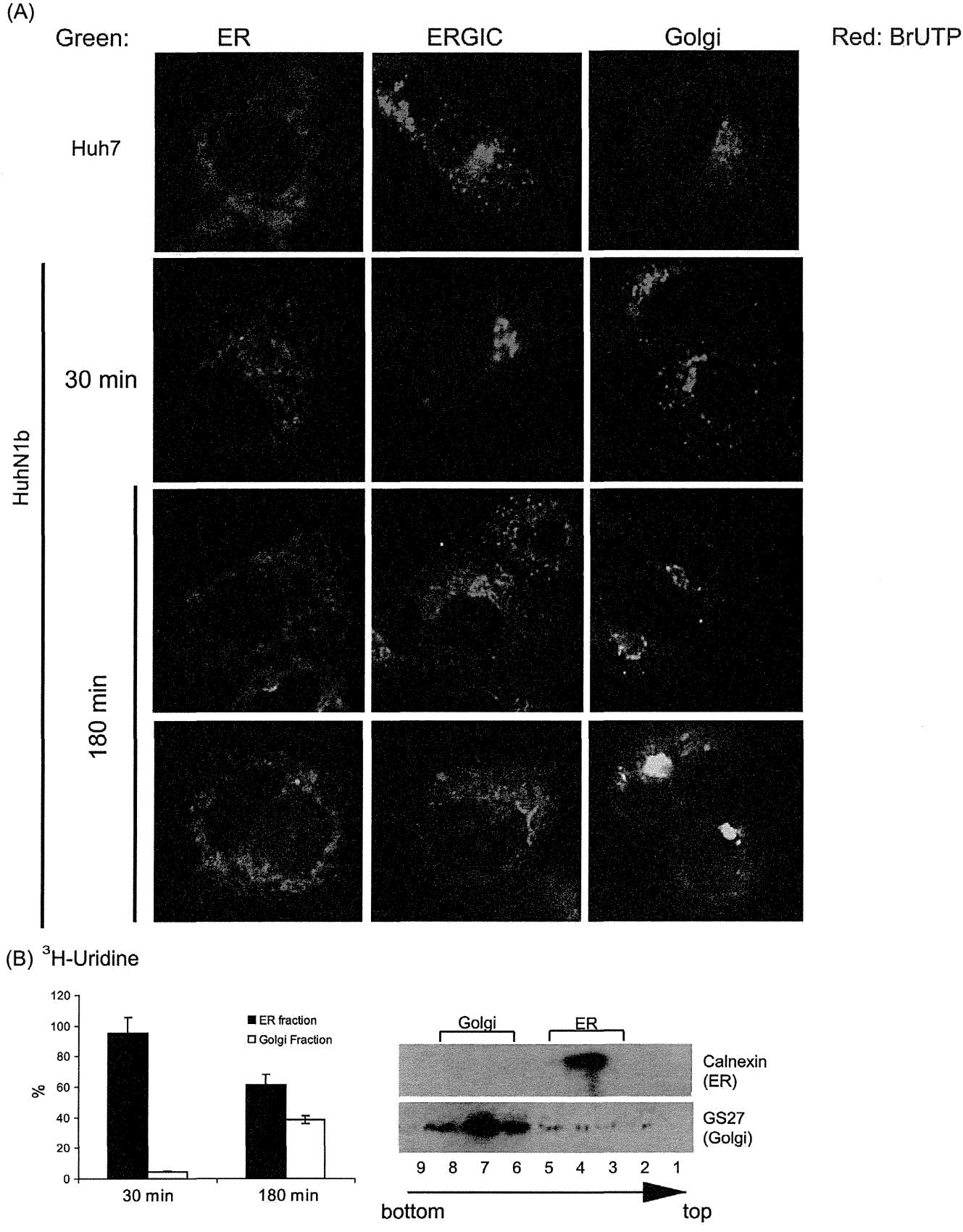

Figure 1. The translocation of newly-synthesized HCV RNA. HCV replicon cells were labeled with BrUTP (A) or ^{3}H-Uridine (B) in the presence of actinomycin D and chased for up to 180 minutes. (A) Immunofluorescence staining with anti-BrdU and other organelle antibodies shows the colocalization of BrU-labeled HCV RNA with ER initially (30 min) and then with Golgi (180 min). (B) Fractionation of ER and Golgi by sucrose gradient.

Fraction numbers and their gradient positions are noted at the bottom. ^{3}H-Uridine-labeled RNA in the ER (fraction 4) and the Golgi (fraction 6–8) fractions were collected, and the radioactivity of ^{3}H-Uridine-labeled RNA was counted. Immunoblotting of ER and Golgi makers demonstrates the separation of ER and Golgi by sucrose gradient fractionation.
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viral proteins occurred near the site of RNA synthesis. We also found that the translation of HCV proteins was dependent on active RNA synthesis: inhibition of RNA synthesis resulted in decreased HCV viral protein synthesis before there was significant decrease in the total amount of HCV RNA, and that the replication-defective HCV RNA could not be translated efficiently *in vivo*. Finally, we found that at least most of the newly synthesized HCV proteins colocalized with the newly synthesized viral proteins. These findings together thus indicate that HCV replication and translation are coupled, in the sense that replication of viral RNA is linked to translation of viral RNA in situ.

Materials and Methods

Cell Lines, HCV Full-length and Subgenomic Constructs

Huh7 or Huh7.5 cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Huh7 cells were obtained from Dr. Sato's lab [20], and Huh7.5 cells were obtained from Dr. Rice' lab [21]. Bicistronic HCV-N1b replicon was derived from the HCV-N strain with a neomycin-phosphotransferase (NPT) gene for selection as described [22]. Huh-Noe cells are stable cells derived from Huh7 cells with NPT expression as described previously [23,24]. Huh-N1b replicon cell line and Huh-Neo cells containing an NPT gene were grown under the same conditions as Huh7 cells using the same media containing 0.5 mg/ml G418.

In vitro Transcription and Electroporation of HCV Full-length and Subgenomic RNA

HCV JFH1 and JFH-GND constructs were obtained from Dr. Wakita's lab (NIID, Japan) [25]. Bicistronic replicon with either firefly luciferase (FFLuc) or Renilla luciferase (RLuc) gene was derived from HCV1bneo [22] by replacing NPT with either FFLuc or RLuc reporter gene. To prepare the template for *in vitro* transcription, the plasmids were digested by Xba I and Mungbean nuclease and gel-purified. For electroporation, Huh7 or Huh7.5 cells were trypsinized, washed and resuspended in serum-free DMEM. HCV replicon RNA or JFH full-length RNA were transcribed *in vitro* by T7 MegaScript (Ambion). A total of 6 to 10 μg of RNA and 10^{7} Huh7 cells were mixed and incubated on ice for 5 minutes and subjected to an electric pulse at 975 μF and 220 V. Cells were immediately transferred to 8 ml of DMEM containing 10% FBS for incubation.

Labeling and Immunofluorescene Staining of De Novo-synthesized Viral RNA and Newly-translated Peptides

Labeling of de novo-synthesized viral RNA, immunofluorescence staining and confocal microscopy were modified from the previously described methods [26]. Briefly, Huh7, Huh7.5 or replicon cells were plated on 8-well chamber slides at density of 1×10^{4} cells per well. Two days after seeding, cells were incubated with actinomycin D (10 μg/ml) for 1 hour to inhibit cellular RNA synthesis, and in some experiments, also with 20 nM of hippuristanol [27] for 1 hour to inhibit eIF4A-dependent protein synthesis, which represents most of the host cell protein synthesis. For immunofluorescence detection of de novo synthesized viral RNA, 2 mM of bromouridine triphosphate (BrUTP) was subsequently transfected into cells at 4°C for 15 min using Fugene 6 transfection reagent according to the manufacturer's instructions (Roche). For live-cell imaging of both RNA and proteins, Cy5-UTP and BODIPY-FL-Lys-tRNA were cotransfected to label nascent HCV RNA and peptides, respectively. For immunofluorescence staining of both nascent viral RNA and proteins, Transcend biotinyl-Lys-RNA (Promega) [28] and BrUTP were instead used. The transfected cells were washed with phosphate-buffered saline (PBS) twice and incubated at 37°C with DMEM supplemented with 10% FBS for different periods of time. After incubation, cells were washed twice with PBS and subsequently fixed with 4% formaldehyde for 1 hr at 4°C. For permeabilization, the cells were treated with 0.1% Triton X-100 in PBS supplemented with 1% FBS for 30 min at RT. Primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and incubated with cells for 1 hr at RT. After three washes in PBS, the cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated or Rhodamine-conjugated secondary antibodies, or Texas-red-conjugated streptoavidin diluted at a 1:100 with PBS containing 5% BSA for 1 hr at RT. Then the cells were washed three times in PBS and mounted in Vectashield (Vector Laboratories).

Analysis of Intracellular Viral RNA by Northern Blotting and Real-time RT PCR

To determine the quantity of RNA by real-time PCR, a single-tube reaction was performed by using the TaqMan EZ RT-PCR Core Reagents (Applied Biosystems). Duplicate reactions for RNA standards and the samples were performed in 20-μl volume using 1 μl of HCV RNA, primers from HCV 5′ non-coding region (5′ GAG TGT CGT GCA GCC TCC A 3′ and 5′ CAC TCG CAA GCA CCC TAT CA 3′) of the HCV 1b sequence [29], and a fluorescent probe [5′ (FAM) CCC GCA AGA CTG CTA GCC GAG TAG TGT TGG (TAMRA) 3′] spanning these two regions. The RT step was performed at 60°C for 50 min, followed by 1 min at 50°C. The amplification condition was as follows: 95°C for 5 min and 50 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 10 sec, and extension at 69°C for 1 min.

Using the ABI Prism 7900 program, standard curves of the assays were obtained automatically by plotting the three hold values against each standard dilution of known concentration (10^{1}–10^{6} copies per reaction) of HCV genotype 1b transcript. The same software was used to calculate the coefficients of regression. Values were normalized to that of GAPDH (Applied Biosystems). Each test was done in triplicate and averages were obtained.

Fractionation of ER and Golgi Membrane

The procedure was based on the published method [30]. Cell lysates were applied to a discontinuous sucrose gradient composed of layers of 2 M, 1.3 M, 1.0 M and 0.6 M sucrose. The ER fraction was concentrated at the interface between 0.6 M and 1.0 M sucrose, and the Golgi fraction was concentrated at the interface between 2 M and 1.3 M. To determine the signal of ^{3}H-Uridine-labeled RNA, the fractions were passed through DE81 membranes to concentrate the labeled RNA. The membranes were then counted by scintillation counter, and the ratio of signals from the ER and Golgi fractions were calculated.

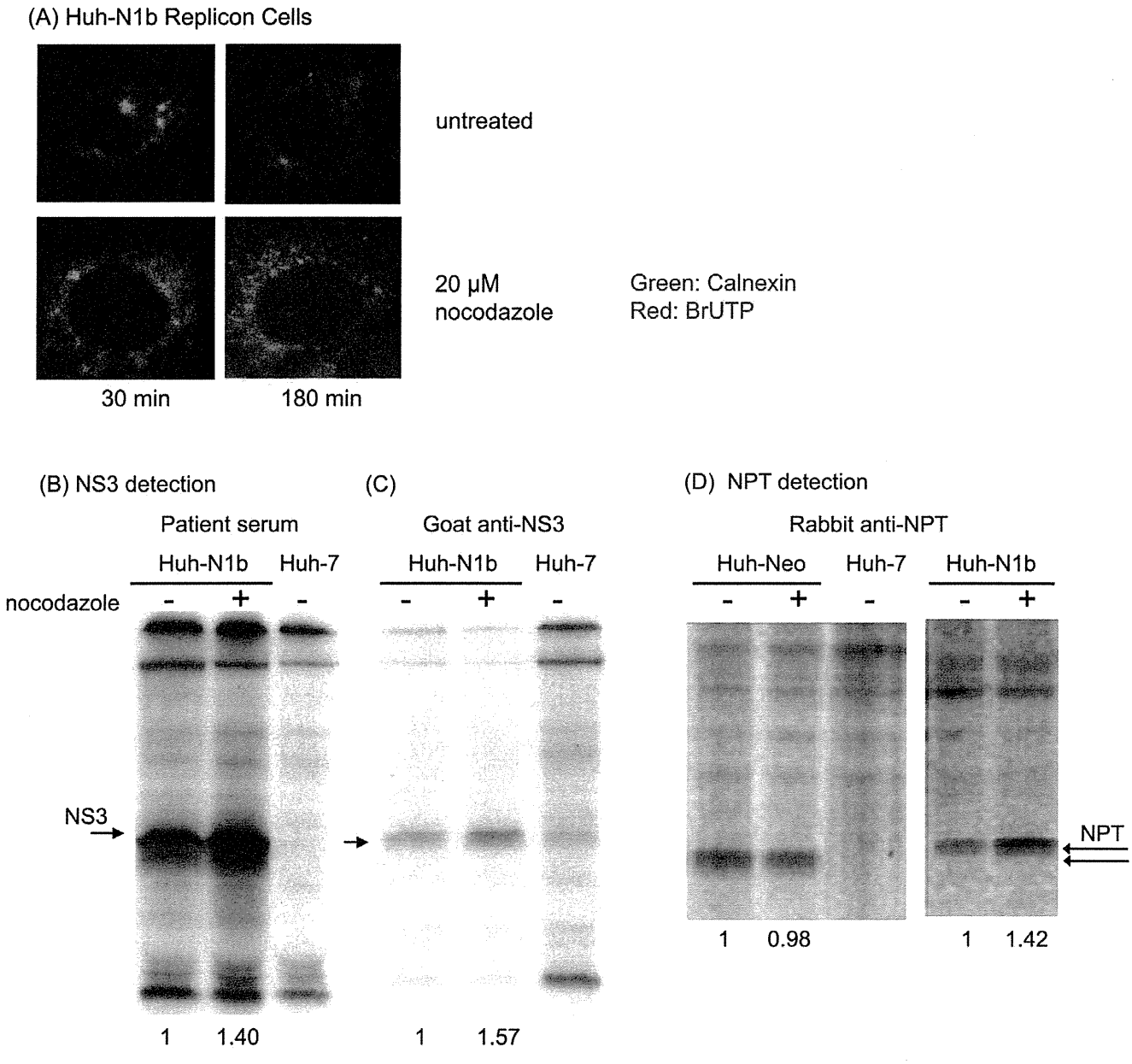


Figure 2. Increase in replicon RNA translation in nocodazole-treated HCV replicon cells. Huh-N1b cells was pre-treated with 20 μM nocodazole for 4 hours and then labeled with BrUTP or with ^{35}S-Methionine. (A) The BrU-labeled RNA remained colocalized with calnexin (an ER amrker) even after 180 min. (B–D) Proteins were immunoprecipitated with (B) HCV patient serum, (C) Goat anti-NS3 antibody, or (D) Rabbit anti-NPT antibody. The immunoprecipitated products were detected by autoradiography. The nocodazole-pretreated Huh-N1b cells showed about 50% increase in NS3 and NPT translated from replicon RNA, whereas NPT translation in the Huh-Neo control cells was not affected by the nocodazole treatment.
doi:10.1371/journal.pone.0043600.g002

Results

The Newly-synthesized HCV RNA Localizes to the ER and Moves Along with Anterograde Vesicle Trafficking

To visualize the replication of HCV RNA, we performed pulse BrUTP labeling in the actinomycin D-treated Huh-N1b replicon cells; under such conditions, only the viral RNA, which depends on RNA-dependent RNA polymerase, is labeled. After 15-minute labeling, the labeled RNA was chased in non-labeled media for 30 minutes to 3 hours, and the subcellular localization of BrU-labeled RNA was detected with anti-BrdU antibody and co-stained with individual organelle markers for ER (Calnexin), ERGIC (ERGIC53), and Golgi apparatus (GS27), respectively (Fig. 1A). Consistent with our previous report, the newly synthesized HCV RNA was initially colocalized with the ER marker (Fig. 1A). However, after 3 hours of chase, the majority of the labeled HCV RNA did not colocalize with the ER marker, but colocalized with the Golgi marker instead (Fig. 1A, bottom panels). We did not observe any significant colocalization between BrU-labeled HCV RNA and ERGIC53. As a control, the BrUTP signals could not be detected in the actinomycin D-treated Huh7 cells, while the immunofluorescence-staining patterns of the ER, ERGIC, and Golgi apparatus appeared similar between Huh7 and Huh-N1b cells (Fig. 1A).

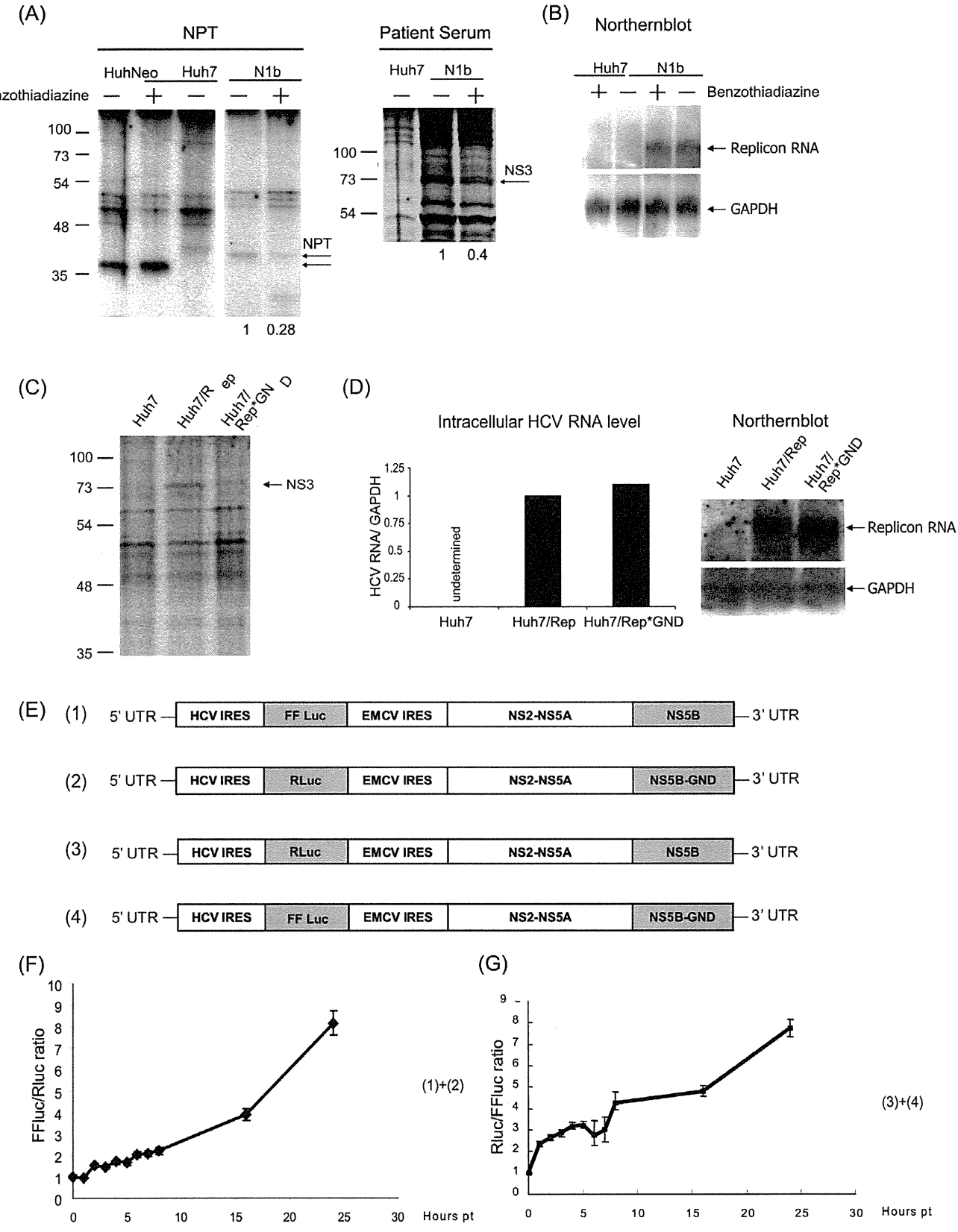


Figure 3. HCV RNA translation is dependent on the RNA transcription. (A), Mock- or Benzothiadiazine-treated Huh-N1b and Huh-Neo cells were labeled by ^{35}S-Methionine for 4 hours, and followed by immunoprecipitation with anti-NPT or anti-NS3 antibodies or sera from hepatitis C patients. The immunoprecipitates were separated by SDS-PAGE and detected by autoradiography. (B) The intracellular replicon RNA was detected by Northern blotting. (C) Huh7 cells transfected with *in vitro* transcribed Rep or the replication-defective Rep*GND RNA were metabolically labeled with ^{35}S-Methionine for 14 hours and followed by immunoprecipitation with anti-NS3 antibody. (D) The amounts of the intracellular HCV RNA in panel (C)

were determined by realtime RT-PCR and Nothern blotting. The relative ratios of the HCV RNA/GAPDH mRNA in the different cells are presented. E) Structures of the bi-cistronic replicon reporter constructs used. Time course studies of the luciferase activity in cells transfected with constructs 1 and 2 (panel (F)) and constructs 3 and 4 (panel (G)) were measured by dual luciferase assay at various time points after transfection. The ratios of the FFluc/Rluc (F) or Rluc/FFluc (G) are presented. Error bars represent +/− standard deviation.
doi:10.1371/journal.pone.0043600.g003

The ER-to-Golgi trafficking of the newly synthesized RNA was further confirmed by biochemical analysis. The actinomycin D-treated Huh-N1b cells were labeled with ^{3}H-uridine for 30 minutes and chased for 30 minutes to 3 hours. The labeled cell lysates were separated into ER and Golgi fractions by ultracentrifugation. Immunoblotting studies showed that the ER and the Golgi apparatus were efficiently separated by this procedure (Fig. 1B, right panel). The relative ratio of ^{3}H-uridine-labeled RNA in the Golgi and the ER significantly increased over time (Fig. 1B). The result suggested that the newly-synthesized HCV RNA was transported from the ER-derived to the Golgi apparatus-derived membranes.

HCV RNA Translation is Increased when Anterograde Vesicle Trafficking is Blocked

The ER-to-Golgi apparatus trafficking is known as the anterograde vesicle trafficking pathway, and can be blocked by nocodazole, which depolymerized microtubules and disrupts Golgi apparatus [31,32]. When BrUTP labeling was performed in the presence of nocodazole, BrU-labeled RNA colocalized with ER (calnexin) even after 3 hours of chase (Fig. 2A). Under these conditions, the morphology of cells was not altered by the treatment. These results suggest that the anterograde vesicle trafficking is involved in the transport of HCV RNA after its synthesis. We then further investigated if this transportation is required for certain steps of the HCV life cycle. Previously it has been shown that prolonged (more than 24 hours) nocodazole treatment inhibits HCV replication and viral production [33]; we thus tested if the nocodazole treatment could affect HCV translation. Huh7 or Huh-N1b (HCV replicon) cells were pre-treated with nocodazole for 4 hours, and then labeled with ^{35}S-Methionine for 4 hours to determine HCV translation activity (Fig. 2B). We determined the amounts of ^{35}S-Methionine-labeled Neomycin-phospho-transferase (NPT) and HCV NS3 proteins, both of which are encoded from the HCV replicon RNA but under the control of separate IRES elements. We found that after a 4-hour nocodazole treatment, the total amount of the labeled NS3 protein, as detected by anti-NS3 or HCV patients' sera, was significantly increased (Fig. 2, B and C). Similar observation was made for the NPT protein translated from the HCV replicon (Fig. 2D). Quantitation of the proteins showed a 40%–50% increase in both NPT and NS3 protein synthesis after a 4-hour nocodazole treatment. Since the translation of these two proteins was under the regulation of different sequence elements, these results suggest that the increase in HCV protein translation was not due to specific enhancement of HCV IRES-mediated translational activity. We also tested the effects of the nocodazole treatment on the NPT synthesis in the neomycin-resistant Huh-Neo cells, in which NPT is expressed from an integrated plasmid DNA. In contrast to HCV replicon cells, NPT synthesis in Huh-Neo cells was not affected by the nocodazole treatment (Fig. 2D,), indicating that the increase in HCV protein synthesis by the nocodazole treatment in HCV replicon cells was not due to enhancement of global translation.

These results are unexpected, raising a possibility that the newly-synthesized HCV RNA may be used for RNA translation in situ, without being transported away from the site of RNA synthesis. This result brought up an intriguing possibility that HCV RNA replication and translation are coupled and take place in the same replication complex.

HCV RNA Translation is Dependent on the Transcriptional Activity of the RNA

To test the idea that the newly synthesized HCV RNA is used for translation in situ, we then investigated whether translation was dependent on active RNA synthesis. We used a specific NS5B polymerase inhibitor, Benzothiadiazine [34,35], to inhibit HCV RNA synthesis and then examined the possible effects, if any, on HCV translation.

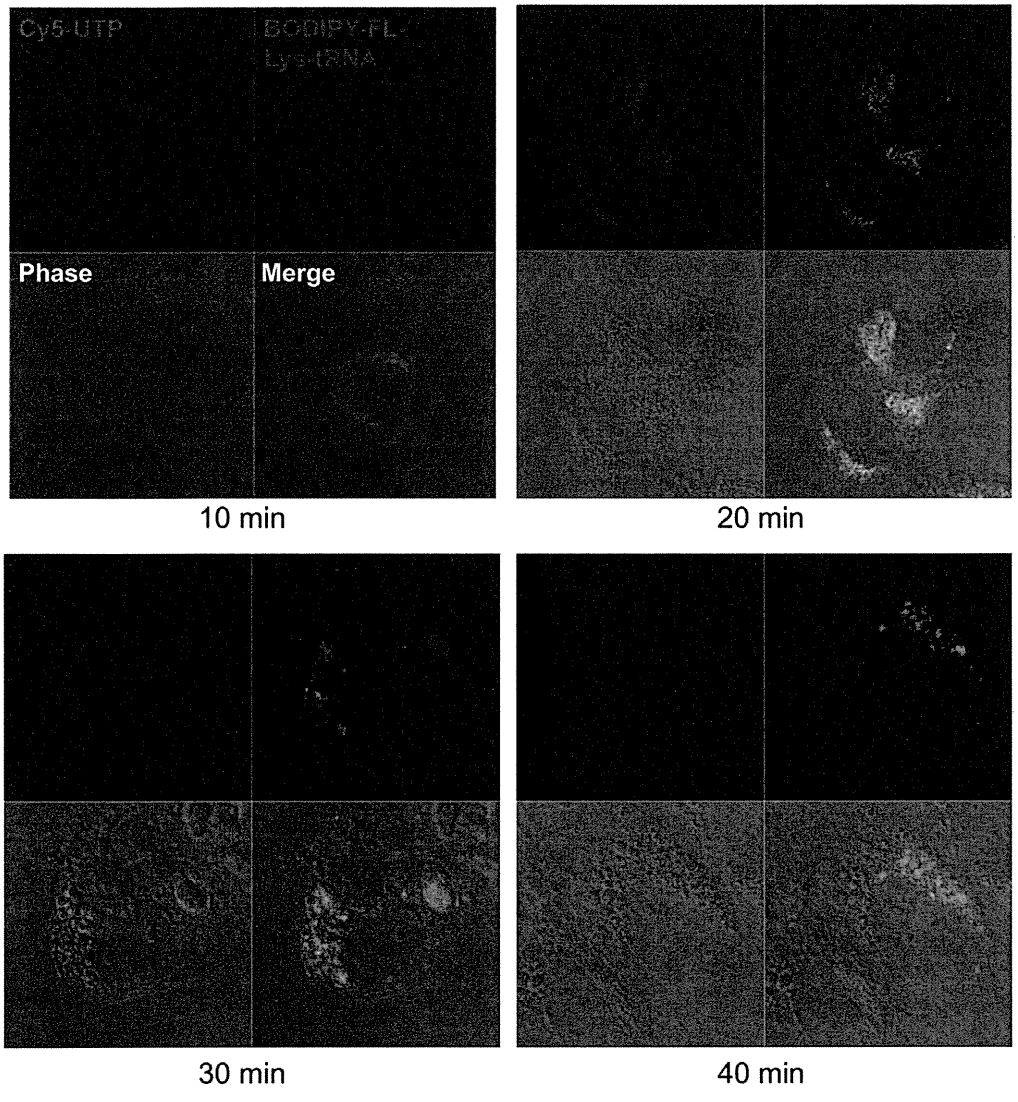
We first determined the efficiency and specificity of the inhibitor on ^{3}H-uridine incorporation (Fig. S1A). Huh7-N1b replicon cells were pretreated with or without Benzothiadiazine for 16 hours and then with actinomycin D for an additional 1 hour prior to ^{3}H-uridine labeling. Under this condition, ^{3}H-uridine is expected to be incorporated into HCV RNA only, but not cellular RNA [30]. The data showed that, in Huh7 cells, actinomycin D almost completely inhibited uridine incorporation. However, in Huh-N1b cells, actinomycin D did not completely inhibit ^{3}H-uridine incorporation; the residual incorporation likely represents HCV RNA synthesis, as confirmed by the autoradiography of the RNA products (Fig. S1A, lower panel). This residual RNA synthesis was inhibited by Benzothiadiazine. Furthermore, the Br-UTP label in Huh-N1b cells was detected as speckles in the perinuclear region; these speckles were not visible when the cells were treated with Benzothiadiazine (Fig. S1B). These results together indicate that Benzothiadiazine inhibits viral RNA synthesis specifically.

We also studied the effects of Benzothiadiazine on the steady-state level of replicon RNA by realtime RT-PCR analysis. The data showed that even after 16 hours of treatment, the total amount of replicon RNA in the cells was not significantly affected (Fig. S1C). Furthermore, the size of HCV RNA remained the same even after 16 hours of Benzothiadiazine treatment. After 2 days of treatment, however, the amounts of the replicon RNA decreased by about 50%. After 5 days, the RNA level dropped to 10% that of the control cells (Fig. S1D). As a comparison, nocodazole, which was reported to inhibit HCV RNA replication [33], had a smaller effect on the amounts of HCV RNA.

Having established the specificity of Benzothiadiazine on HCV RNA synthesis, we then labeled the Benzothiadiazine-treated cells with ^{35}S-Met and immunoprecipitated HCV proteins from the cell extracts (Fig. 3A). At 4 hour post-treatment, the amounts of newly synthesized HCV NS3 and NPT proteins were significantly decreased after the Benzothiadiazine treatment (Fig. 3A), while the amount and size of HCV RNA were not significantly affected, as determined by realtime RT-PCR and Northern blot (Fig. 3B). In contrast, in Huh-Neo cells, the Benzothiadiazine treatment did not affect the translation of NPT (Fig. 3A), indicating that Benzothiadiazine specifically inhibited translation of HCV RNA.

We further used a replication-defective replicon RNA (GND mutation in the NS5B region) to assess if active RNA replication of HCV is required for HCV protein translation. A separate experiment using an *in vitro* translation system showed that both wild-type HCV replicon and its GND mutant RNAs could produce equivalent amounts of NS2 to NS5B proteins (data not shown), indicating that the open reading frames in these RNAs are intact. The wild-type HCV replicon (Rep) and its GND mutant

HCV(JFH1)-infected Huh7.5 cells



Huh7.5 cells

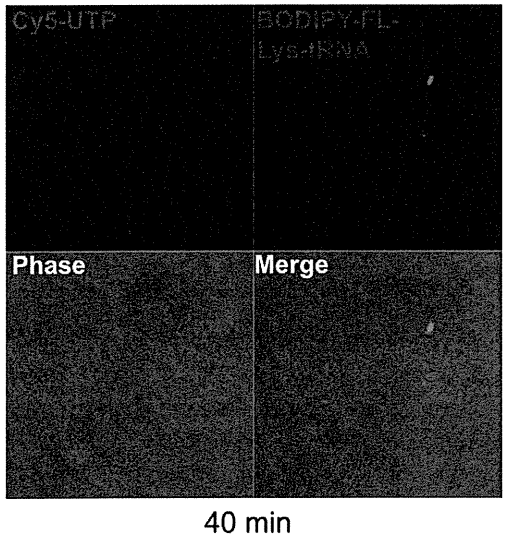


Figure 4. Double-labeling of newly-synthesized HCV RNA and newly synthesized viral peptides in JFH1-infected Huh-7.5 cells. Huh7.5 cells were infected with HCV JFH-1 strain for 2 days, and then were labeled with Cy5-UTP and BODIPY-FL-Lys-tRNA in the presence of actinomycin D and hippuristanol, which inhibit host RNA and protein synthesis, respectively. The cells were kept in 37°C chamber supplied with CO_2 for live cell imaging on a Zeiss LSM 510 laser scanning confocal microscope. Images were taken after 10–40 minutes of chase. Newly-synthesized HCV RNA was the first to be detected (as shown in red) and was in a perinuclear pattern. Newly-translated HCV viral peptides (as shown in green) were detected at later time points, completely co-localized with the sites of RNA synthesis. No significant amount of Cy5-UTP and BODIPY-FL-Lys-tRNA labeling could be detected in naïve Huh7.5 cells (as a negative control) in the presence of actinomycin D and hippuristanol.
doi:10.1371/journal.pone.0043600.g004

(Rep*GND) RNAs were then transfected into Huh7 cells, and the transfected cells were metabolically labeled with ^{35}S-Met for 14 hours to detect protein syntheses by immunoprecipitation with anti-NS3 or -NPT antibodies. The result showed that, the GND mutant yielded very little NS3 as compared with the corresponding wildtype replicon RNA (Fig. 3C). The amounts of wildtype and mutant RNAs were equivalent at 14 hours post RNA electroporation (Fig. 3D). This result suggested that HCV RNA replication enhanced the efficiency of HCV RNA translation, but could not rule out the possibilities that this enhanced translation was due to quicker degradation of the Rep*GND mutant and/or a higher copy number of the Rep RNA as a result of RNA replication. To further investigate if replication-competent HCV replicon RNA was preferentially translated, we compared the translation activity of a bicistronic Firefly luciferase replicon RNA (Luc-Rep) and the comparable but replication-defective Renilla luciferase replicon GND mutant (RLuc-RepGND) (Fig. 3E). Both constructs were first tested by *in vitro* translation assay to ensure that the both reporters were functional (data not shown). Immediately after transfection into Huh7 cells, both luciferase activities were equivalent initially; however, the FFLuc/RLuc ratio increased over time (Fig. 3F). The reverse paired replicons, RLuc-Rep and Luc-GND, also gave a similar result (Fig. 3G). We observed 2–4 folds more luciferase activities translated from the replication-competent replicon RNA than those from the GND mutants at 8 hr post-transfection (Fig. 3F & G), at which time the incoming RNAs had not yet been degraded, indicating that the replication-competent HCV RNA was preferentially translated.

We further performed a similar study using the infectious HCV clone JFH1 and its replication-defective mutant, JFH1-GND, in a time-course study of HCV protein translation. Huh7 cells were transfected with JFH1 or JFH1/GND RNA, labeled with ^{35}S-Met during the 0–8, 8–16, or 16–24 hours post-transfection, and followed by immunoprecipitation. The amounts of these two RNAs at 24 hours post-transfection were almost the same (Fig. S2B). However, only the infectious JFH RNA, but not its GND

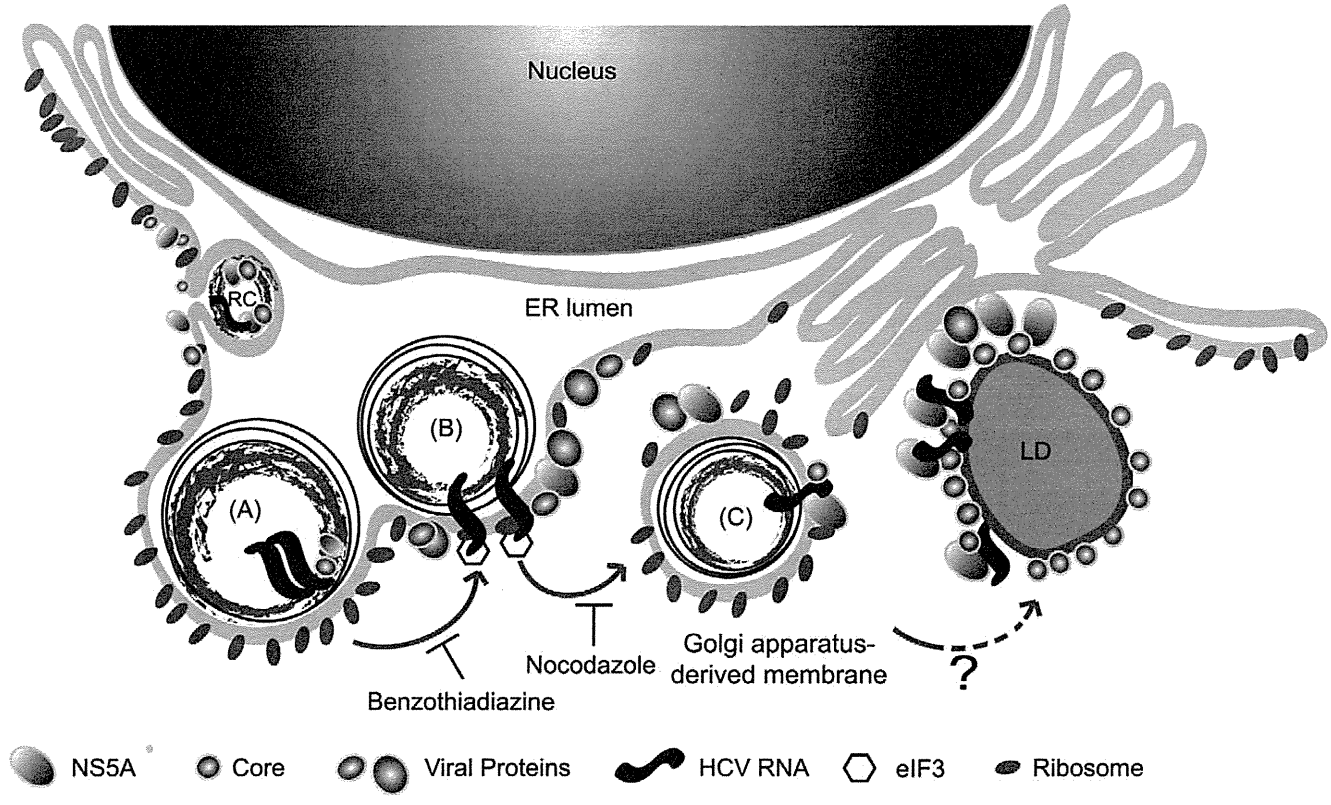


Figure 5. The proposed model of coupled replication/translation of HCV RNA. A proposed model of HCV replication-translation complex "replicasome". As reported, HCV replication complexes are assembled at ER, and then bud into the ER lumen. Consequently, (A) HCV RNA replication is first initiated in the multi-layered vesicle structure derived from the ER membrane. (B) The newly synthesized HCV RNA is translated around the ER-derived vesicle [39], where there are membrane-associated ribosomes. Benzothiadiazine blocks HCV RNA transcription and therefore decreases translation. (C) The newly-synthesized HCV RNA is later transported away from ER; nocodazole inhibits this transportation. HCV RNA is then transported to Golgi-derived membrane and/or then the lipid droplet (LD) for packaging and assembly of virus particles.
doi:10.1371/journal.pone.0043600.g005

mutant, yielded detectable amounts of NS3 and NS5A at 16–24 hours posttransfection (Fig. S2A). The kinetics of HCV protein synthesis corresponded well with the previously reported kinetics of HCV RNA synthesis following HCV (JFH1) RNA transfection [36]. The result further supported the conclusion that the replication of HCV RNA is required for competent HCV RNA translation.

We next assessed if the replication and translation of HCV RNA occurred in the same subcellular localizations. Huh7.5 cells were infected with HCV (JFH1); at 2 days after infection, the cells were labeled with Cy5-UTP and BODIPY-FL-lys-tRNA in the presence of hippuristanol and actinomycin D, which blocked eIF4A-dependent translation and host RNA transcription, respectively [27,37]. Under such a condition, Cy5-UTP will label newly synthesized HCV RNA, while BODIPY-FL-lys-tRNA will label only the newly synthesized HCV proteins since HCV translational initiation does not require eIF4A, which is blocked by hippuristanol [38]. A control experiment showed that neither dyes labeled the uninfected Huh7.5 cells (Fig. 4, lower panel). JHF-1-infected Huh7.5 cells were labeled with Cy5-UTP and BODIPY-FL-lys-tRNA for 15 minutes and then chased in unlabeled media from 10 to 40 minutes. The Cy5-U label could be detected at the early time point (10 min.) in the perinuclear region; BODIPY-FL-Lys-tRNA-labeled peptides were detected sparingly at this time, but gradually increased in intensity at later time points (Fig. 4). The earlier detection of Cy5-U label than BODIPY-FL-lys-tRNA label may have been due to the more efficient incorporation and more sensitive detection of the former label. Strong BODIPY-FL-lys-tRNA labeling signals were detected after 30 minutes of chase. Significantly, all of the BODIPY-FL-lys-tRNA labeled peptide colocalized with Cy5-U-labeled RNA at all the time points studied (Fig. 4). These findings suggest that HCV protein translation occurs on the newly synthesized RNA, and thus is likely coupled with RNA synthesis. From all the results above, we conclude that HCV RNA replication activity is a prerequisite to the efficient translation of HCV RNA.

Discussion

The mechanisms of replication and translation of HCV RNA have been extensively studied in the past few years. However, the exact subcellular localization of HCV RNA replication and translation is still unclear. Evidence has previously been presented that HCV RNA replication occurs on the detergent-resistant membrane (DRM) possibly derived from the ER [23,39]. In this report, the newly synthesized RNA was shown to be transported by the anterograde vesicle transport pathway. The microtubule-dependent mobility of newly-synthesized HCV RNA or the replication complex has also been described elsewhere [40]. Our data in this study further showed that the nocodazole treatment inhibited the transportation of the newly-synthesized RNA from the ER-derived replication complex to Golgi but did not inhibit the initiation of HCV RNA replication, since BrUTP labeling of HCV RNA occurred normally in the presence of nocodazole (Fig. 2). Intuitively, the newly-synthesized HCV RNA is expected to be transported to the site of the cellular translation machinery, similar to the case for cellular mRNAs, which are synthesized in the nucleus and transported to the cytoplasmic translation machinery for protein synthesis. However, we instead found that the movement of the HCV RNA from the ER to Golgi was not required for HCV translation, suggesting that the newly synthesized HCV RNA is used for translation near the site of HCV RNA synthesis before being transported away. Furthermore, we showed that active RNA replication was a prerequisite for efficient HCV translation. This conclusion was demonstrated using four different approaches, including studying the effects of an HCV RNA polymerase inhibitor on HCV protein translation (Fig. 3A), comparing the translation efficiencies of wildtype and replication-defective replicons (Fig. 3C) and those of infectious and non replicating JFH strain of HCV (Fig. S2A), and also by determining the relative translation efficiencies of the replicating and non-replicating dual luciferase reporter plasmids (Figs. 3F–G). Finally, we showed that the newly synthesized viral proteins almost completely colocalized with the newly synthesized viral RNA, suggesting that the sites of HCV RNA replication and protein translation nearly overlap. This mechanism of coupled RNA replication and translation may explain the previous findings that many cellular proteins, such as PTB [9,26], La antigen [10,13] and SYNCRIP [11,14], are involved in both the replication and translation in the HCV life cycle. The close proximity of these two machineries will allow for ready switches between translation and replication.

Although coupling of translation and RNA replication has been reported for many RNA viruses [16,17,41,42]_ENREF_37, the HCV case appears to be unique. For example, translation and replication of poliovirus RNA are coupled, but in the sense that RNA transcription is dependent on viral translation in *cis* [16]. Insertion of an early termination codon resulted in lower efficiency of poliovirus RNA replication. The translation and replication are regulated by the binding of different cellular or viral proteins to the 5? UTR of poliovirus RNA [43–45]. Also, the microtubule-dependent movement of poliovirus viral RNA is associated with the replication activity of viral RNA [46]. While the inactive replication complexes reside at microtubule-organizing center (MTOC), the replicating viral RNA is localized at the perinuclear sites [46]. Thus, the RNA movement is required for poliovirus replication, in contrast to the situation with HCV. In HCV, nocodazole did not inhibit viral RNA replication; also, the newly-synthesized HCV RNA failed to exit from ER after the nocodazole treatment and yet, protein translation increased; thus, the cytoskeleton-assisted movement of the newly-synthesized HCV RNA is not required for RNA translation. Thus, in HCV, the observed movement of the viral RNA from the ER-derived to the Golgi-derived membrane appears to be required for other steps of HCV replication, rather than protein translation. Due to the fact that the viral structural proteins are absent in the HCV replicon cells, this RNA movement is likely mediated by viral NS proteins, such as NS5A, which has been reported to target Golgi apparatus [47]. In a kinetics study examining the appearance of the newly synthesized HCV RNA and HCV proteins in the HCV (JFH-1)-infected cell, we also found that all of the newly synthesized proteins were at the site of newly synthesized RNA (Fig. 4). Thus, there appears to be a replication complex that carries out both replication and translation. This concept is novel to the known mechanisms of RNA virus translation and transcription.

These findings raised an important issue, namely, how the initial viral translation is carried out, since, as a positive-strand RNA virus, the very initial round of translation from the incoming HCV viral genome has to take place before viral RNA replication can occur. Conceivably, the free viral RNA genome generated by uncoating of the incoming virion in the endosome (or from the transfected viral RNA or replicons) can associate with ribosomes on the rough ER and be translated in an RNA replication-independent manner. Such translation is likely of low efficiency, but is sufficient to support first round of HCV RNA translation. These initial viral protein products and RNAs will then be encased into the membranous replication complex and become part of the

replication-translation machinery. The latter process will then become the main mechanism of HCV replication-translation.

In summary, we propose the following pathway for HCV RNA replication and translation (Fig. 5). Previous studies have shown that HCV RNA replication takes place in an ER-derived membranous vesicle [39]. The newly-synthesized viral RNA will be translated immediately after being synthesized in or around the vesicle. Benzothiadiazine treatments inhibited HCV RNA transcription and therefore inhibited HCV RNA translation as well (Fig. 5, A–B). After translation has occurred, HCV RNA is then transported via anterograde vesicle trafficking pathway away from the ER to a membrane compartment associated with the Golgi apparatus (Fig. 5C). Several other reports suggest that HCV RNA may be transported along with viral proteins (core and NS5A) to lipid droplets, where viral assembly takes place [48]; however, the kinetics of this process is unclear. Further studies in dissecting HCV RNA transportation during HCV infection will be valuable for understanding HCV life cycle comprehensively. In conclusion, this replication and translation machinery constitutes the viral "replicasome", which may reflect the membranous webs observed previously.

Supporting Information

Figure S1 Benzothiadiazine treatment in Huh-N1b replicon cells. Huh7 and Huh-N1b cells pre-treated with actinomycin D, Benzothiadiazine (NS5B inhibitor), or combination of both for 4 hours, and then labeled with ^{3}H-Uridine (A) or BrUTP (B). RNA synthesis was detected by RNA precipitation followed by scintillation counting (A), by immunofluorescence staining (B), or by real-time RT-PCR (C, D). Autoradiography of the H^{3}-uridine-labeled RNA was also performed (bottom, panel A). The relative amounts of intracellular HCV RNA after the various treatments are shown in (C, D). Benzothiadiazine specifically inhibited viral transcription, but did not affect the intracellular replicon RNA levels under this condition (C). (D), long-term Benzothiadiazine treatment (over 2 days) significantly decreased the replicon RNA levels.
(TIF)

Figure S2 Preferential translation of JFH1 wildtype over the GND mutant HCV RNA. (A) A time-course study of HCV NS protein translation in cells transfected with JFH1 or its GND mutant. The cells were labeled with S^{35}-methionine from 0–8, 8–16 and 16–24 hours posttransfection and followed by immunoprecipitation with anti-NS3 or HCV patient serum. NS3 and NS5A were detected by immunoprecipitation and separated by SDS-PAGE. (B) The corresponding intracellular HCV RNA levels in (A). The intracellular RNA levels at 24 hour post-transfection were determined by real-time RT-PCR.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HML ML. Performed the experiments: HML HA. Analyzed the data: HML JO ML. Contributed reagents/materials/analysis tools: HML HA KM. Wrote the paper: HML ML.

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Trans-complemented hepatitis C virus particles as a versatile tool for study of virus assembly and infection

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ABSTRACT

In this study, we compared the entry processes of *trans*-complemented hepatitis C virus particles (HCVtcp), cell culture-produced HCV (HCVcc) and HCV pseudoparticles (HCVpp). Anti-CD81 antibody reduced the entry of HCVtcp and HCVcc to almost background levels, and that of HCVpp by approximately 50%. Apolipoprotein E-dependent infection was observed with HCVtcp and HCVcc, but not with HCVpp, suggesting that the HCVtcp system is more relevant as a model of HCV infection than HCVpp. We improved the productivity of HCVtcp by introducing adapted mutations and by deleting sequences not required for replication from the subgenomic replicon construct. Furthermore, blind passage of the HCVtcp in packaging cells resulted in a novel mutation in the NS3 region, N1586D, which contributed to assembly of infectious virus. These results demonstrate that our plasmid-based system for efficient production of HCVtcp is beneficial for studying HCV life cycles, particularly in viral assembly and infection.

Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic liver diseases (Hoofnagle, 2002). HCV is an enveloped virus of the family *Flaviviridae*, and its genome is a positive-strand RNA consisting of the 5′-untranslated region (UTR), an open reading frame encoding viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and the 3′-UTR (Suzuki et al., 2007).

Host–virus interactions are required during the initial steps of viral infection. It was previously reported that CD81 (Bartosch et al., 2003a, b; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (Bartosch et al., 2003a, b; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. CD81 interacts with HCV E2 via a second extracellular loop (Bartosch et al., 2003a, b; Hsu et al., 2003) and its role in the internalization process was confirmed (Cormier et al., 2004; Flint et al., 2006). It has also been shown that infectious HCV particles produced in cell cultures (HCVcc) exist as apolipoprotein E (ApoE)-enriched lipoprotein particles (Chang et al., 2007) and that ApoE is important for HCV infectivity (Owen et al., 2009).

Investigation of HCV had been hampered by difficulties in amplifying the virus in vitro before development of robust cell culture systems based on JFH-1 isolates (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Retrovirus-based HCV pseudoparticles (HCVpp), in which cell entry is dependent on HCV glycoproteins, have been used to study virus entry (Bartosch et al., 2003a; Hsu et al., 2003). Vesicular stomatitis virus (VSV)-based pseudotypic viruses bearing HCV E1 and E2 and replication-competent recombinant VSV encoding HCV envelopes have also been available as surrogate models for studies of HCV infection (Mazumdar et al., 2011; Tani et al., 2007).

It was recently shown that HCV subgenomic replicons can be packaged when structural proteins are supplied in *trans* (Adair et al., 2009; Ishii et al., 2008; Masaki et al., 2010; Steinmann et al., 2008). These *trans*-complemented HCV particles (HCVtcp) are infectious, but support only single-round infection and are unable to spread. Establishment of flexible systems to efficiently produce HCVtcp should contribute to studying HCV assembly, in particular encapsidation of the viral genome, and entry to cells with less stringent biosafety and biosecurity measures. Although single-round infection can be achieved by using the HCVcc system with receptor knock-out

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cells, the single-round HCVcc system is not suitable for studying virus entry. We previously described plasmid-based production of HCVcc and HCVtcp (Masaki et al., 2010). Here, we demonstrated that HCVtcp production can be enhanced by introducing the previously reported cell-culture adaptive mutations and by deleting sequences not essential for replication in the subgenomic replicon construct. By providing genotype 1b-derived core-to-p7 in addition to intragenotypic viral proteins, chimeric HCVtcp were generated. Furthermore, blind passage of HCVtcp in the packaging cells resulted in the identification of a novel cell culture-adaptive mutation in NS3 that enables us to establish the efficient production of HCVtcp with structural proteins from various strains. Taken together, our system for producing single-cycle infectious HCV particles should be useful in the study of entry and assembly steps of the HCV life cycles. This technology may also have potential to be the basis for the safer vaccine development.

Results

Enhancement of HCVtcp production by adaptive mutations in E2, p7 and NS2 and by deleting sequences not essential for replication from replicon construct

In our HCVtcp system, the RNA polymerase I (Pol I)-driven replicon plasmid, which carries a dicistronic subgenomic luciferase reporter replicon of JFH-1 strain with a Pol I promoter and terminator (pHH/SGR-Luc), as well as a plasmid containing core-NS2 cDNA under the CAG promoter (pCAGC-NS2) were used (Masaki et al., 2010). In an effort to improve the yield of HCVtcp production, cell culture-adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) which were previously selected from serial passage of HCVcc (Russell et al., 2008) were introduced into the core-NS2 expression plasmid (Fig. 1A) (residues are numbered

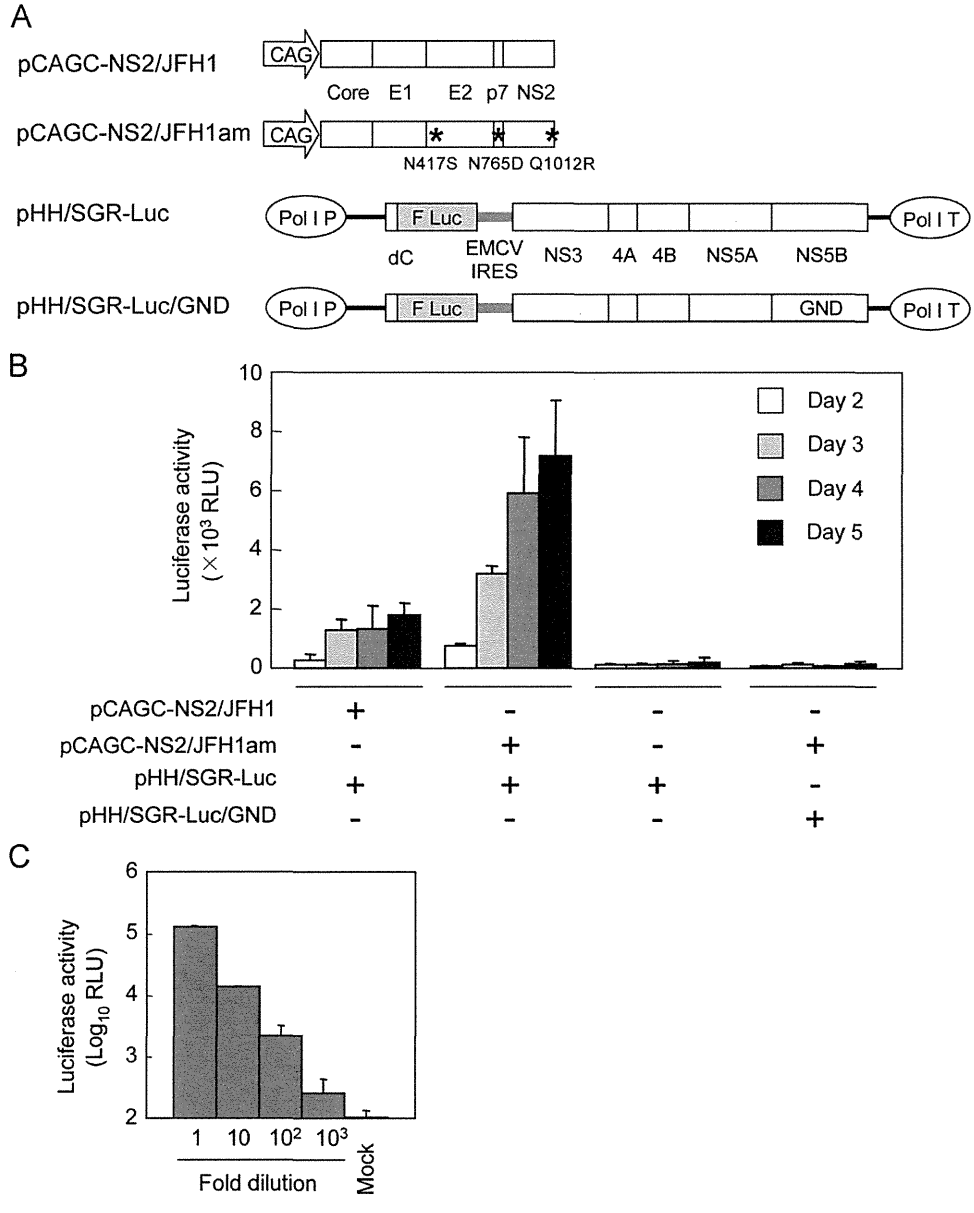

Fig. 1. HCVtcp production by two-plasmid transfection. (A) Schematic representation of plasmids is shown. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. The internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) is denoted as gray lines. Adaptive mutations are indicated as asterisks. F Luc: firefly luciferase gene; CAG: CAG promoter; Pol I P: RNA polymerase I promoter; Pol I T: RNA polymerase I terminator; GND: replication-deficient GND mutation. (B) Luciferase activity in Huh7.5.1 cells inoculated with supernatant from cells transfected with indicated plasmids at the indicated time points. Data are averages of triplicate values with error bars showing standard deviations. (C) Luciferase activity in cells inoculated with serially diluted HCVtcp.

according to positions within the JFH-1 polyprotein). Supernatants of cells transfected with plasmids (Fig. 1A) were collected and were used to infect Huh7.5.1 cells, which were analyzed by luciferase assay. Introduction of adaptive mutations (pCAGC-NS2/JFH1am) resulted in more than 4-fold higher production of HCVtcp at 5 day post-transfection, as compared to wild-type (WT) (pCAGC-NS2/JFH1) (Fig. 1B), indicating that the adaptive mutations contribute to enhancing HCVtcp production. To confirm that luciferase activity levels in HCVtcp-infected cells are correlated with the number of infectious particles, Huh7.5.1 cells were inoculated with serial dilutions of HCVtcp. Luciferase activity was well correlated with viral load (Fig. 1C), indicating that luciferase assay in HCVtcp-infected cells can be used to quantify HCV infection.

In order to further explore the efficient production of HCVtcp, we generated replicon constructs that lack the luciferase gene or include the partial coding sequences for structural proteins instead of reporter (Fig. 2A). Replication of each replicon in plasmid-transfected cells was then assessed by Western blotting (Fig. 2B). Among the constructs tested, NS5B levels were lowest in cells expressing pHH/SGR-Luc. NS5B levels in cells replicating other replicons appeared to be comparable. Cells were infected with supernatants of cells transfected with each replicon plasmid, along with pCAGC-NS2/JFH1am, followed by infectious unit assay (Fig. 2C). The highest production of HCVtcp was obtained from cells transfected with pHH/SGR, where the luciferase sequence was deleted from pHH/SGR-Luc, thus suggesting that deletion of the sequence not essential for RNA replication in the replicon may contribute to enhancing HCVtcp production.

Production of chimeric HCVtcp by providing heterologous core-p7

In order to elucidate whether *trans*-encapsidation of JFH-1 replicon can be achieved by providing core-p7 from other HCV strains, core-NS2 plasmids were constructed (Fig. 3A). In these plasmids, core through the N-terminal 33 aa of NS2, which contains transmembrane domain 1 of NS2, was derived from either H77c (genotype 1a), THpa (genotype 1b), Con1 (genotype 1b) or J6 (genotype 2a) strain. Residual NS2 was derived from JFH-1, as described previously (Pietschmann et al., 2006). HCVtcp was efficiently produced by core-p7 of J6 and THpa strains, but its production was less efficient in the case of Con1 strain. *Trans*-packaging was not detectable when core-p7 of H77c strain was used (Fig. 3C). Among HCV strains tested, difference in luciferase activity levels in HCVtcp-infected cells (Fig. 3C) were in agreement with that in the viral RNA levels in the culture supernatants of the transfected cells (Fig. 3B). Although the efficacy of *trans*-complementation was variable among strains, chimeric HCVtcp can be generated by providing genotype 1b-derived core-p7 in addition to intragenotypic viral proteins, and was used in subsequent studies.

ApoE- and CD81-dependent infection by HCVtcp

There is accumulating evidence that apolipoproteins, particularly ApoE, contribute to HCV production and infectivity (Chang et al., 2007; Owen et al., 2009). To determine whether ApoE is involved in infection of target cells by HCVtcp, we infected cells in the presence of increasing concentrations of anti-ApoE antibody.

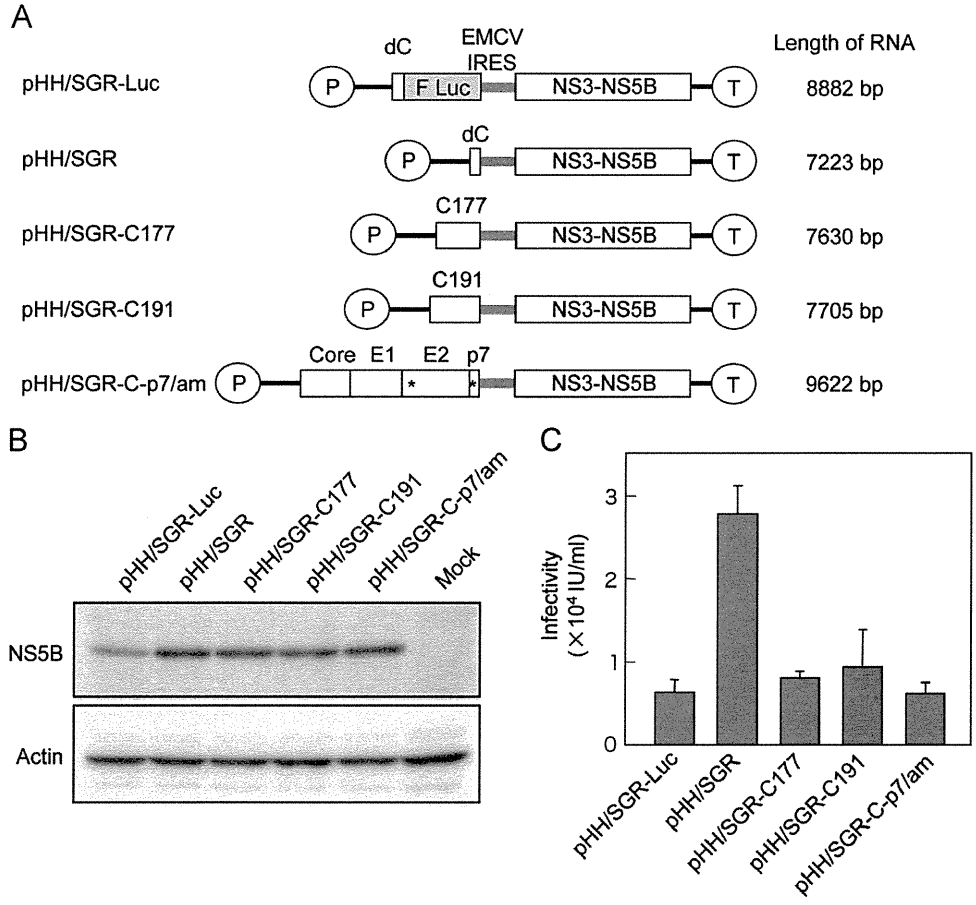

Fig. 2. Production of HCVtcp with different replicon constructs. (A) Schematic representation of plasmids used for production of HCVtcp. Deduced length of transcribed RNA from each construct is shown on the right. HCV polyproteins from JFH-1 strain are indicated by open boxes. HCV UTRs are indicated by bold lines. The EMCV IRES is denoted by gray bars. Adaptive mutations are indicated by asterisks. F Luc: firefly luciferase gene; P: RNA polymerase I promoter; T: RNA polymerase I terminator. (B) Detection of NS5B and actin in Huh7.5.1 cells transfected with indicated plasmids at 4 day post-transfection. (C) Infectivity of culture supernatants from cells transfected with indicated replicon plasmids along with pCAGC-NS2/JFH1am at 4 day post-transfection.

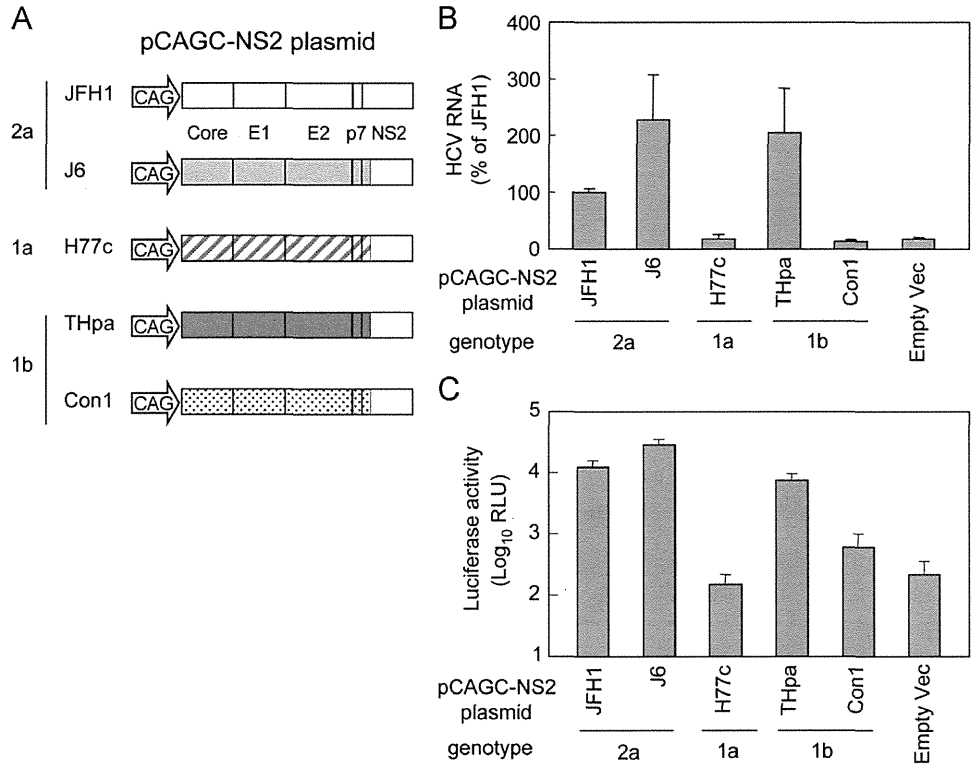

Fig. 3. HCVtcp production with structural proteins from various strains. (A) Schematic representation of plasmids used. HCV polyproteins of JFH-1, J6, H77c, THpa and Con1 strain are shown in the open box, bright gray box, box with diagonal lines, dark gray box and dotted box, respectively. (B) Relative levels of HCV RNA in the supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc. (C) Luciferase activity in cells inoculated with supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc at 4 day post-transfection.

pCAGC-NS2/THpa and pCAGC-NS2/JFH1am were used as core-NS2 plasmids for HCVtcp production carrying core-p7 derived from genotypes 1b and 2a (HCVtcp-1b and HCVtcp-2a, respectively). HCVpp derived from JFH-1 and VSVpp were generated and used for comparison. Infection with HCVtcp-1b or HCVtcp-2a was blocked by anti-ApoE antibody in a dose-dependent manner. In contrast, anti-ApoE antibody did not affect infection with HCVpp and VSVpp (Fig. 4A).

The CD81 dependence of infection was also compared between HCVtcp and HCVpp (Fig. 4B). Anti-CD81 antibody inhibited the entry of HCVtcp-1b, HCVtcp-2a, and HCVpp in a dose-dependent manner. The antibody had no effect on VSVpp infection. HCVtcp infection appears to be more sensitive to anti-CD81 antibody when compared with HCVpp infection; more than 60% inhibition was observed at 0.08 μg/mL anti-CD81 antibody for HCVtcp-1b and HCVtcp-2a, whereas approximately 50% inhibition was observed for HCVpp at 2 μg/mL antibody. Neutralization of HCVcc by anti-ApoE and anti-CD81 antibodies was also determined. Antibodies blocked HCVcc infection (Fig. 4C and D), as observed with HCVtcp. These results suggest that ApoE, as well as CD81, play an important role in HCVtcp infection. Thus, HCVtcp may be more useful for evaluating the HCV entry process than HCVpp.

Identification of novel culture-adaptive mutation in NS3 by serial passage of HCVtcp in packaging cells

The HCVtcp system was further applied to analyses of genetic changes during serial passages in target cells. As an initial attempt, supernatants of cells co-transfected with pCAGC-NS2/JFH1am and pHH/SGR were inoculated into Huh7.5.1 cells transiently transfected with pCAGC-NS2/JFH1am. However, infectious titer was lost after repeated inoculation, likely due to low HCVtcp titers and low efficiency of plasmid transduction (data not shown). To overcome this, we utilized recombinant adenovirus vectors (rAdVs) to provide core-NS2. As we were not able to obtain rAdV directly expressing core-NS2, conditional transgene expression based on a Cre-loxP strategy was employed (Kanegae et al., 1995). We constructed an rAdV containing core-NS2 gene downstream of a stuffer DNA flanked by a pair of loxP sites (AxCALNLH-CNS2). When cells were doubly infected with AxCALNLH-CNS2 and the Cre-expressing rAdV, AxCANCre (Kanegae et al., 1995), the Cre-mediated excisional deletion removed the stuffer DNA, resulting in core-NS2 expression under control of the CAG promoter (Fig. 5A). As expected, tightly regulated production of HCVtcp was observed. The cells infected with AxCANCre and AxCALNLH-CNS2 along with transduction of pHH/SGR-Luc produced HCVtcp at high levels. Production of HCVtcp was undetectable when either AxCANCre or AxCALNLH-CNS2 was not infected (Fig. 5B). The Cre-mediated rAdV expression system appears to have yielded considerably higher production of HCVtcp when compared with the settings for plasmid co-transfection.

Supernatants from cells in which core-NS2 was expressed using rAdVs and the subgenomic RNA derived from pHH/SGR replicated were inoculated into cells infected with AxCALNLH-CNS2 and AxCANCre (Fig. 6A). Blind passage was performed by sequentially transferring culture supernatants to cells infected with the above rAdVs. The two independent 10 blind passages (p10) showed virus titers of $>1\times10^6$ IU/mL, which were markedly higher than those of the passage 0 (p0) stock cultures (4×10^4 IU/mL). Side-by-side infection analysis revealed that the HCVtcp p10 #1 achieved a virus titer approximately 36 times higher than that of HCVtcp p0 on the packaging cells at 6 day post-infection (Fig. 6B). Sequencing of the entire replicon in the supernatants at p10 in two independent experiments revealed

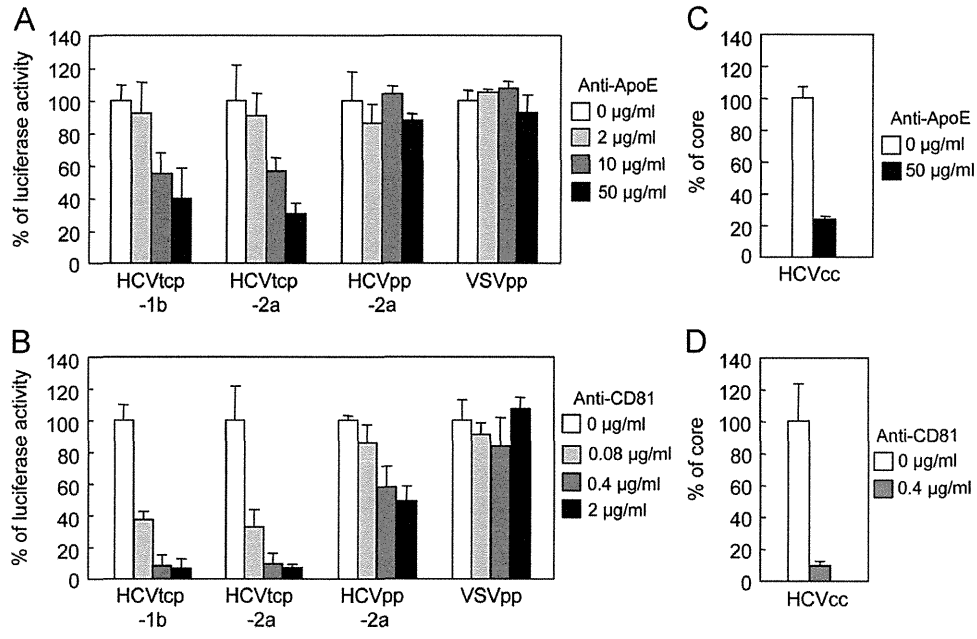

Fig. 4. Effects of anti-ApoE and anti-CD81 antibodies on HCV entry. (A) Aliquots of virus sample were incubated with increasing concentrations of anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells. Luciferase activity was determined at 72 h post-infection and is expressed relative to activity without antibodies (white bar). (B) Huh7.5.1 cells were preincubated for 1 h with increasing concentrations of anti-CD81 antibodies, followed by inoculating virus samples. Luciferase activity was determined and expressed as shown in (A). (C) Aliquots of HCVcc were incubated with anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells at an MOI of 0.05. Intracellular core levels were quantitated at 24 h post-infection and are expressed relative to levels without antibodies (white bar). (D) Huh7.5.1 cells were preincubated for 1 h with anti-CD81 antibodies. HCVcc infection and measurement of core proteins were performed as indicated in (C).

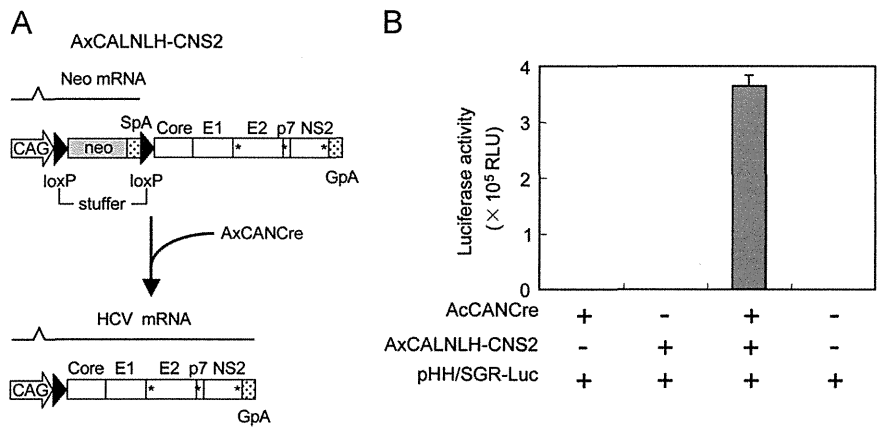

Fig. 5. Transgene activation mediated by rAdVs expressing Cre recombinase under control of CAG promoter. (A) Cre recobminase expressed by AxCANCre recognizes a pair of its target sequences loxP in AxCALNLH-CNS2, and removes the stuffer region resulting in expression of HCV core-NS2 polyprotein by CAG promoter. CAG: CAG promoter; SpA: SV40 early polyA signal; GpA: rabbit b-globin poly(A) signal. (B) Luciferase activity in Huh7.5.1 cells inoculated with 4-day post-transfection culture supernatant from cells transfected with pHH/SGR-Luc, and then infected with indicated rAdVs.

that both passaged HCVtcp had an identical nonsynonymous mutation in the NS3 region (N1586D) (Fig. 6C).

In order to examine the role of NS3 mutation identified on HCV RNA replication and on HCVtcp production, the N1586D mutation was introduced into pHH/SGR-Luc. Luciferase activities of the N1586D-mutated replicon were apparently lower than those of the WT-replicon, thus suggesting that the NS3 mutation reduced viral RNA replication (Fig. 7A). HCV RNA levels in the supernatants of cells transfected with WT- or mutant replicon plasmid along with pCAGC-NS2/JFH1am and luciferase activity in cells inoculated with supernatants from the transfected cells were then determined (Fig. 7B). The viral RNA level secreted from cells replicating the N1586D-mutated replicon was lower than that from cells replicating WT replicon (Fig. 7B, left). By contrast, a significantly higher infectivity of HCVtcp produced from the mutant replicon-cells was observed, as compared to WT replicon-cells (Fig. 7B, right), suggesting that the adaptive mutation increased the specific infectivity (almost 9-fold) of the virus particles. To further determine whether the N1586D mutation affects infectious viral assembly and/or virus release, we used the CD81-negative Huh-7 subclone, Huh7-25 (Akazawa et al., 2007), which may produce infectious particles, but is not susceptible to HCV entry due to a lack of CD81 expression, therefore allowing us to examine viral assembly and release without the influence of reinfection by produced HCVtcp. Measurement of intracellular and extracellular HCVtcp indicated that Huh7-25 cells replicating the N1586D-mutated replicon produced more infectious virus than WT in both supernatants and cell lysates (Fig. 7C). Thus, it can be concluded that the N1586D mutation contributes to enhanced infectious viral assembly, not RNA replication. We could not exclude the possibility that N1586D mutation affects virus release, since the mutation enhanced extracellular virus titers more than did the intracellular titer.

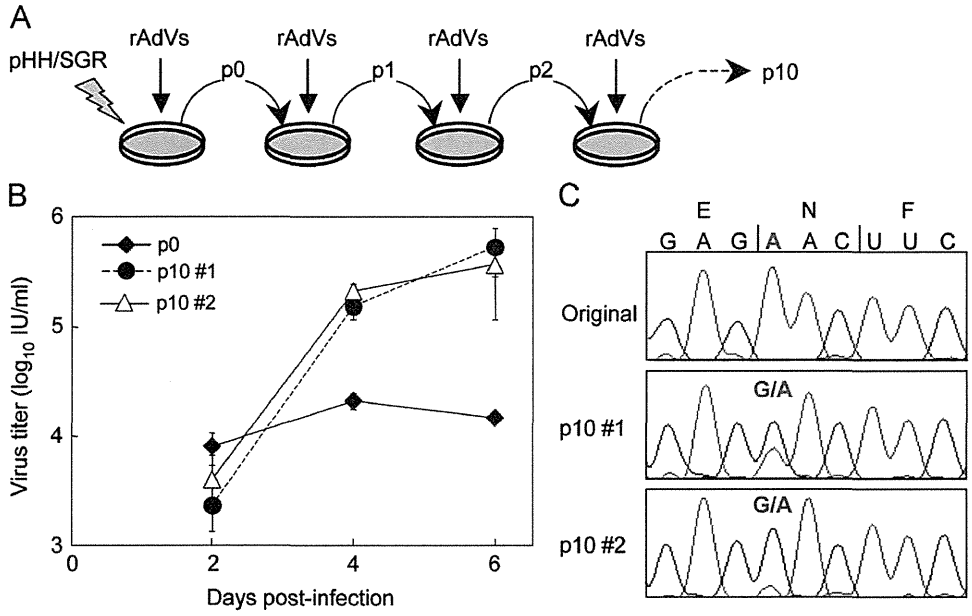

Fig. 6. Genotypic changes in HCVtcp following blind passage. (A) Experimental procedure for blind passage of HCVtcp. Huh7.5.1 cells were transfected with pHH/SGR and were doubly infected with AxCANCre and AxCALNLH-CNS2. Culture fluids were collected and were inoculated into cells infected with AxCANCre and AxCALNLH-CNS2. These procedures were repeated 10 times with two independent samples (#1 and #2). (B) Growth curves of HCVtcp p0 and p10 on Huh7.5.1 cells expressing core-NS2. Cells were infected with HCVtcp at an MOI of 0.05, and medium was collected at the indicated time points and subjected to titration. (C) Nucleotide sequences of original and blind-passaged replicons from HCVtcp. Nucleotides of mutated position are shown in red and bold.

The impact of the N1586D mutation on production of intra- and intergenotypic HCVtcp chimeras was also investigated. The N1586D mutation in the replicon enhanced the production of chimeric HCVtcp by providing core-p7 from all strains examined, although not statistically significant in THpa, and Con1 strains (Fig. 7D). Finally, to determine whether the N1586D mutation was responsible for enhancing HCVcc production, this mutation was introduced into pHHJFH1, which carries the full-length wild-type JFH-1 cDNA (Masaki et al., 2010), yielding pHHJFH1N1586D. The virus titer obtained from cells transfected with the pHHJFH1N1586D was significantly higher than that of WT (Fig. 7E), thus demonstrating that the N1586D mutation enhances yields of HCVcc, in addition to HCVtcp.

Discussion

Single-round infectious viral particles generated by *trans*-packaging systems are considered to be valuable tools for studying virus life cycles, particularly the steps related to entry into target cells, assembly and release of infectious particles. However, limited HCV strains have been applied for the efficient production of HCVtcp to date. In this study, we improved the HCVtcp system in order to enhance the productivity of infectious particles. Production of chimeric HCVtcp by providing genotype 1b-derived core-p7, in addition to intragenotypic viral proteins, was also confirmed. Furthermore, we exploited the system to investigate genetic changes during serial passage of target cells and identified a novel cell culture-adaptive mutation in NS3, which also contributes to enhance the productivity of HCVtcp.

HCVpp (Bartosch et al., 2003a; Hsu et al., 2003) has proven to be a valuable surrogate system by which the study of viral and cellular determinants of the viral entry pathway is possible. Early steps of HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp (Lavie et al., 2007). However, as HCVpp is generated in non-hepatic cells such as the human embryo kidney cells 293T, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc. Hepatocytes play a role in maintaining lipid homeostasis in the body by assembling and secreting lipoproteins, including VLDL. It is highly likely that HCV exploits lipid synthesis pathways, as there is a tight link between virion formation and VLDL synthesis. Down-regulation of ApoE considerably reduces HCV production (Benga et al., 2010; Chang et al., 2007; Hishiki et al., 2010; Jiang and Luo, 2009; Owen et al., 2009). Infectivity of HCVcc is also neutralized by anti-ApoE antibodies (Chang et al., 2007). These data suggest that ApoE is important for HCV infectivity. Furthermore, Niemann-Pick C1-like 1 (NPC1L1), involving cholesterol uptake receptor, was recently identified as a host factor for HCV entry (Sainz et al., 2012). Knockdown of NPC1L1 had no effect on the entry of HCVpp whereas HCVcc entry was impaired, possibly due to different cholesterol content of these particles. Here, we found that the anti-ApoE antibody neutralized infection by HCVtcp and HCVcc, but not by HCVpp (Fig. 4A and C), thus suggesting that biogenesis and/or secretion pathways of VLDL are involved in HCVtcp similarly to HCVcc, but not in HCVpp.

We also observed that infectivity of HCVtcp and HCVcc is more efficiently neutralized by the anti-CD81 antibody, as compared to that of HCVpp (Fig. 4B and D). It has recently been reported that E2 of HCVcc contained both high-mannose-type and complex-type glycans, whereas most of the glycans on HCVpp-associated E2 were complex-type, which is matured by Golgi enzymes (Vieyres et al., 2010). Mutational analysis of the N-linked glycosylation sites in E1/E2 demonstrated that several glycans on E2 may affect the sensitivity of HCVpp against antibody neutralization, as well as access of CD81 to its binding site on E2 (Helle et al., 2010). The differences in sensitivity between HCVtcp and HCVpp to neutralization by anti-CD81 antibody observed here may be due to differences in carbohydrate composition of HCV glycoproteins during expression and processing of E1/E2 in cells and morphogenesis of HCVtcp and HCVpp.

By analyzing the various replicons for *trans*-packaging, we observed the highest production of HCVtcp with replicons from pHH/SGR, which lacked sequences not essential for RNA

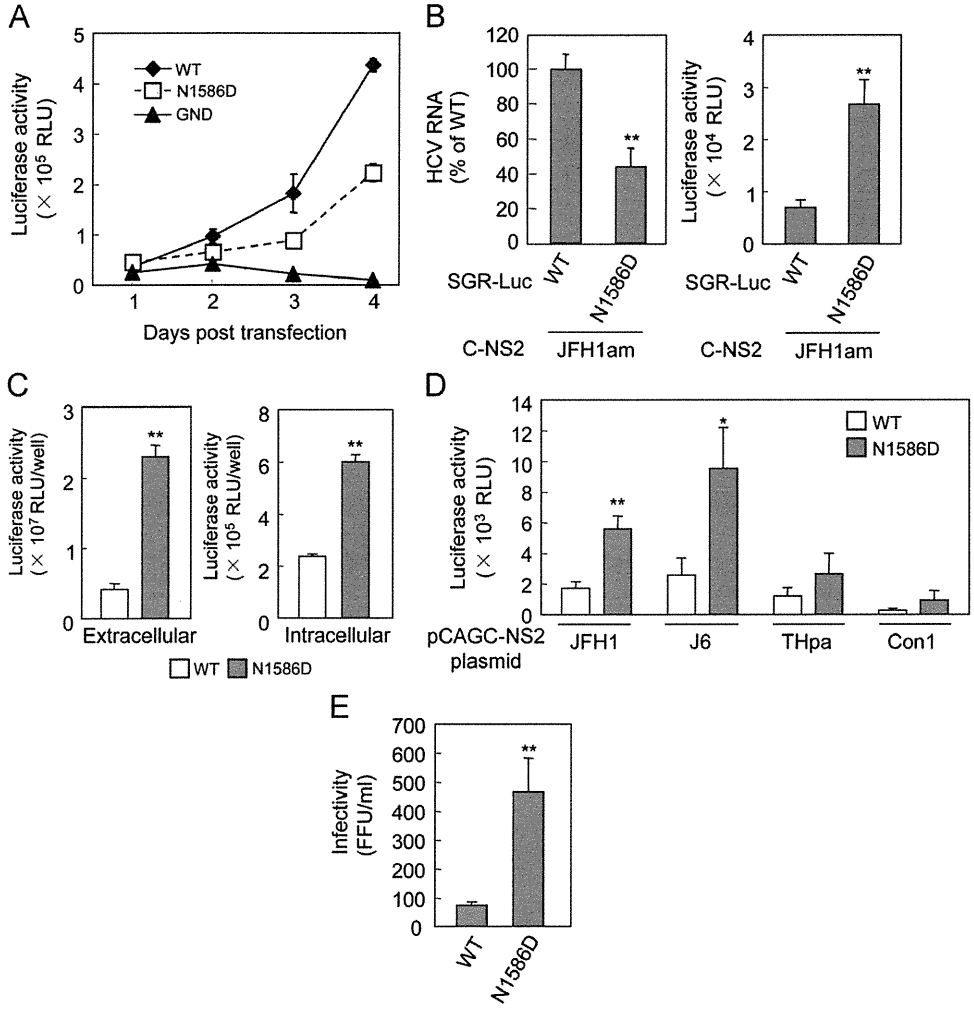

Fig. 7. Effects of N1586D mutation on RNA replication and production of HCVtcp or HCVcc. (A) RNA replication of replicons in cells transfected with pHH/SGR-Luc (WT) or N1586D mutant. Luciferase activities at 1 to 4 day post-transfection were determined. (B) Relative levels of HCV RNA in the supernatants from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am were shown in the left panel. Luciferase activities in cells inoculated with supernatants from cells transfected with indicated plasmids at 4 day post-transfection were shown in the right panel. (C) Luciferase activity in cells inoculated with supernatant and cell lysates from Huh7-25 cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am at 5 day post-transfection. (D) Luciferase activity in cells inoculated with culture supernatant from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with indicated core-NS2 plasmids at 4 day post-transfection. (E) Infectivity of supernatant from cells transfected with pHH/JFH1 (WT) or its derivative plasmid containing N1586D mutation at 6 day post-transfection. Statistical differences between WT and N1586D were evaluated using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.005$ vs. WT.

replication, while less efficient productivity was observed from pHH/SGR-Luc, pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am (Fig. 2C). Differences in the replication efficiency of the replicon do not appear to be a major determinant for HCVtcp productivity, at least in the present settings, as all replicon constructs except pHH/SGR-Luc replicated at similar levels, as confirmed by Western blotting (Fig. 2B). Although the shorter viral genome sequence may offer advantages over the longer sequence, further investigation is required in order to understand the molecular mechanisms underlying viral genome packaging. By comparing pHH/SGR vs. pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, it is likely that the expression of the structural protein in *cis* does not increase HCVtcp production when sufficient amounts of structural proteins are supplied in *trans*.

Blind passage of HCVtcp in packaging cells infected with rAdVs providing core-NS2 enabled us to identify a novel culture-adaptive mutation in NS3. The N-terminal third of NS3 forms a serine protease, together with NS4A, and its C-terminal two-thirds exhibits RNA helicase and RNA-stimulated NTPase activities. In addition, similarly to flaviviruses (Kummerer and Rice, 2002; Liu et al., 2002), it is now apparent that HCV NS3 is also involved in viral morphogenesis (Han et al., 2009; Ma et al., 2008), although its precise role and underlying molecular mechanism(s) have not fully been elucidated. Two cell-culture adaptive NS3 mutations which are involved in HCV assembly have been identified. The Q1251L mutation in helicase subdomain 1 resulted in approximately 30-fold higher production of HCV without affecting NS3 enzymatic activities (Ma et al., 2008). The M1290K adaptive mutation was also located in subdomain 1 of the NS3 helicase (Han et al., 2009). The N1586D mutation identified here was located in subdomain 3 of helicase. Analogous to Q1251L and M1290K, the N1586D mutation enhanced the infectious viral assembly by increasing specific infectivity without affecting the efficiency of viral RNA replication. Considering the possibility that NS3 plays a role in linking between the viral replicase and assembly sites (Jones et al., 2011), it is likely that NS3 helicase is one of the determinants for interaction with the structural proteins. Our results, together with earlier studies, suggest that chimeric and defective mutations as well as supplying the viral components in *trans*, function as selective pressures in virion assembly.

In summary, we have established a plasmid-based reverse genetics for efficient production of HCVtcp with structural

proteins from various strains. Single-round infectious HCVtcp can complement the HCVcc and HCVpp systems as a valuable tool for the study of HCV life cycles.

Materials and methods

Cells

Huh7 derivative cell line Huh7.5.1 and Huh7-25 were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

Plasmids

Plasmids pHHJFH1, pHH/SGR-Luc, pHH/SGR-Luc/GND and pCAG/C-NS2 were as described previously (Masaki et al., 2010). In this study, plasmid pCAG/C-NS2 was designated as pCAGC-NS2/JFH. The plasmid pCAGC-NS2/JFHam having adaptive mutations in E2 (N417S), p7 (N765D), and NS2 (Q1012R) in pCAGC-NS2/JFH was constructed by oligonucleotide-directed mutagenesis. These mutations were also introduced in pHHJFH1, resulting in pHHJFH1am. To generate core-NS2 expression plasmids with different strains of HCV, the cDNA coding core to the first transmembrane region of NS2 (33 amino acids) in pCAGC-NS2/JFH was replaced with the corresponding sequence of the J6 (Lindenbach et al., 2005), H77c (Yanagi et al., 1997), THpa (Shirakura et al., personal communication) and Con1 (Koch and Bartenschlager, 1999) strains. The THpa sequence contained the P to A mutation at 328 aa at E1 in the original TH strain. To generate pHH/SGR, pHH/SGR-Luc was digested with MluI and PmeI, followed by Klenow enzyme treatment and self-ligation to delete the luciferase coding sequence. To generate pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, cDNA coding the partial core and luciferase in pHH/SGR-Luc were replaced with coding sequences for mature core (177aa), full-length core (191aa) or core-p7 polyprotein containing adaptive mutations in E2 and p7, respectively. The selected NS3 mutation (N1586D) was introduced into pHH/SGR-Luc and pHHJFH1 by oligonucleotide-directed mutagenesis.

Generation of viruses

HCVcc and HCVtcp were generated as described previously (Masaki et al., 2010). For the production of HCVpp-2a, plasmid pcDNAdeltaC-E1-E2(JFH1)am having adaptive mutations in E2 (N417S) in pcDNAdeltaC-E1-E2(JFH1) (Akazawa et al., 2007) was constructed by oligonucleotide-directed mutagenesis. Murine leukemia virus pseudotypes with VSV G glycoprotein expressing luciferase reporter (VSVpp) were generated in accordance with previously described methods (Akazawa et al., 2007; Bartosch et al., 2003a).

Luciferase assay

Huh7.5.1 cells were seeded onto a 24-well plate at a density of 3×10^4 cells/well 24 h prior to inoculation with reporter viruses. Cells were incubated for 72 h, followed by lysis with 100 μL of lysis buffer. Luciferase activity of the cells was determined using a luciferase assay system (Promega, Madison, WI). All luciferase assays were performed in triplicate.

Quantification of HCV infectivity and HCV RNA

To determine the titers of HCVtcp and HCVcc, Huh7.5.1 cell monolayers prepared in multi-well plates were incubated with dilutions of samples and then replaced with media containing 10% FBS and 0.8% carboxymethyl cellulose. Following incubation for 72 h, monolayers were fixed and immunostained with rabbit polyclonal anti-NS5A antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen), and stained foci or individual cells were counted and used to calculate a titer of focus-forming units (FFU)/mL for spreading infections or infectious units (IU)/mL for non-spreading infections. For intracellular infectivity, the cell pellet was resuspended in culture media, and cells were lysed by four freeze–thaw cycles. Cell debris was pelleted by centrifugation for 5 min at 4000 rpm. Supernatant was collected and used for titration. To determine the amount of HCV RNA in culture supernatants, RNA was extracted from 140 μL of culture medium by QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37 °C for 1 h. Extracted RNA was further purified by using an RNeasy Mini Kit, which includes RNase-free DNase digestion (QIAGEN). Copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously (Wakita et al., 2005).

Antibodies

Mouse monoclonal antibodies against actin (AC-15) and CD81 (JS-81) were obtained from Sigma (St. Louis, MO) and BD Biosciences (Franklin Lakes, NJ), respectively. Goat polyclonal antibody to ApoE (LV1479433) was obtained from Millipore (Tokyo, Japan). Anti-NS5A and anti-NS5B antibodies were rabbit polyclonal antibody against synthetic peptides.

Neutralization assay

For neutralization experiments with anti-CD81 antibody, Huh7.5.1 cells were incubated with dilutions of anti-CD81 antibody for 1 h at 37 °C. Cells were then infected with viruses for 5 h at 37 °C. For neutralization experiments with anti-ApoE antibody, viruses were incubated with various concentrations of anti-ApoE antibody at room temperature for 1 h and cells were infected with viruses for 5 h at 37 °C. Following infection, supernatant was removed and cells were incubated with culture medium, and luciferase activity was determined at 3 day post-infection for HCVtcp and pseudotyped viruses. For neutralization experiments with HCVcc generated with pHHJFH1am, a multiplicity of infection (MOI) of 0.05 was used for inoculation, and intracellular core protein levels were monitored by ELISA (Ortho Clinical Diagnostics) at 24 h post-infection.

Immunoblotting

Transfected cells were washed with PBS and incubated with lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% triton X-100). Lysates were then sonicated for 5 min and were added to the same volume of SDS sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE, and transferred to PVDF membrane. After blocking, membranes were probed with first antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; PIERCE, Rockford, IL), in accordance with the manufacturer's protocols.

Generation of recombinant adenoviruses

rAdV, AxCANCre, expressing Cre recombinase tagged with nuclear localization signal under CAG promoter was prepared as described previously (Baba et al., 2005). The target rAdV AxCALNLH-CNS2 expressing HCV core-NS2 polyprotein with adaptive mutations in E2, p7 and NS2 was generated as follows. Cosmid pAxCALNLwit2 is identical to pAxCALNLw (Sato et al., 1998), except that both the terminal sequences of the rAdV genome are derived from pAxCAwit2 (Fukuda et al., 2006). The core-NS2 fragment obtained from pCAGC-NS2/JFH1am by StuI-EcoRI digestion and subsequent Klenow treatment was inserted into the SwaI site of pAxCALNLwit2. The resultant cosmid pAxCALNLH-CN2it2 was digested with PacI and transfected into 293 cells to generate rAdV AxCALNLH-CNS2.

Preparation of packaging cells for HCVtcp

Huh7.5.1 cells were coinfected with AxCANCre at an MOI of 1 and AxCALNLH-CNS2 at an MOI of 3 for expression of JFH-1 core-NS2 polyprotein containing the adaptive mutations in E2, p7 and NS2.

RNA preparation, RT-PCR and sequencing

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription with random hexamer and Superscript III reverse transcriptase (Invitrogen). Three fragments of HCV cDNAs that cover the entire HCV subgenomic replicon genome, were amplified by nested PCR with TaKaRa Ex Taq polymerase (Takara, Shiga, Japan). Amplified products were separated by agarose gel electrophoresis, and were used for direct DNA sequencing.

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