density was determined as a function of the measured area of the section examined. Quantification from three fields taken at random allowed an estimation of 45% of the efficacy of transfection.

Real-time RT-PCR Analysis

Total cellular RNA was extracted using the TRIZOL reagent, according to the manufacturer's instructions (Invitrogen). HCV RNA was quantified by one-step qRT-PCR. Reverse-transcription, amplification and real-time detection of PCR products were performed with 5 μ l total RNA samples, using the SuperScript III Platinum one-step qRT-PCR kit (Invitrogen) and an AbiPrism 7700 machine. The sequence of the primers used for HCV RNA amplification are: 5'-TGCGGAACCGGTGAGTACA-3' and 5'-CGGGTTGATCCAAGAAAGGA-3', together with the internal probe 5'-FAM-CGGAATGCCAGGACGACCGG-3'-TAMRA. IFN- β RNA, Renilla luciferase RNA and Firefly luciferase RNA were quantified by a two-step qRT-PCR assay. The reverse transcription step was performed on 1 μ g of total RNA. Quantitative PCR was performed using an AbiPrism 7700 machine, with a SYBR GREEN PCR Master Mix (Applied Biosystems). IFN- β was amplified using the primers: 5'-TGCATACCTGAAGGCCAAG-3' and 5'-AAGCAATTGTCCAGTCCCA-3'; Renilla luciferase was amplified using the primers: 5'-ATCATCCCTGATCTGATCGG-3' and 5'-GGAGTAGTGAAGGCCAGAC-3' and Firefly luciferase with the primers: 5'-GAAGAGATACGCCCTGGTTCCT-3' and 5'-TGTCACCTCGATATGTGCATC-3'. Standard curves were

established as described [40] using 10-fold serial dilutions of plasmids containing IFN- β , R-luc or F-luc amplicons. The results were normalized to the amount of GAPDH RNA using the GAPDH control kit from EuroGentec.

Immunoprecipitation and Immunoblot analysis

Cells were washed once with phosphate-buffered saline and scraped into lysis buffer (50 mM TRIS-HCl, pH 7.5, 140 mM NaCl, 5% glycerol, 1% CHAPS) that contained cocktails of phosphatase and protease inhibitors (Complete, Roche Applied Science). The protein concentration was determined by the Bradford method. For immunoprecipitation, lysates were incubated at 4°C overnight with monoclonal anti-PKR 71-10 antibody or anti-eIF2 α antibody, and then in the presence of A/G-agarose beads (Santa Cruz Biotechnology) for 60 minutes. The beads were washed three times, and the precipitated proteins were extracted at 95°C using SDS-PAGE sample buffer. Protein electrophoresis was performed on standard SDS-PAGE or NuPAGE gels (Invitrogen). Proteins were respectively transferred onto polyvinylidene difluoride membranes (BioRad) or nitrocellulose membranes (Biorad), and probed with specific antibodies. Immunoblots were revealed using either the ECL Plus Western Blotting Detection System (Amersham Biosciences) or chemiluminescence. In the latter case, nitrocellulose membranes were blocked in Odyssey Blocking Buffer (Rockland) for one hour and incubated with primary antibodies in Odyssey Blocking Buffer with gentle shaking. They were then incubated for 35 mn in the presence of appropriate secondary antibodies labelled with IRDye 800 (Rockland Immunochemicals), with gentle shaking and protection from light. Fluorescent immunoblot images were acquired and quantified by using an Odyssey scanner and the Odyssey 1.2 software (Li-Cor Biosciences).

Chemical treatments

The cell-permeable peptide containing the DRBM of double-stranded RNA-dependent protein kinase (PRI peptide [18]; Sanofi-Aventis, Vitry sur Seine, France; dissolved in water), and the C16 compound ([17]; Calbiochem; solubilized in DMSO), were provided by Jacques Hugon. Huh7.25.CD81 cells were infected by JFH1 for 11 hrs, washed once with phosphate buffered saline and then exposed to serum-free DMEM containing either 200 μ M of C16 or 30 μ M of PRI peptide. In the latter case, the cells received fresh aliquotes of the PRI peptide (30 μ M final concentration) every hour until the end of treatment to maximise the effect of the peptide. At different times after infection, cells were lysed and analysed by reporter assay or real-time RT-PCR as described above.

Supporting Information

Figure S1 Specific inhibition of TK-Renilla luciferase activity 12 hrs post-infection with HCV. The graphs represent the R-luc activity normalized to R-luc RNA in the cell extracts corresponding to the experiment described in Figure 3, in which Huh7.25.CD81 cells (A) or Huh7.5 cells (B) have been infected with either SeV (top) or HCV (bottom). Error bars represent the mean \pm S.D. for triplicates.

Found at: doi:10.1371/journal.pone.0010575.s001 (1.19 MB TIF)

Figure S2 Inhibition of TK-Renilla luciferase activity 12 hrs post-infection with HCV in the presence of an IRES either from HCV or from EMCV. The graphs represent R-luc activity normalized to R-luc RNA in the cell extracts corresponding to the experiment described in Figure 6, in which Huh7.25.CD81 cells have been transfected with 400 ng of CAT-IRES^{HCV}-LUC (A)

or 50 ng of CAT-IRESMCMV-LUC (B), together with the pRL-TK-RLUC plasmid (40 ng) and the HA-TRIM25 expressing plasmid (100 ng). Error bars represent the mean \pm S.D. for triplicates.

Found at: doi:10.1371/journal.pone.0010575.s002 (0.15 MB TIF)

Figure S3 Silencing of endogenous PKR abrogates HCV-mediated inhibition of TK-Renilla luciferase activity. The graphs represent R-luc activity normalized to R-luc RNA in cell extracts corresponding to the experiment described in Figure 7, where Huh7.25.CD81 cells were first transfected with 25 nM of siRNA directed against PKR or with 25 nM of control siRNA and then transfected 24 hrs later with the pGL2-IFN β -FLUC/pRL-TK-RLUC reporter plasmids and the TRIM25 expressing plasmid. 24 hrs post-transfection, the cells were infected with JFH1 at an m.o.i. of 0.2. Error bars represent the mean \pm S.D. for triplicates. Found at: doi:10.1371/journal.pone.0010575.s003 (0.71 MB TIF)

Figure S4 Pharmacological inhibitors of PKR abrogate the HCV-mediated inhibition of TK-Renilla luciferase activity. The

graphs represent R-luc activity normalized to R-luc RNA in cell extracts corresponding to the experiment described in Figure 9A, in which Huh7.25.CD81 cells were first transfected with the pGL2-IFN β -FLUC/pRL-TK-RLUC reporter plasmids and the TRIM25 expressing plasmid. 24 hrs post-transfection, the cells were infected with JFH1 at an m.o.i. of 0.2. At 11 hrs post-infection, cells were exposed to 200 μ M of C16 or 30 μ M of the PRI peptide. Error bars represent the mean \pm S.D. for triplicates. Found at: doi:10.1371/journal.pone.0010575.s004 (0.99 MB TIF)

Acknowledgments

We thank Katherine Kean for editing the manuscript.

Author Contributions

Conceived and designed the experiments: NA SD EFM. Performed the experiments: NA SD PM. Analyzed the data: NA PM PM AG TW EFM. Contributed reagents/materials/analysis tools: AB KK PM DG JH AG DA TW. Wrote the paper: NA EFM.

References

- Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, et al. (2006) 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 311: 991–997.
- Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, et al. (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437: 1167–1172.
- Gack MU, Shin YC, Joo CH, Urano T, Liang C, et al. (2007) TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446: 916–920.
- Yoneyama M, Fujita T (2009) RNA recognition and signal transduction by RIG-I-like receptors. *Immunol Rev* 227: 54–65.
- Sumpter R, Jr., Loo YM, Foy E, Li K, Yoneyama M, et al. (2005) Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 79: 2689–2699.
- Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M, Jr. (2008) Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 453: 523–527.
- Loo YM, Owen DM, Li K, Erickson AK, Johnson CL, et al. (2006) Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc Natl Acad Sci U S A* 103: 6001–6006.
- Lau DT, Fish PM, Sinha M, Owen DM, Lemon SM, et al. (2008) Interferon regulatory factor-3 activation, hepatic interferon-stimulated gene expression, and immune cell infiltration in hepatitis C virus patients. *Hepatology* 47: 799–809.
- Cheng G, Zhong J, Chisari FV (2006) Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. *Proc Natl Acad Sci U S A* 103: 8499–8501.
- Sadler AJ, Williams BR (2007) Structure and function of the protein kinase R. *Curr Top Microbiol Immunol* 316: 233–292.
- Blight KJ, McKeating JA, Rice CM (2002) Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 76: 13001–13014.
- Akazawa D, Date T, Morikawa K, Murayama A, Miyamoto M, et al. (2007) CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. *J Virol* 81: 5036–5045.
- Garcia MA, Mcurs EF, Esteban M (2007) The dsRNA protein kinase PKR: virus and cell control. *Biochimie* 89: 799–811.
- Terenin IM, Dmitriev SE, Andreev DE, Shatsky IN (2008) Eukaryotic translation initiation machinery can operate in a bacterial-like mode without eIF2. *Nat Struct Mol Biol* 15: 836–841.
- Bakay L, Rifo RS, Ricci EP, Decimo D, Ohlmann T (2009) Structural and functional diversity of viral IRESes. *Biochim Biophys Acta*. 1789: 512–517.
- Hugon J, Paquet C, Chang RC (2009) Could PKR inhibition modulate human neurodegeneration? *Expert Rev Neurother* 9: 1155–1157.
- Jammi NV, Whibly LR, Beal PA (2003) Small molecule inhibitors of the RNA-dependent protein kinase. *Biochem Biophys Res Commun* 308: 50–57.
- Nekhai S, Bottaro DP, Woldehawariat G, Spellerberg A, Petryshyn R (2000) A cell-permeable peptide inhibits activation of PKR and enhances cell proliferation. *Peptides* 21: 1149–1156.
- Garaiogorta U, Chisari FV (2009) Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. *Cell Host Microbe* 6: 513–522.
- Saunders LR, Barber GN (2003) The dsRNA binding protein family: critical roles, diverse cellular functions. *Faseb J* 17: 961–983.
- Dar AC, Dever TE, Sicheri F (2005) Higher-order substrate recognition of eIF2 α by the RNA-dependent protein kinase PKR. *Cell* 122: 887–900.
- Manche L, Green SR, Schmedt C, Mathews MB (1992) Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol* 12: 5238–5248.
- Nallagatla SR, Hwang J, Toroney R, Zheng X, Cameron CE, et al. (2007) 5'-triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science* 318: 1455–1458.
- Daher A, Laraki G, Singh M, Melendez-Pena CE, Bannwarth S, et al. (2009) TRBP control of PACT-induced phosphorylation of protein kinase R is reversed by stress. *Mol Cell Biol* 29: 254–265.
- Binder M, Kochs G, Bartenschlager R, Lohmann V (2007) Hepatitis C virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. *Hepatology* 46: 1365–1374.
- Shimoike T, McKenna SA, Lindhout DA, Puglisi JD (2009) Translational insensitivity to potent activation of PKR by HCV IRES RNA. *Antiviral Res* 83: 228–237.
- Wang C, Pflugheber J, Sumpter R, Jr., Sodora DL, Hui D, et al. (2003) Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication. *J Virol* 77: 3898–3912.
- Kang JI, Kwon SN, Park SH, Kim YK, Choi SY, et al. (2009) PKR protein kinase is activated by hepatitis C virus and inhibits viral replication through translational control. *Virus Res* 142: 51–56.
- Yan XB, Battaglia S, Boucreux D, Chen Z, Brechot C, et al. (2007) Mapping of the interacting domains of hepatitis C virus core protein and the double-stranded RNA-activated protein kinase PKR. *Virus Res* 125: 79–87.
- Brocard M, Paulous S, Komarova AV, Deveaux V, Kean KM (2007) Evidence that PTB does not stimulate HCV IRES-driven translation. *Virus Genes* 35: 5–15.
- Dreux M, Gastaminza P, Wieland SF, Chisari FV (2009) The autophagy machinery is required to initiate hepatitis C virus replication. *Proc Natl Acad Sci U S A* 106: 14046–14051.
- Langland JO, Cameron JM, Heck MC, Jancovich JK, Jacobs BL (2006) Inhibition of PKR by RNA and DNA viruses. *Virus Res* 119: 100–110.
- Gale M, Jr., Blakely CM, Kwieciszewski B, Tan SL, Dossert M, et al. (1998) Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Molecular & Cellular Biology* 18: 5208–5218.
- Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM (1999) Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 285: 107–110.
- Pavio N, Taylor DR, Lai MMC (2002) Detection of a novel glycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. *J Virol* 76: 1265–1272.
- Sumpter R, Jr., Wang C, Foy E, Loo YM, Gale M, Jr. (2004) Viral evolution and interferon resistance of hepatitis C virus RNA replication in a cell culture model. *J Virol* 78: 11591–11604.
- Hinson ER, Cresswell P (2009) The antiviral protein, viperin, localizes to lipid droplets via its N-terminal amphipathic α -helix. *Proc Natl Acad Sci U S A*. 106: 20152–20157.
- Strable L, Marq JB, Brini A, Hausmann S, Kolakofsky D, et al. (2007) Activation of the beta interferon promoter by unmutated Sendai virus infection requires RIG-I and is inhibited by viral C proteins. *J Virol* 81: 12227–12237.
- Kalliampakou KI, Kalamvoki M, Mavromara P (2005) Hepatitis C virus (HCV) NS5A protein downregulates HCV IRES-dependent translation. *J Gen Virol* 86: 1015–1025.

10. Breiman A, Grandvaux N, Lin R, Ottone C, Akira S, et al. (2005) Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J Virol* 79: 3969–3978.
11. Lin R, Lacoste J, Nakhaei P, Sun Q, Yang L, et al. (2006) Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-1A proteolytic cleavage. *J Virol* 80: 6072–6083.
12. Laurent AG, Krust B, Galabru J, Svab J, Hovanessian AG (1985) Monoclonal antibodies to interferon induced 68,000 Mr protein and their use for the detection of double-stranded RNA dependent protein kinase in human cells. *Proc Natl Acad Sci U S A* 82: 4341–4345.

RNA Polymerase Activity and Specific RNA Structure Are Required for Efficient HCV Replication in Cultured Cells

Asako Murayama^{1,2}, Lei Yun Weng^{2,3}, Tomoko Date¹, Daisuke Akazawa^{1,3}, Xiao Tian², Tetsuro Suzuki¹, Takanoobu Kato¹, Yasuhito Tanaka⁴, Masashi Mizokami⁵, Takaji Wakita^{1*}, Tetsuya Toyoda^{2*}

1 Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, **2** Unit of Viral Genome Regulation, Key Laboratory of Molecular Virology & Immunology, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, People's Republic of China, **3** Pharmaceutical Research Lab, Toray Industries, Inc., Kanagawa, Japan, **4** Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Aichi, Japan, **5** Research Center for Hepatitis and Immunology, Kohnodai Hospital, International Medical Center of Japan, Chiba, Japan

Abstract

We have previously reported that the NS3 helicase (N3H) and NS5B-to-3'X (N5BX) regions are important for the efficient replication of hepatitis C virus (HCV) strain JFH-1 and viral production in HuH-7 cells. In the current study, we investigated the relationships between HCV genome replication, virus production, and the structure of N5BX. We found that the Q377R, A450S, S455N, R517K, and Y561F mutations in the NS5B region resulted in up-regulation of J6CF NS5B polymerase activity *in vitro*. However, the activation effects of these mutations on viral RNA replication and virus production with JFH-1 N3H appeared to differ. In the presence of the N3H region and 3' untranslated region (UTR) of JFH-1, A450S, R517K, and Y561F together were sufficient to confer HCV genome replication activity and virus production ability to J6CF in cultured cells. Y561F was also involved in the kissing-loop interaction between SL3.2 in the NS5B region and SL2 in the 3'X region. We next analyzed the 3' structure of HCV genome RNA. The shorter polyU/UC tracts of JFH-1 resulted in more efficient RNA replication than J6CF. Furthermore, 9458G in the JFH-1 variable region (VR) was responsible for RNA replication activity because of its RNA structures. In conclusion, N3H, high polymerase activity, enhanced kissing-loop interactions, and optimal viral RNA structure in the 3'UTR were required for J6CF replication in cultured cells.

Citation: Murayama A, Weng L, Date T, Akazawa D, Tian X, et al. (2010) RNA Polymerase Activity and Specific RNA Structure Are Required for Efficient HCV Replication in Cultured Cells. *PLoS Pathog* 6(4): e1000885. doi:10.1371/journal.ppat.1000885

Editor: Michael Gale Jr., University of Washington, United States of America

Received: October 21, 2009; **Accepted:** March 30, 2010; **Published:** April 29, 2010

Copyright: © 2010 Murayama et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: A.M. is supported by the Japan Health Sciences Foundation and Viral Hepatitis Research Foundation of Japan. This work was supported by grants from the Chinese Academy of Sciences (O514P51131 and KSCX1-YW-10-03), the Chinese 973 project (2009CB522504) and the Chinese National Key Project (2008ZX1000Z-14) to T.T., and a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labor and Welfare of Japan, and from the Ministry of Education, Culture, Sports, Science and Technology and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation to T.W. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: wakita@nih.go.jp (TW); tttoyoda@sibs.ac.cn (TT)

† These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) contains a positive-stranded RNA genome and belongs to the *Flaviviridae* family [1]. Chronic HCV infection affects more than 130 million people worldwide [2]. The HCV RNA genome is approximately 9.6 kb in length and contains a long open reading frame that encodes a polyprotein of approximately 3,010 amino acids. This polyprotein is processed into at least 10 polypeptides by host and viral proteases [3,4]. The 5'-untranslated region (UTR) contains a highly conserved internal ribosome entry site (IRES) that is 341 nucleotides long [5]. The 3'UTR is known to contain a variable region (VR), a poly pyrimidine "U/C" (polyU/UC) tract, and a 98-base X-region (3'X tail) [6]. The second stem loop of the X region interacts with the NS5B/SL3 cis-acting replication element (CRE) and may contribute to initiation of negative strand RNA synthesis [7].

JFH-1 belongs to genotype 2a and is the only strain that can efficiently replicate and produce virions in HuH-7 and HuH-7-derived cell lines [8,9,10]. When the structural protein-coding regions of the non-replicating HCV strains were fused to the non-

structural protein-coding region and 3'UTR of JFH-1, replication was initiated and virions were produced in HuH-7-derived cells [10,11]. In order to analyze the mechanisms underlying the robust replication of JFH-1, we compared JFH-1 with J6CF. J6CF shares approximately 90% sequence homology with JFH-1 but does not replicate in HuH-7 cells. Analysis of JFH-1/J6CF chimeras demonstrated that the NS3 helicase-coding region (N3H) and the NS5B-to-3'X (N5BX) region of JFH-1 conferred replication activity to J6CF in HuH-7 cells [12]. Mutations in the N3H region are expected to affect helicase activity, while mutations in the NS5B-to-3'X region may affect polymerase and replication activity through secondary or higher order structures of the RNA. We have also previously reported that JFH-1-type mutations in the NS5B region enhanced genotype 1b RdRP activity *in vitro* [13]. Thus, JFH-1-type mutations in the NS5B region of J6CF are hypothesized to enhance J6CF RdRP activity. As mentioned above, the 3'UTR of the HCV genome consists of a VR, polyU/UC tracts of various lengths and a highly conserved 3'X tail. Deletion of the VR was reported to allow replication in both cultured cells [14] and in the chimpanzee [15]. The

Author Summary

Hepatitis C virus (HCV) is a major cause of chronic liver disease. Chronic HCV infection affects more than 130 million people worldwide. An efficient cell culture system is indispensable for HCV research and the development of antiviral strategies, including antiviral drugs and vaccines. Using one HCV strain, JFH-1, we have developed a novel cell culture system that, for the first time, has allowed for both the production of infectious HCV and the analysis of the HCV life cycle. To date, JFH-1 is the only HCV strain that replicates efficiently in cultured cells. Understanding the mechanisms underlying replication of JFH-1 in cultured cells is important and advantageous for the development of antiviral strategies. In the present study, we demonstrate that high polymerase activity, enhanced kissing-loop interactions between the NS5B and 3'X regions, and optimal viral RNA structure of the 3' UTR are required for the efficient replication of JFH-1 and viral production in cultured cells. Our data provides information that will prove essential for the establishment of replication-competent variants of HCV strains that are currently replication incompetent in cultured cells. This study also contributes to a better understanding of the mechanisms underlying persistent HCV infections.

minimum length of polyU/UC tract required for replication has also been previously determined [14,16].

In the current study, we examined RNA polymerase activity and the RNA structures of the NS5B and 3'UTR that contribute to HCV replication, and determined the essential domains required for robust HCV RNA replication in cultured cells.

Materials and Methods

Cell culture

HuH-7 cells [17] and Huh-7.5.1 cells [9] were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO₂ conditions.

Construction of plasmids encoding a C-terminal 12xHis tagged HCV RdRP lacking 21 C-terminal amino acids

HCV JFH-1 and J6CF RdRP without the C-terminal 21 amino acid hydrophobic sequence were PCR amplified from pJFH1 [8] and pJ6CF (a kind gift from Jens Bukh) [15], respectively. Primer sequences for mutagenesis are listed in Table S1. Following digestion with *Xba*I and *Xho*I, DNA fragments were cloned into the *Nhe*I and *Xho*I sites of pET21b (Novagen, Madison, WI), resulting in pET21bHCVJFH-1RdRpwt and pET21bHCVJ6-CFRdRpwt. pET21bHCVJFH-1RdRpwt and pET21bHCVJ6-CFRdRpwt were then digested with *Xba*I and *Xho*I and the RdRP fragments cloned into the same restriction sites of pET28a, resulting in pET21(KM)JFH-1RdRpwt and pET21(KM)J6CFRdRpwt, respectively.

Mutation analysis of J6CF and JFH-1 RdRP

JFH-1-type substitutions (S377R, A450S, S455N, R517K, and Y561F in the NS5B region; amino acid numbers are based on the AA relative numbering [18]) were introduced into J6CF RdRP and J6CF-like substitutions (S450A, N455S, K517R, F561Y, and F561I) and D318A were introduced into JFH-1 RdRP using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primer sequences for mutagenesis are listed in Table S1. Sequences were confirmed by nucleotide sequencing.

Expression, purification, and *in vitro* transcription of HCV RdRP

pET21(KM)JFH-1RdRpwt, pET21(KM)J6CFRdRpwt, and their mutants were expressed with pGEX-HSP90α [13] in *Escherichia coli* Rosetta/pLysS (Novagen). RdRP was then purified as previously described [13], with the exception that protein induction was undertaken at 18°C for 4 h. *In vitro de novo* transcription was performed as described previously [13]. Briefly, following 30 min pre-incubation without ATP, CTP, or UTP, 0.1 μM HCV RdRP was incubated in 50 mM Tris/HCl (pH 8.0), 200 mM monopotassium glutamate, 3.5 mM MnCl₂, 1 mM DTT, 0.5 mM GTP, 50 μM ATP, 50 μM CTP, and 5 μM [α -³²P]UTP, 0.02 μM RNA template (SL12-1S) and 100 U/ml human placental RNase inhibitor at 29°C for 90 min. [³²P]-RNA products were subjected to PAGE (6% gel, 8 M urea). The resulting autoradiograph was analyzed with a Typhoon trio plus image analyzer (GE Healthcare, Piscataway, NJ). The radio isotope count of 184 nt RNA product of each mutant RdRPs was measured and compared to that of JFH-1 RdRP wt in the same PAGE.

Subgenomic-replicon constructs

pSGR-J6/N3H+5BSLX-JFH1/Luc was constructed by replacement of the 5BSL-to-3'X fragment (9211 to 9678 of JFH-1) generated by PCR with the corresponding fragment of pSGR-J6/N3H+3'UTR-JFH1/Luc [12]. Constructs with substitutions in NS5B region were generated as follows; mutations were introduced by PCR-based mutagenesis and *Xho*I-*Xba*I-restricted fragments were exchanged with the corresponding fragment of pSGR-J6/N3H+5BSLX-JFH1/Luc or pSGR-J6/N3H+3'UTR-JFH1/Luc [12]. To generate the constructs used for the analyses of the 3'UTR, VR fragments (9415–9479 of JFH-1 and J6CF) or polyU/UC fragments (9480–9579 of JFH-1 and 9480–9606 of J6CF) were generated by PCR and replaced with the corresponding fragment of pSGR-J6/N3H+5BSLX-JFH1/Luc. To generate the constructs with substitutions in the VR or 3'SL2, mutations were introduced by PCR-based mutagenesis and *Sgr*AI-*Xba*I-restricted fragments were exchanged with the corresponding fragment of pSGR-J6/N3H+5BSLX-JFH1/Luc. Primer sequences for mutagenesis are listed in Table S1.

Full-length genomic HCV constructs

Plasmids used in the analysis of genomic RNA replication and core production were constructed from pJ6/N3H+N5BX-JFH1 [12] and pJ6CF [15]. pJ6/N3H+5BSLX-JFH1 was constructed by replacement of the corresponding sequence with the 5BSL-to-3'X fragment (9211 to 9678 of JFH-1) generated by PCR. pJ6/N3H+3'UTR-JFH1 was constructed by using the N3H region [*Cla*I (3929) - *Eco*T22I (5293)] and 3'UTR [*Sul*I (9415) - *Xba*I (9678)] of JFH-1 to replace the corresponding sequences of pJ6CF. Mutagenesis was performed as described above.

RNA synthesis and transfection

RNA synthesis and transfection were performed as described previously [8,12]. Briefly, plasmids were linearized with *Xba*I, treated with Mung Bean Nuclease (New England Biolabs, Ipswich, MA) and purified. Linearized, purified DNA was then used as a template for *in vitro* RNA synthesis using the MEGAscript T7 kit (Ambion, Austin, TX) in accordance with the manufacturer's instructions. Synthesized RNA was treated with DNase I (Ambion) followed by purification using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The quality of the synthesized RNA was examined via agarose gel electrophoresis. Ten micrograms of *in vitro*

synthesized RNA was used for each electroporation. Trypsinized HuH-7 cells or Huh-7.5.1 cells (3×10^6 cells) were washed with Opti-MEM 1 (Invitrogen, Carlsbad, CA) and resuspended in Cytomix buffer [19]. RNA was then combined with 400 μ l of cell suspension and the mixture was transferred to an electroporation cuvette (Bio-Rad, Hercules, CA). The cells were then pulsed at 260 V and 950 μ F using the Gene Pulser II apparatus (Bio-Rad). Transfected cells were immediately transferred to 6-well plates containing culture medium and incubated at 37°C under standard 5% CO₂ conditions.

Luciferase reporter assay

Luciferase activity of the JFH-1 subgenomic replicon and chimeras in HuH-7 cells were measured as described previously [12,20]. Briefly, 5 μ g of transcribed RNA was transfected into 3×10^6 HuH-7 cells by electroporation. Transfected cells were immediately resuspended in culture medium and seeded into 6-well culture plates. Cells were then harvested at 4, 24, and 48 h after transfection and lysed with 200 μ l of Cell Culture Lysis Reagent (Promega, Madison, WI). Debris was removed by centrifugation. Luciferase activity was quantified using a Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany) and a Luciferase Assay System (Promega). Assays were performed three times independently, with each value corrected for transfection efficiency as determined by measuring luciferase activity 4 h after transfection. Data are presented as relative light units (RLU).

Quantification of HCV core protein

To estimate the concentration of HCV core protein in the culture medium, we harvested supernatants at the indicated time points. The supernatant was then passed through a filter with a 0.22- μ m pore size (Millipore, Bedford, MA) and subjected to the chemiluminescence enzyme immunoassay (Lumipulse II HCV core assay, Fujirebio, Tokyo, Japan) in accordance with the manufacturer's instructions.

Infection of cells with secreted HCV and determination of infectivity

Culture medium from RNA transfected cells was collected at 72 hours post-transfection. Huh7.5.1 cells were seeded at a density of 1×10^4 cells per well in poly-D-lysine coated 96-well plates (CORNING, Corning, NY). On the following day, the collected culture media were serially diluted and used for inoculation of the seeded cells, and the plates were incubated for another 3 days at 37°C. The cells were fixed in methanol for 15 min at -20°C, and the infected foci were visualized by immunofluorescence as described below.

Cells were blocked for 1 hour with BlockAcc (Dainippon Sumitomo Pharma, Osaka, Japan), then washed with PBS, followed by incubation with anti-core antibody at 50 μ g/ml in BlockAcc. After incubation for 1 hour at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) in BlockAcc. The cells were then washed and examined using fluorescence microscopy (Olympus, Tokyo, Japan). Infectivity was quantified by counting the infected foci and expressed as focus forming units per milliliter (ffu/ml).

Chemicals and radio isotope

Nucleotides were purchased from GE, [α -³²P]UTP from New England Nuclear (Boston, MA), and human placental RNase inhibitor and restriction enzymes from TaKaRa (Shiga, Japan).

Statistical analysis

Significant differences were evaluated using the Student's *t*-test. $p < 0.05$ was considered significant.

RNA secondary structure prediction

RNA secondary structure prediction was performed using Mfold software [21].

Results

As we have reported previously, the NS3 helicase and the NS5B-to-3'X regions of JFH-1 are important to confer replication competence to J6CF, a replication-incompetent strain [12]. Of these two regions, NS5B-to-3'X of JFH-1 is the most important to replication-competence. The NS5B region encodes RdRP, and the JFH-1-version of this polymerase may have high activity and be crucial to replication-competence. The requirement of 3'UTR of JFH-1 suggested that the RNA structure in this region is important for efficient genome replication. To understand the mechanisms of efficient replication of JFH-1 in HuH-7 cells, we focused on the NS5B-to-3'X region because the NS3 helicase region of JFH-1 had relatively minor effects on replication of J6CF derivatives [12]. In order to identify the important protein domains within RdRP required for efficient virus replication, we compared the RNA polymerase activity of HCV J6CF RdRP to that of JFH-1 RdRP using three assays, *in vitro* transcription with purified RdRP, *in vivo* virus RNA replication, and *in vivo* virus production. To identify the important sequences or structures in the NS5B-to-3'X region involved in efficient replication, we analyzed the effect of sequence differences in this region on replication of the viral genome.

Comparison of RNA polymerase activity *in vitro*

By comparing the sequence of RdRP of JFH-1 (GenBank Accession No. AB047639), J6CF (AF177036), other 2a strains (AB047640 - 5, AY746460, AF238481 - 5, AF169002 - 5), a 1a strain (H77: AF009606), and four 1b strains (Con1: AJ238799, AB080299, AY045702, M58335), we found 14 amino acid variants unique to JFH-1 RdRP (57T, 130P, 131Q, 150A, 377R, 405I, 435V, 450S, 455N, 474M, 479H, 517K, 561F and 571S). We focused on five JFH-1-type amino acid substitutions (Q377R, A450S, S455N, R517K, and Y561F) that have been shown to increase the polymerase activity of 1b RdRP [13]. We introduced these JFH-1-type amino acid substitutions into J6CF RdRP, individually and in combination, to test their effects on polymerase activity. We also tested a J6CF RdRP variant with a R517K substitution because it was included in the J6/N3H+5BSLX-JFH1 replicon (see below), although it did not enhance the polymerase activity of 1b RdRP *in vitro* [13].

The RdRPs of HCV JFH-1 and J6CF and mutant variants were purified as indicated in the Materials and Methods and Fig. S1A. The polymerase activity of wild-type (wt) and mutant RdRPs was measured using a *de novo* transcription system (Fig. 1 and Fig. S1B). The activity of J6CF RdRP was $7.0 \pm 0.6\%$ of that of JFH-1. Similar to results seen with 1b RdRP substitution variants, the single amino acid substitutions Q377R, A450S, S455N, R517K, and Y561F resulted in increased polymerase activity of J6CF RdRP (25.5 ± 1.5 , 27.7 ± 1.0 , 53.1 ± 0.9 , 16.9 ± 3.5 and $16.7 \pm 2.5\%$ of JFH-1 RdRP wt, respectively). However, combining double and triple amino acid substitutions did not demonstrate any additive or synergistic effects on the *in vitro* polymerase activity (Fig. 1).

JFH-1 RdRP variants with individual J6CF-type amino acid substitutions, including R377Q, S450A, N455S, K517R, and F561Y, were also examined *in vitro*. With the exception of N455S,

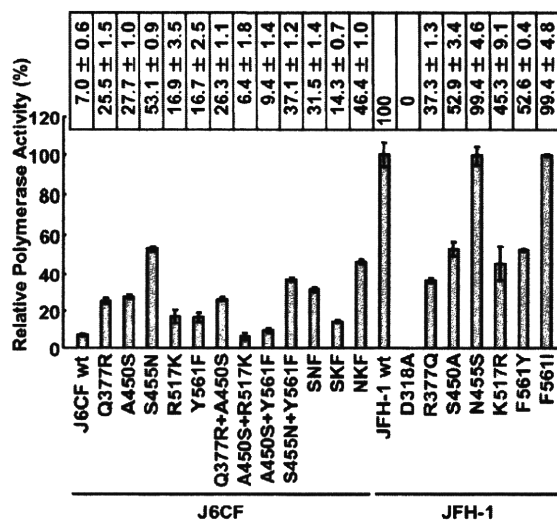


Figure 1. Relative HCV RNA polymerase activity of JFH-1 and J6CF wild-type and mutant RdRP. HCV RdRP activity was measured using the purified HCV RdRP (Fig. S1A) and the average RdRP activity and the standard deviation (error bar) relative to that of JFH-1 RdRP wt were calculated from three independent experiments (Representative gel images are shown in Fig. S1B). The relative activity values are presented above the graph. SNF, A450S+S455N+Y561F; SKF, A450S+R517K+Y561F; NKF, S455N+R517K+Y561F. doi:10.1371/journal.ppat.1000885.g001

all other J6CF-type amino acid substitutions reduced the activity of JFH-1 RdRP, with levels ranging from 37.3 to 52.9% of the activity from wt JFH-1 RdRP (Fig. 1). The N455S variant maintained polymerase activity similar to that of JFH-1 wt. The JFH-1 D318A variant has a mutation in the active site of RdRP and showed no polymerase activity, confirming our *in vitro* transcription system.

JFH-1-type amino acid residues in the NS5B region restored the replication activity of the J6CF-based replicon

In order to test whether the JFH-1-type amino acid substitutions into the NS5B region of J6CF that enhanced polymerase activity *in vitro* enabled the replication of J6CF in cultured cells, we used the subgenomic J6CF replicon harboring the NS3 helicase region and 3'UTR of JFH-1 (J6/N3H+3'UTR-JFH1-Luc; Fig. 2A) as a reference construct. This replicon could replicate in cultured cells but exhibited less than 1% of the JFH-1 replication activity [12]. In order to test the effect of JFH-1 type amino acids on replication, we introduced the five substitutions that increased polymerase activity of J6CF RdRP *in vitro* (Q377R, A450S, S455N, R517K, and Y561F, see Fig. 2B) into the subgenomic replicon J6/N3H+3'UTR-JFH1-Luc and analyzed their effects on RNA replication. Among these JFH-1-type amino acid substitutions, Y561F was the most effective ($23.2 \pm 3.5\%$ of J6/N3H+N5BX-JFH1-Luc; Fig. 2C), while A450S, S455N, and R517K exhibited only a small effect on the replication activity ($7.1 \pm 0.6\%$, $3.0 \pm 0.5\%$, and $5.5 \pm 1.0\%$ of J6/N3H+N5BX-JFH1-Luc, respectively; Fig. 2C). The Q377R mutation demonstrated no effect on replication (Fig. 2C). We next tested the effects of Y561F in combination with each of the other substitutions. We found that A450S, S455N, and R517K mutations enhanced the replication activity of Y561F ($59.1 \pm 6.1\%$, $43.9 \pm 6.6\%$, and

$57.9 \pm 4.6\%$ of J6/N3H+N5BX-JFH1-Luc, respectively; Fig. 2C). We also tested the effects of triple mutations and found that the replication activity of the SNF (A450S+S455N+Y561F) and SKF (A450S+R517K+Y561F) mutants demonstrated $86.8 \pm 6.0\%$ and $112.2 \pm 7.9\%$ replication activity of J6/N3H+N5BX-JFH1-Luc, respectively (Fig. 2C). In addition, we did not observe any significant differences between replicon activity of these mutants and that of J6/N3H+N5BX-JFH1-Luc. A combination of four mutations (SNKF; A450S+S455N+R517K+Y561F) resulted in similar activity as SKF ($115.2 \pm 11.7\%$ of J6/N3H+N5BX-JFH1-Luc; Fig. 2C). These results indicated that Y561F represented the most effective JFH-1-type mutation required for efficient replication, and that SKF and SNKF were sufficient to support replication activity equivalent to that of the replicon with the entire NS5B and 3' UTR of JFH-1 (J6/N3H+N5BX-JFH1-Luc). The additive effects of the JFH-1-type NS5B substitutions on the replicon differed from results obtained with the *in vitro* polymerase activity assay.

Next, we examined whether these substitutions were sufficient for full-genome RNA replication and virus production. We used Huh-7.5.1 cells to assess virus production because Huh-7.5.1 is highly permissive for HCV propagation [9]. We found that J6/N3H+3'UTR-JFH1-Luc showed weak replication activity (Fig. 2C), and the core protein was not detectable in the culture medium of J6/N3H+3'UTR-JFH1-transfected cells (Fig. 3B). The constructs expressing A450S, S455N, or R517K substitution variants demonstrated only very low core levels in the supernatant, while the construct expressing the Y561F mutation underwent RNA replication and produced the core protein (Y561F; $15.5 \pm 3.0\%$ of J6/N3H+N5BX-JFH1; Fig. 3B). Double mutants containing the Y561F mutation were found to produce greater amounts of core protein than the Y561F single mutant (A450S+Y561F, $57.4 \pm 3.3\%$; S455N+Y561F, $45.9 \pm 4.0\%$; and R517K+Y561F, $61.9 \pm 5.8\%$ of J6/N3H+N5BX-JFH1; Fig. 3B). The triple mutant SNF (A450S+S455N+Y561F) produced more core protein than the double mutants ($75.7 \pm 12.0\%$ of J6/N3H+N5BX-JFH1; Fig. 3B). In addition, we observed that the core production from the SKF and SNKF mutant RNA-transfected cells was similar to the levels produced by J6/N3H+N5BX-JFH1 ($111.5 \pm 8.8\%$ and $119.0 \pm 5.1\%$ of J6/N3H+N5BX-JFH1, respectively; Fig. 3B). We also measured infectivity of the supernatants from the mutant RNA-transfected cells at 72h after transfection (Fig. 3B). The levels of infectious titers correlated with the core levels among the tested constructs in this experiment. These results indicated that the SKF substitutions in the C-terminal region of NS5B were sufficient to elevate viral RNA replication and viral production.

Extra complementary sequence at the 5BSL3.2 kissing-loop interaction site of JFH-1 was essential for efficient replication

We observed a discrepancy between the *in vitro* RNA polymerase activity assay and the genome replication assay in the effects of the amino acid substitutions (Figs. 1 and 2C). Y561F was the most effective JFH-1-type amino acid substitution in the replication assay, while S455N was the most effective in the *in vitro* polymerase activity assay. As the kissing-loop interaction between 5BSL3.2 and 3'X are important for RNA replication and amino acid (aa) 561 encoding nucleotides are involved in the stem-loop 3.2 in the NS5B region (5BSL3.2) [7,16,22], we hypothesized that the cis-factor (genome structure) may also affect RNA replication in the cells. Thus, we constructed the subgenomic replicon J6/N3H+5BSLX-JFH1-Luc and the full genome construct J6/N3H+5BSLX-JFH1 that contained the NS3 helicase region and the 5BSL3-to-3'X region

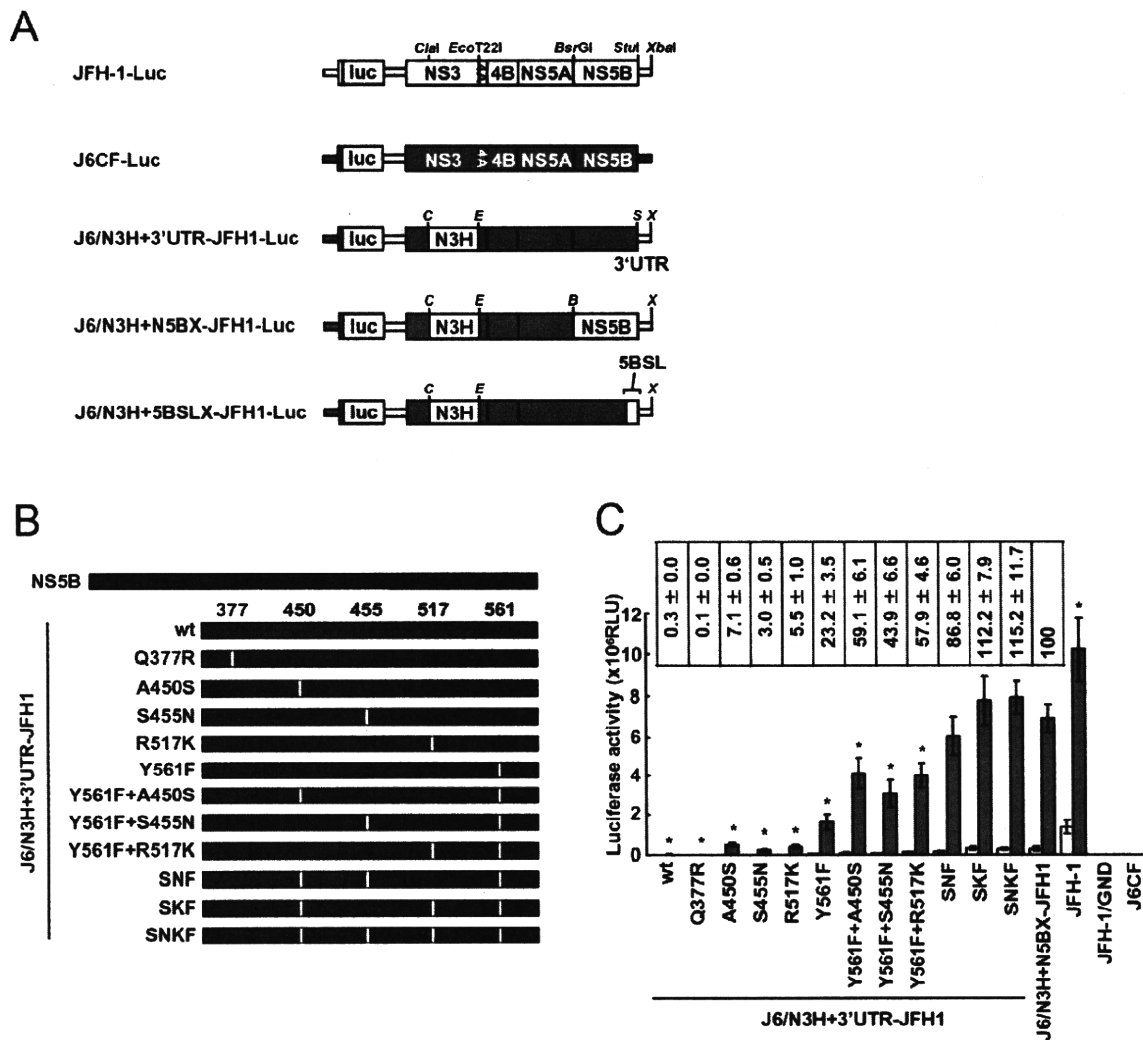


Figure 2. Luciferase activity of J6CF backbone replicons containing substitutions in the NS5B region. (A) Schematic structures of wt JFH-1 and J6CF constructs and the chimeric subgenomic replicons containing a J6CF backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. C, *Clal*; E, *EcoT221*; B, *BsrGI*; S, *StuI*; X, *XbaI*; wt, wild-type. (B) Schematic diagram of the mutations introduced into J6/N3H+3'UTR-JFH1-Luc and J6/N3H+3'UTR-JFH1. (C) Replication activity of J6CF-based replicons. Subgenomic RNA was synthesized *in vitro* from wild-type or chimeric replicon constructs. Transcribed subgenomic RNA (5 μ g) was then electroporated into HuH-7 cells and the cells harvested at 4, 24, and 48 h after transfection. The harvested cells were lysed, and the luciferase activity in the cell lysates was measured. The assays were performed three times independently and the results expressed as luciferase activities (RLU). Each value was corrected for transfection efficiency as determined by measuring the luciferase activity 4 h after transfection. Data are presented as the mean \pm standard deviation for luciferase activity at 24 h (white bars) and 48 h (gray bars) after transfection. Asterisks indicate significant differences relative to the replication activity of J6/N3H+N5BX-JFH1 ($p < 0.05$) at 48 h and the values represent the relative values against J6/N3H+N5BX-JFH1 at 48 h after transfection. SNF, A450S+S455N+Y561F; SKF, A450S+R517K+Y561F; SNKF, A450S+S455N+R517K+Y561F. doi:10.1371/journal.ppat.1000885.g002

(nucleotide (nt) 9211 to 9678) of JFH-1 (Figs. 2A and 3A), and determined their replication activity and virus production level. As presented in Figure 4B, the J6/N3H+5BSLX-JFH1-Luc construct demonstrated similar replication activity to that of J6/N3H+N5BX-JFH1-Luc 48h post-transfection (92.9 \pm 7.5% of J6/N3H+N5BX-JFH1; Fig. 4B). Moreover, both J6/N3H+N5BX-JFH1 and J6/N3H+5BSLX-JFH1 released similar levels of core protein into the supernatant (Fig. 3B).

We next analyzed the effects of mutations in the J6/N3H+5BSLX-JFH1 construct. The 5BSL region of this construct

contains three amino acid differences from J6CF (R517K, Y561F, and L571S). R517K and Y561F were important in the *in vitro* polymerase activity assay (Fig. 1). We did not assess aa 571 *in vitro* because it was deleted to purify HCV RdRP. The replication activities of J6/N3H+5BSLX-JFH1-Luc with K517R or F561Y were found to be 28 \pm 2.7% and 14 \pm 2.0% of J6/N3H+5BSLX-JFH1-Luc, respectively, confirming the importance of these JFH-1-type amino acids for replication (Fig. 4B). J6/N3H+5BSLX-JFH1-Luc with S571L revealed similar replicon activity as the J6/N3H+5BSLX-JFH1-Luc (108 \pm 7.8% of J6/N3H+5BSLX-JFH1

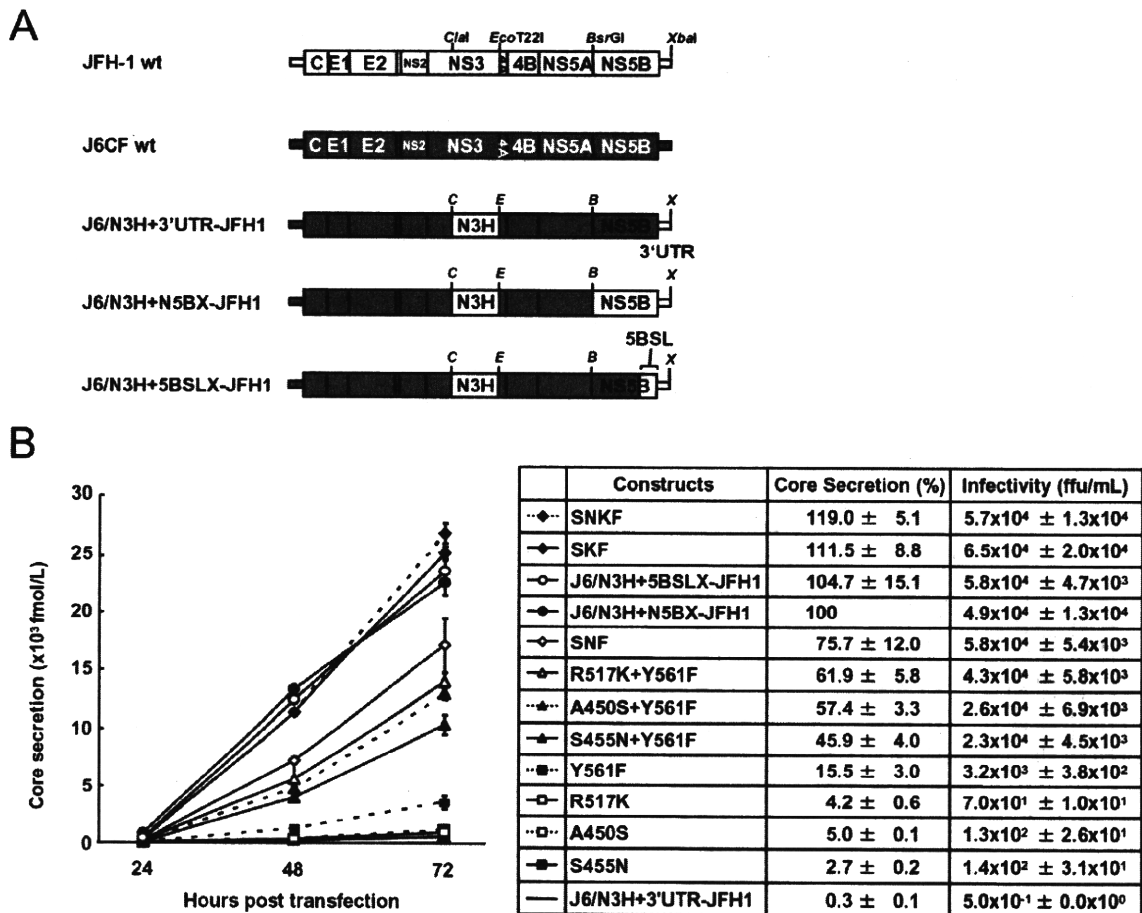


Figure 3. Analysis of transient replication of genomic chimeric HCV RNA. (A) The structure of full-length chimeric HCV RNA. Each chimeric full-length construct was prepared by the replacement of the indicated restricted fragments. The restriction enzyme recognition sites used for the plasmid constructions are indicated. C, *Clal*; E, *EcoT22I*; B, *BsrGI*; S, *StuI*; X, *XbaI*; wt, wild-type. (B) HCV core protein production in the culture medium from RNA-transfected cells. Transcribed wild-type or chimeric full-length HCV RNA (10 μ g) was transfected into Huh-7.5.1 cells. Culture medium was harvested at 4, 24, 48, and 72 h after transfection. The amount of core protein in the harvested culture medium was measured using a HCV core chemiluminescence enzyme immunoassay (Lumipulse II HCV core assay). The assays were performed three times independently, and the data are presented as the mean \pm standard deviation. Values in the right panel represent the relative core values against J6/N3H+N5BX-JFH1 at 72 h after transfection and infectious titers of the media from chimeric HCV RNA-transfected cells at 72h after transfection determined using Huh7.5.1 cells. SNF, A450S+S455N+Y561F; SKF, A450S+R517K+Y561F; SNKF, A450S+ S455N+R517K+Y561F. doi:10.1371/journal.ppat.1000885.g003

wt; Fig. 4B). These results indicated the importance of 517K and 561F but not 571F in the 5BSL region of JFH-1 in efficient RNA replication. The codon encoding aa 561 possibly affects RNA structure, as it is located in the loop of stem-loop 3.2 in NS5B (5BSL3.2) and overlaps sequences important to the kissing-loop interaction with the stem loop 2 of the 3'X SL2 [22]. Although we demonstrated that aa 561F was more effective than 561Y in RdRP activity *in vitro* (Fig. 1), it remains possible that the nucleotide mutation located at the codon of aa 561 affected the RNA structure and genome replication, as the replication activity of J6/N3H+3'UTR-JFH1-Luc with Y561F was the highest of all the clones with JFH-1 type single amino acids in the NS5B region (Fig. 2C). To investigate the effects of these mutations on RNA structure, we made mutants with nucleotide substitutions at the codon of aa 561 (Fig. 4F). The codon encoding aa 561 was UUU (Phe) for JFH-1 and UAU (Tyr) for J6CF. The third base of the

codon overlaps with the kissing sequence [22]. In order to maintain the 5BSL3.2 stem loop structure and the kissing interaction between 5BSL3.2 and 3'X SL2, the third base should be U (nt 9349 of JFH-1). JFH-1 may exhibit additional interactions between 9348U of 5BSL3.2 and 9619A of 3'X SL2 to enhance kissing-loop interaction. To assess this hypothesis, we fixed the second base (9348) as U, and the first base (9347) was altered from U to A, G or C. The G and C substitutions were predicted to disrupt the important loop structure of 5BSL3.2 using Mfold and considered to affect replication activity. We next investigated the effects of U to A substitution (AUU, F561I) in an *in vitro* assay. F561I was introduced into JFH-1 RdRP and its RdRP activity was 99.4 \pm 4.8% of the wt, demonstrating that an F to I mutation did not affect polymerase activity (Fig. 1). We also examined the effects of the F561I mutation on RNA replication in the cells, and it revealed that it had similar replication activity as the wt,

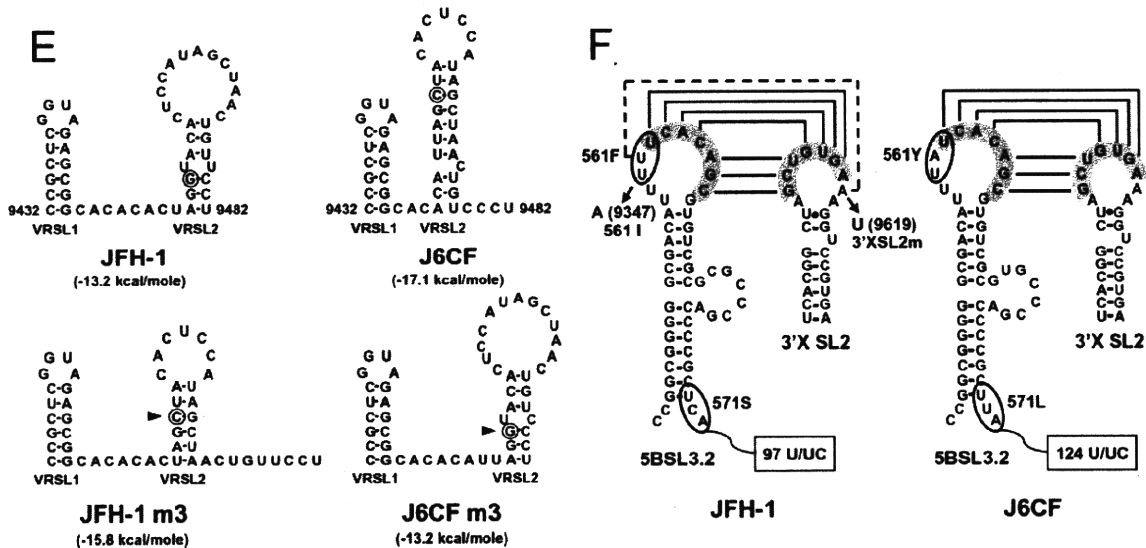
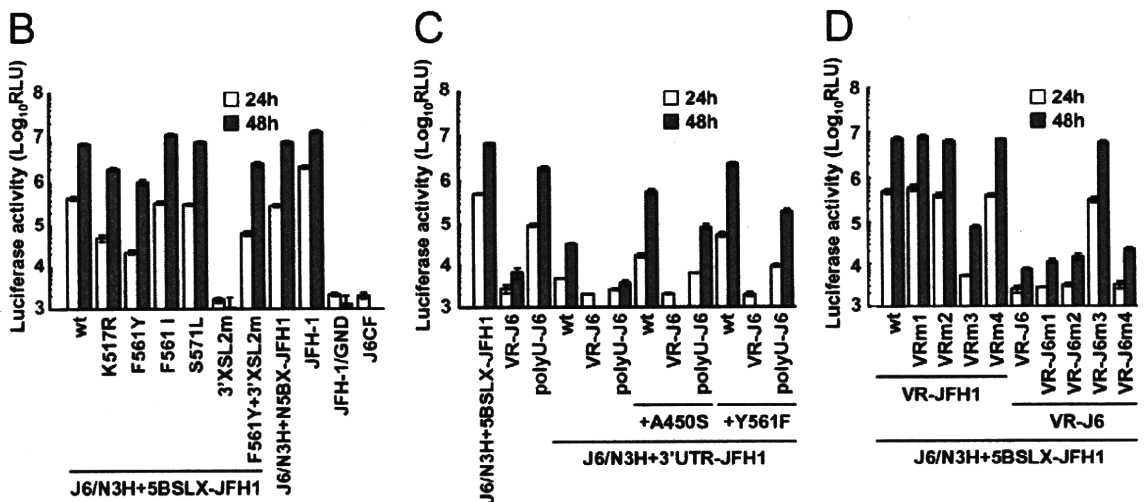
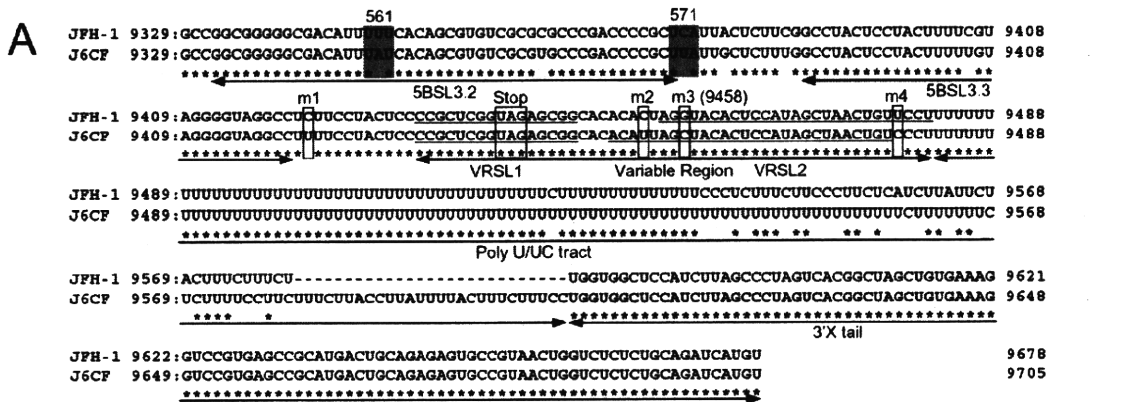


Figure 4. Replication activity of J6CF-based replicons containing variants or substitutions. (A) Comparison of the nucleotide sequence of 5BSL3.2 to 3'X of JFH-1 and J6CF. Boxes indicate nucleotide differences in VR and stop codon. Shaded boxes indicate non-synonymous variants in this region. 5BSL3.2, 5BSL3.3, Variable Region (VR), Poly U/UC tract, and 3'X tail are indicated by double-headed arrows in the figure. Stem-loop structures of VR (VRSL1 and VRSL2) are underlined. Asterisk; conserved nucleotides between JFH-1 and J6CF. (B, C, D) Replication activity of J6CF-based replicons. Five micrograms of *in vitro* synthesized RNA was electroporated into HuH-7 cells and the cells were harvested at 4, 24, and 48 h after transfection. The harvested cells were then lysed, and the luciferase activity in the cell lysates was measured. The assays were performed three times independently, and the results were expressed as luciferase activities (RLU). Data are presented as the mean \pm standard deviation for luciferase activity at 24 h (white bars) and 48 h (gray bars) after transfection. (E) The predicted secondary structure of the VR. The RNA secondary structures of JFH-1, JFH-1 m3, J6CF, and J6CF m3 were predicted by Mfold. The stem-loop structure 1 (VRSL1) and 2 (VRSL2) are indicated. Nucleotide 9458 is circled and the mutated nucleotides are indicated by arrowheads. (F) Schematic structures of the 5BSL3.2 and X tail. The predicted stem loop structure of 5BSL3.2 and SL2 of 3'X of JFH-1 and J6CF strains are indicated. The sequences forming kissing interaction with 3'X SL2 [22] are shaded. Codons encoding aa 561 and 571 are circled and the mutated sequences are indicated. The reported kissing-loop interactions are indicated by the connecting lines. The predicted interaction of the JFH-1 strain is indicated by the dotted connecting line.
doi:10.1371/journal.ppat.1000885.g004

confirming that this mutation exhibited no effect on RNA replication in cultured cells (Fig. 4B). These results demonstrated that both Phe and Ile could be substituted at aa 561 and revealed the importance of the precise RNA structure of this region. Finally, we introduced an A to U mutation at nt 9619 in the 3'X SL2 that was complementary to the second base of the codon encoding 561F (9348) to alter the kissing-loop interaction (Fig. 4F; 3'XSL2m). We observed a significant reduction in 3'XSL2m replication activity (Fig. 4B; 3'XSL2m). However, when 3'XSL2m was combined with the F561Y mutation that was expected to recover the kissing-loop interaction, replicon activity was restored (Fig. 4B; F561Y+3'XSL2m). These results indicated that the extra complementary sequence at the kissing-loop interaction site of 5BSL3.2 was important for the efficient RNA replication of JFH-1. The extra complementary sequence may enhance the kissing loop interactions. We also tested the effect of the Y561F substitution on replicons of other genotypes, H77S (GT1a) and HCV-N (GT1b). While the Y561F substitution increased replication activity in both genotype 1 strains (Text S1 and Fig. S3), the Y561F effect on the genotype 1 strains was much smaller than its corresponding effect on J6CF.

A shorter poly U/UC sequence in the JFH-1 strain favored replication

We next compared the sequences of the poly U/UC tracts of the 3'UTRs of JFH-1 and J6CF. The poly U/UC tract of JFH-1 was 27 nucleotides shorter than that of J6CF (Figs. 4A and F). The polyU stretch of the pJ6CF plasmid that we used was six nucleotides shorter than that of the original J6CF sequence reported ([15], GenBank: AF177036). In order to analyze the effects of poly U/UC length on HCV replication, the poly U/UC region of J6/N3H+5BSLX-JFH1-Luc was replaced with that of J6CF and was designated as polyU-J6. The replicon activity of J6/N3H+5BSLX-JFH1-Luc with polyU-J6 was approximately four times lower than that of the J6/N3H+5BSLX-JFH1-Luc (Fig. 4C). This result showed that longer polyU/UC region lengths of J6CF were not favorable for efficient replication.

JFH-1 type structure of the variable region was advantageous for efficient replication

When we compared the VR sequences of the 3'UTRs of JFH-1 and J6CF, we found that four nucleotides are different between the VRs of JFH-1 and J6CF and that substitution of the VR from JFH-1 with that of J6CF of J6/N3H+5BSLX-JFH1 resulted in a 1000-fold decrease in replication activity (Fig. 4C, VR-J6). Mfold analysis of predicted RNA secondary structure of the VR in JFH-1 and J6CF suggests that there are two stem-loop structures in the VR. The first stem loop (VRSL1) structure is identical in JFH-1 and J6CF, but the loop of the second stem-loop (VRSL2) is larger in JFH-1 than in J6CF (Fig. 4E). Analysis of the effects of these

nucleotide mutations on RNA structure revealed that only the third mutation (m3 at 9458 in Fig. 4A) is predicted to alter the structure of VRSL2 (Fig. 4E). The m3 G substitution into J6CF VR generated a predicted structure identical to that of JFH-1 VRSL2 resulting in identical VR structures (Fig. 4E). The m3 C substitution altered the structure of JFH-1 VR to the J6CF type (Fig. 4E). Substitutions of other nucleotides did not change the predicted structures (Data not shown). We then analyzed the effects of the mutations on replication activity. The m3 C substitution in JFH-1 VR was found to reduce replication activity 100-fold of the J6/N3H+5BSLX-JFH1-Luc (Fig. 4D; VRm3), whereas other substitutions (Fig. 4A; m1, m2 and m4) did not reduce replication activity at all (Fig. 4D; VRm1, VRm2 and VRm4). In contrast, the construct containing the J6CF VR with m3 G substitution completely restored replication activity (Fig. 4D; VR-J6m3). Other JFH-1 type nucleotide did not restore replication activity (Fig. 4D; VR-J6m1, VR-J6m2 and VR-J6m4). These results were in agreement with the stem-loop structure prediction of VR (Fig. 4E), demonstrating that the JFH-1 VR increased RNA replication. These results suggested the importance of VR secondary structure. Next, we tested if the effect of VR of JFH-1 was restricted to NS5B of JFH-1 or not. We constructed replicons with NS5B of J6CF and tested the effect on replication. The replication activities of the replicon with entire NS5B of J6CF (J6/N3H+3'UTR-JFH1), J6/N3H+3'UTR-JFH1 with A450S or Y561F (J6/N3H+3'UTR-JFH1+A450S, J6/N3H+3'UTR-JFH1+Y561F, respectively) were enhanced by the VR of JFH-1 (see polyU-J6 of each constructs in Fig. 4C) and not enhanced by the VR of J6CF (see VR-J6 of each constructs in Fig. 4C). These results indicated that the VR structure of JFH-1 was preferable for both JFH-1- and J6CF-derived NS5B and this effect was independent of the enhanced kissing-loop interaction (compare J6/N3H+3'UTR-JFH1 wt and A450S vs. Y561F in Fig. 4C).

Discussion

It has been demonstrated previously that HCV JFH-1, the only strain that replicates and produces virions efficiently in cell culture systems, expresses high replication activity without adaptive mutations [8]. We have previously reported that the N3H and N5BX regions of JFH-1 were able to rescue replication of the genotype 2a replicons [12]. The NS3 helicase and N5BX regions have been shown to be important to the virus production in HuH-7 cells. We have continued this line of experiment in the current study by focusing on RdRP activity and the genome structure in the 5BSL3.2 (CRE) to 3'X region. Following these aims, we were able to define the amino acids, nucleotides, and structural elements of JFH-1 required to confer replication competence and replication efficiency to the closely related J6CF.

In the present study, we identified five JFH-1-type amino acid residues in NS5B (Q377R, A450S, S455N, R517K, and Y561F) important for HCV replication by the *in vitro* polymerase activity assay and *in vivo* assays using replicons and full length HCV RNA. These amino acid residues are all in the thumb domain of HCV RdRP. All of these JFH-1-type substitutions increased the polymerase activity of J6CF RdRP. J6CF-type amino acids substitution into JFH-1 RdRP, including R377Q, S450A, K517R, and F561Y, reduced polymerase activity, while the N455S substitution demonstrated similar activity to the JFH-1 wt. The A450S and S455N substitutions resulted in the most significant enhancement of 1b [13] and J6CF RdRP (Fig. 1), respectively. aa 450 is located at the tip of the β -hairpin, while aa 455 is located close to the lower portions of the β -hairpin that may control the entry of the RNA template [13]. Both the β -hairpin (aa 450 to 455) and the β -strand (aa 560 to 565) of the thumb domain play an important role in RNA binding due to their extensive hydrogen-bonding network [23]. The β -hairpin has been shown to prevent the recruitment of the primer-template complex into the RNA-binding site to ensure accurate initiation from the 3' end of the template [24,25]. A450S and S455N are thought to possibly affect J6CF RdRP structure by changing the spacing of the nucleic acid binding pocket occluded by the β -hairpin. As JFH-1 N455S did not decrease the polymerase activity of JFH-1, the thumb domain of JFH-1 may be optimized to control the position or movement of the β -hairpin. Simister *et al.* have recently reported that the higher *in vitro* polymerase activity of JFH-1 was due to a higher *de novo* initiation efficiency that may be due to a closed conformation of the JFH-1 polymerase [26]. Eight amino acid mutations in NS5B of JFH-1 are hypothesized to be responsible for the conformational differences in the NS5B sequences JFH-1 and the 2a consensus [26]. However, these amino acids did not overlap with the mutations that we identified to be important for replication. Taken together, these two studies suggested that the thumb structure surrounding the β -hairpin is important to RdRP activity [26]. We only tested six of 29 amino acid differences and other mutations are possibly important to RdRP activity. However, SKF and SNKF slightly increased replication activity compared to the replicon with entire NS5B of JFH-1 (Fig. 2C). These results suggest that there may be some JFH-1-type variants in NS5B region that inhibit the replication activity of JFH-1. The JFH-1 and J6CF 5BSL regions (Fig. S2) differ in three amino acids. The JFH-1-type substitution R517K and Y561F increased replication, while the variation at aa 571 did not affect replication. This means that there are no JFH-1 variants in 5BSL region that inhibit replication activity. However, some other mutations which were not tested outside of 5BSL region may inhibit replication. Taken together, we considered that is why the replicon with 5BSLX of JFH-1 had almost the same replication activity as the replicon with entire NS5B region of JFH-1.

After comparing the activating effects of A450S and S455N vs. R517K and Y561F in the *in vitro* polymerase, *in vivo* RNA replication and virus production assays, we hypothesized that amino acids 517 and 561 likely control HCV genome replication via interactions with additional host and viral factors, including the NS3 helicase and 3'UTR. A450S enhances polymerase activity alone, while R517K and Y561F enhance genome transcription and replication activity via additional factors. The aa 455 and 517 are known to be located at the surface of the polymerase, and these mutations may affect interactions with the proteins that play important roles in RNA replication.

The combination of A450S, R517K, and Y561F substitutions conferred replication activity to the replicon with J6CF RdRP. The results of the core production were in agreement with the

results from the replicon assay and suggested that these amino acid mutations affected only RNA replication and did not affect the additional steps in the virus life cycle within the cells, such as virus particle assembly and virus secretion.

We did, however, observe a discrepancy between the effects of the mutations on *in vitro* RNA polymerase activity and *in vivo* RNA replication and virus production activities. The S455N mutation conferred the highest levels of activity on J6CF RdRP *in vitro*, while Y561F conferred the highest replication and virus production activities on J6/N3H+3'UTR-JFH1 in the cells. We did not observe any combination effects of the substitutions in the *in vitro* polymerase assays, while strong combination effects of the substitutions were observed on replication and core production *in vivo*. In addition, the combination of only three substitutions (SKF; A450S, R517K, and Y561F) was enough to increase HCV replication to levels similar to that of the construct harboring both the entire NS5B region and the 3'UTR of JFH-1. We did not observe any combination effects of the substitutions in the *in vitro* polymerase assays using 1b RdRP [13]. However, a discrepancy between polymerase activity *in vitro* and replication activity was also reported for GTP binding site mutants [27].

Discrepancies between the results from *in vitro* polymerase activity assays and *in vivo* replication assays may arise because of differences in the assay systems. In an *in vitro* polymerase assay, only enzymatic activity can be determined, while an *in vivo* assay of replication activity does not necessarily represent the only polymerase activity. Many viral and host factors may be involved in the RNA replication step in the cells. If a HCV replication assay using entirely reconstituted components were possible, we could compare the isolated effect of different polymerase variants on polymerase activity.

In addition to RdRP activity, host and viral factors, including *cis*-acting RNA structures in the 3'-genome must be considered in HCV replication in cells. In fact, we found a JFH-1-type nucleotide variant in NS5B region important to maintain the genome structure in the *in vivo* assay; this *cis*-acting factor could not have been identified using the *in vitro* polymerase assay. The SKF triple substitution contains the 561F variant that is important for enhanced kissing-loop interaction and high polymerase activity, suggesting that the effects of the SKF combination *in vivo* are rather due to the enhanced kissing-loop interaction.

We also analyzed the 5BSL3.2 and 3'XSL2 structures required for kissing-loop interactions, as aa 561 is in the loop domain of 5BSL3.2 and the activation effect of Y561F in the *in vivo* replicon assay was larger than in the *in vitro* polymerase assay. In order to test the effects of JFH-1-type variants of 5BSL3.2 on replication, we substituted the amino acids located downstream of the 5BSL3-to-3'X region (nt 9211 to 9678) from JFH-1 into the J6CF construct carrying the JFH-1-type NS3 helicase (J6/N3H+5BSLX-JFH1). The J6/N3H+5BSLX-JFH1 exhibited similar replication and virus production levels to J6/N3H+NS5B-X-JFH1. We initially focused on the amino acid differences between JFH-1 and J6CF in the region spanning between JFH-1 5BSL-to-3'X because this region was able to complement the entire JFH-1 NS5B-to-3' X region. We identified three amino acid differences (517, 561, and 571) in the 5BSLX regions of JFH-1 and J6CF. We then introduced J6CF-type substitutions into the 5BSL3.2 region of JFH-1 RdRP. The J6CF-type substitution in JFH-1 5BSL3.2 region at positions 517 and 561, but not 571, resulted in a reduction in replication. These findings were consistent with the results of the *in vitro* polymerase assay. RNA polymerase activity *in vitro* was analyzed using the Δ C21-molecule (1–570) and JFH-1 RdRP that did not contain 571S demonstrated high levels of polymerase activity, indicating that 571S may not be important for

its high polymerase activity. The codon encoding aa 517 is located outside of the 5BSL3.2 region, suggesting that this mutation only affected polymerase activity. The codon encoding aa 561 and aa 571 are within the 5BSL3.2 region. The codon encoding aa 561 is located within the loop of the 5BSL3.2, while the codon encoding aa 571 is in the spacer region located between 5BSL3.2 and 5BSL3.3. The nucleotide mutations resulting the K517R and S571L aa substitutions were predicted to maintain 5BSL3.2 RNA secondary structures similar to that of JFH-1 using Mfold analysis [21].

Since there was the possibility that Y561F mutation affected both RdRp protein activity and genomic RNA structures, we tested the effect of nucleotide substitutions in the aa 561 codon on replication. The third nucleotide (9349U) contained within the codon encoding aa 561 is conserved among the different genotypes and essential for the kissing-loop interaction [16,22]. The second nucleotide (nt 9348) of JFH-1 is a U, while that of J6CF is an A. The first nucleotide (nt 9347) of the codon should be either an A or a U, because these nucleotides are required to maintain the loop structure. Thus, a Phe (JFH-1), Tyr (J6CF and 1b), or Ile (9347A) may reside at position 561. As JFH-1 RdRp F561I retained identical activity levels to the wt (561F), hydrophobic amino acids appeared to be required in this position to maintain the high polymerase activity. Since the predicted secondary structures of 5BSL3.2 were identical for JFH-1 and J6CF, both Phe located at position 561 and the nucleotide sequence UUU in JFH-1 were essential for the high replication activity in cultured cells.

The conserved sequences of the kissing-loop interaction were UCACAGC (nt 9349–9355) in 5BSL3.2 and GCUGUGA (nt 9612–9618) in 3'X SL2. In the case of JFH-1, the nucleotide located at position 9348 was U and the nucleotide located at position 9619 was A, resulting in extended kissing-loop interaction sequence in JFH-1. When we introduced a mutation into the 3'X SL2 region (nt 9619) that was expected to abolish the extra base pair next to the interaction site, replication activity was significantly decreased. In addition, a combination of the F561Y and 3'X SL2m substitutions, expected to restore the extra base pair between nt 9348 and nt 9619, restored replication. Replication level of this double substitution was slightly lower than that of the wt constructs, possibly due to the preference for Phe at 561 over Tyr for genome replication. Mfold analysis also revealed that RNA secondary structure was not affected following the introduction of these substitutions. U at nucleotide position 9348 was previously identified in various HCV strains registered in GenBank [7]. Taken together, these findings suggested that the strong kissing-loop interaction of the JFH-1 genome supports efficient genome replication in HuH-7 cells. We also tested the effect of Y561F substitution in two other genotypes, H77S (G11a) and HCV-N (G11b). While the Y561F substitution increased replication activity in both genotype 1 strains, the Y561F effect on the genotype 1 strains was much smaller than its corresponding effect on J6CF. These results may indicate that the levels of Y561F effect for viral RNA replication are different among the genotypes. These results may also indicate that the Y561F substitution enhanced replication of strains with a substantial replication capacity. In case of J6CF, the Y561F effect was only observed with N3H region and VR of JFH-1 (Fig. 4C, compare VR-J6 and polyU-J6 of J6/N3H+3'UTR-JFH1+Y561F). This result suggested that the Y561F effect was difficult to detect with replication-incompetent clones or clones with weak replication, and also suggested that other mutations or regions are important to replicate genotype 1 replicon efficiently. Therefore, we need more efficient replicating clone of genotype 1 to determine the effect and importance of this mutation on genotype 1 strains.

We next analyzed the effects of 3'UTR structure on replication and demonstrated that the polyU/UC of JFH-1 was 27 nucleotides shorter than that of J6CF. The shorter polyU/UC and the RNA structure of the VR of JFH-1 appeared enhance efficient replication. When using the activated RdRp (SKF) and the optimal RNA structure of the 3' genome together with the JFH-1 NS3 helicase, we found that J6CF, which did not replicate in cells, was successfully converted to a replicating virus. The VR sequence is generally not conserved, even among strains within the same genotype, and the effects of VR on HCV replication remain controversial [14,15,28]. Our data revealed that the VR of JFH-1 was more favorable than that of J6CF for replication. Substitution of the VR from JFH-1 to J6CF significantly reduced replication levels 1000-fold. This dramatic change in replication activity was likely due to alterations in the RNA structure with a mutation at nt 9458. The predicted RNA structure of the VR and replication activity of the constructs containing substitutions or mutations to the VR were completely correlated. It is therefore very likely that cellular and viral factors interact with the HCV genome in this region, and that the specific nucleotide sequence and higher structure of the VR may be essential for these interactions. There is a possibility of genetic interaction between the VR and NS5B region. These kinds of interaction may also affect on polymerase activity.

The length of the polyU/UC tract appeared to be flexible and even differed within the same genotype. Even though JFH-1 and J6CF shared an identical 3'X, the JFH-1 poly U/UC tract (nt 9483–nt 9579) was 27 U shorter than that of J6CF (nt 9483–nt 9606). Thus, we examined whether the polyU/UC tract could be exchanged between JFH-1 and J6CF. The J6/N3H+5BSLX-JFH1 variant that contained the J6CF polyU/UC exhibited a four-fold reduction in replication, demonstrating that the polyU/UC did indeed affect replication. Several published papers have investigated the effects of length on the polyU/UC region [14,15,16]. Several viral and cellular proteins have also been reported to interact with the polyU sequence [29,30,31,32,33,34,35,36]. The preferential length and nucleotide sequence of the polyU/UC may be determined by interaction with these factors.

In conclusion, we found that high RdRp activity, enhanced kissing-loop interaction between 5BSL3.2 and 3'X SL2, optimal VR structure and a shorter polyU/UC tract in JFH-1 contributed to the high levels of HCV RNA replication and virus production in cultured cells. As NS3 helicase region of JFH-1 is also important for replication and viral production of J6CF, the replication enhancing mechanism of NS3 helicase region should be analyzed.

Supporting Information

Figure S1 (A). Purified HCV J6CF and JFH-1 mutant RNA polymerases. HCV RdRp variants were purified as indicated in the Materials and Methods section. Five pmol of RdRp were applied on 10% SDS-PAGE and stained with Coomassie brilliant blue. The designations of HCV J6CF and JFH-1 wt and mutants are indicated above the PAGE. M; molecular weight marker (Takara), and the position is indicated on the left. (B). Representative PAGE of *in vitro* transcription of HCV J6CF and JFH-1 mutant RNA polymerases. *In vitro de novo* transcription was performed as indicated in the Materials and Method section. [³²P]-RNA products were applied on 6% PAGE containing 8 M urea. The autoradiography was analyzed by Typhoon trio plus image analyzer. The radio isotope count of 184 nt RNA product was measured and compared to that of JFH-1 RdRp wt in the same PAGE. The designations of HCV J6CF and JFH-1 wt and mutants are indicated above the PAGE. M; [³²P]-25 base DNA

ladder (Takara), and the position is indicated on the left. The position of 184 nt RNA product is indicated on the right.
Found at: doi:10.1371/journal.ppat.1000885.s001 (0.45 MB TIF)

Figure S2 Comparisons of the amino acid sequence of NS5B of JFH-1 and J6CF. The 5BSL region is indicated with a box.
Found at: doi:10.1371/journal.ppat.1000885.s002 (0.13 MB TIF)

Figure S3 Effect of Y561F substitution on replication activity of genotype 1 replicons. Replication activity of genotype 1a (H77S: (A)) and 1b (HCV-N:(B)) replicons. Subgenomic RNA was synthesized *in vitro* from wild-type or chimeric replicon constructs. Transcribed subgenomic RNA (5 µg) was then electroporated into HuH-7 cells and the cells serially harvested 4, 24, and 48 h after transfection. The harvested cells were lysed and the luciferase activity of the cell lysates was measured. The assays were performed three times independently, and the results expressed as luciferase activities (RLU). Luciferase activity is expressed as the change in RLU (n-fold) relative to the luciferase activity 4 h after transfection. Each value was corrected for transfection efficiency as determined by measuring the luciferase activity 4 h after transfection. Data are presented as the mean ± standard deviation for luciferase activity.

References

- Lemon S, Walker C, Alter M, Yi M (2007) Hepatitis C virus. In: Knipe D, Howley P, eds. *Fields Virology* 5 ed. Philadelphia PA: Lippincott-Raven Publishers. pp 1233–1304.
- Wasley A, Alter MJ (2000) Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 20: 1–16.
- Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM (1993) Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* 67: 1385–1395.
- Hijikata M, Mizushima H, Tanji Y, Komoda Y, Hirowatari Y, et al. (1993) Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc Natl Acad Sci U S A* 90: 10773–10777.
- Tsukiyama-Kohara K, Izuka N, Kohara M, Nomoto A (1992) Internal ribosome entry site within hepatitis C virus RNA. *J Virol* 66: 1476–1483.
- Tanaka T, Kato N, Cho MJ, Shimotohno K (1995) A novel sequence found at the 3' terminus of hepatitis C virus genome. *Biochem Biophys Res Commun* 215: 744–749.
- You S, Stump DD, Branch AD, Rice CM (2004) A cis-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. *J Virol* 78: 1352–1366.
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, et al. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11: 791–796.
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, et al. (2005) Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* 102: 9294–9299.
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, et al. (2005) Complete replication of hepatitis C virus in cell culture. *Science* 309: 623–626.
- Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, et al. (2006) Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* 103: 7408–7413.
- Murayama A, Date T, Morikawa K, Akazawa D, Miyamoto M, et al. (2007) The NS3 helicase and NS5B-to-3'X regions are important for efficient hepatitis C virus strain JFH-1 replication in HuH7 cells. *J Virol* 81: 8030–8040.
- Weng L, Du J, Zhou J, Ding J, Wakita T, et al. (2009) Modification of hepatitis C virus 1b RNA polymerase to make a highly active JFH1-type polymerase by mutation of the thumb domain. *Arch Virol* 154: 763–773.
- Fricke P, Bartenschlager R (2002) Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. *J Virol* 76: 3326–3338.
- Yanagi M, Purcell RH, Emerson SU, Bukh J (1999) Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* 262: 250–263.
- You S, Rice CM (2008) 3' RNA elements in hepatitis C virus replication: kissing partners and long poly(U). *J Virol* 82: 181–195.
- Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J (1982) Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res* 42: 3838–3863.
- Kuiken C, Combet C, Bukh J, Shin IT, Deleage G, et al. (2006) A comprehensive system for consistent numbering of HCV sequences, proteins and epitopes. *Hepatology* 44: 1355–1361.
- van den Hoff MJ, Moorman AF, Lamers WH (1992) Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res* 20: 2902.
- Kato T, Date T, Miyamoto M, Sugiyama M, Tanaka Y, et al. (2005) Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. *J Clin Microbiol* 43: 3679–3684.
- Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31: 3406–3415.
- Fricke P, Boudet J, Simorre JP, Bartenschlager R (2005) Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *J Virol* 79: 380–392.
- Leveque VJ, Johnson RB, Parsons S, Ren J, Xie C, et al. (2003) Identification of a C-terminal regulatory motif in hepatitis C virus RNA-dependent RNA polymerase: structural and biochemical analysis. *J Virol* 77: 9020–9028.
- Zhong W, Ferrari E, Lesburg CA, Maag D, Ghosh SK, et al. (2000) Template/primer requirements and single nucleotide incorporation by hepatitis C virus nonstructural protein 5B polymerase. *J Virol* 74: 9131–9143.
- Hong Z, Cameron CE, Walker MP, Castro C, Yao N, et al. (2001) A novel mechanism to ensure terminal initiation by hepatitis C virus NS5B polymerase. *Virology* 285: 6–11.
- Simister P, Schmitt M, Geitmann M, Wicht O, Danielson UH, et al. (2009) Structural and functional analysis of hepatitis C virus strain JFH1 polymerase. *J Virol*.
- Cai Z, Yi M, Zhang C, Luo G (2005) Mutagenesis analysis of the rGTP-specific binding site of hepatitis C virus RNA-dependent RNA polymerase. *J Virol* 79: 11607–11617.
- Arumugawami V, Remenyi R, Kanagavel V, Sue EY, Ngoc Ho T, et al. (2008) High-resolution functional profiling of hepatitis C virus genome. *PLoS Pathog* 4: e1000182. doi:10.1371/journal.ppat.1000182.
- Kanai A, Tanabe K, Kohara M (1995) Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. *FEBS Lett* 376: 221–224.
- Luo G, Hamatake RK, Mathis DM, Racela J, Rigat KL, et al. (2000) De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. *J Virol* 74: 851–863.
- Huang L, Hwang J, Sharma SD, Hargitai MR, Chen Y, et al. (2005) Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. *J Biol Chem* 280: 36417–36428.
- Gontarek RR, Gutshall LL, Herold KM, Tsai J, Sathie GM, et al. (1999) hnRNP C and polypyrimidine tract-binding protein specifically interact with the pyrimidine-rich region within the 3'NTR of the HCV RNA genome. *Nucleic Acids Res* 27: 1457–1463.
- Luo G (1999) Cellular proteins bind to the poly(U) tract of the 3' untranslated region of hepatitis C virus RNA genome. *Virology* 256: 105–118.
- Petrik J, Parker H, Alexander GJ (1999) Human hepatic glyceraldehyde-3-phosphate dehydrogenase binds to the poly(U) tract of the 3' non-coding region of hepatitis C virus genomic RNA. *J Gen Virol* 80 (Pt 12): 3109–3113.
- Spangberg K, Wiklund L, Schwartz S (2001) Binding of the La autoantigen to the hepatitis C virus 3' untranslated region protects the RNA from rapid degradation *in vitro*. *J Gen Virol* 82: 113–120.
- Spangberg K, Wiklund L, Schwartz S (2000) HuR, a protein implicated in oncogene and growth factor mRNA decay, binds to the 3' ends of hepatitis C virus RNA of both polarities. *Virology* 271: 378–390.