Efficient infectious cell culture systems of the hepatitis C virus prototype strains HCV-
1 and H77

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Abstract

The first discovered and sequenced hepatitis C virus (HCV) genome and the first in vivo infectious HCV clones originated from the HCV prototype strains HCV-1 and H77, respectively, both widely used in research of this important human pathogen. In the present study, we developed efficient infectious cell-culture systems for these genotype 1a strains by using the HCV-1/SF9_A and H77C in vivo infectious clones. We initially adapted a genome with the HCV-1 5’UTR-NS5A and the JFH1 NS5B-3’UTR (5-5A recombinant), including the genotype 2a-derived mutations F1464L/A1672S/D2979G (LSG), to grow efficiently in Huh7.5 cells, thus identifying the E2 mutation S399F. Combination of LSG/S399F and reported TNcc(1a)-adaptive mutations A1226G/Q1773H/N1927T/Y2981F/F2994S promoted adaptation of the full-length HCV-1 clone. An HCV-1 recombinant with seventeen mutations (HCV1cc) replicated efficiently in Huh7.5 cells, and produced supernatant infectivity titers of $10^{4.0}$ focus-forming-units (FFU)/ml. Eight of these mutations were identified from passaged HCV-1 viruses, and the A970T/I1312V/C2419R/A2919T mutations were essential for infectious particle production. Using CD81-deficient Huh7 cells, we further demonstrated the importance of A970T/I1312V/A2919T or A970T/C2419R/A2919T for virus assembly and that the I1312V/C2419R combination played a major role in virus release. Using a similar approach, we found that NS5B mutation F2994R identified here from culture-adapted full-length TN-viruses and a common NS3-helicase mutation (S1368P) derived from viable H77C and HCV-1 5-5A recombinants initiated replication and culture-adaptation of H77C containing LSG and TNcc(1a)-adaptive mutations. An H77C recombinant harbouring nineteen mutations (H77Cc) replicated and spread efficiently after transfection and subsequent infection of naïve Huh7.5 cells, reaching titers of $10^{3.5}$ and $10^{4.4}$ FFU/ml, respectively.

Keywords: plus-strand RNA virus, infection, full-length genome, adaptive mutations, genotype 1a, HCV
**Importance**

Hepatitis C virus (HCV) was discovered in 1989 with the cloning of the HCV-1 genome. In 1997, two molecular clones of H77, the other HCV prototype strain, were shown to be infectious in chimpanzees, but not *in vitro*. HCV research was hampered by a lack of infectious cell-culture systems, which became available only in 2005 with the discovery of JFH1 (genotype 2a), a genome that could establish infection in Huh7.5 cells. Recently, we developed *in vitro* infectious clones for genotype 1a(TN), 2a(J6), and 2b(J8, DH8, and DH10) strains by identifying key adaptive mutations. Globally, genotype 1 is the most prevalent. Studies using HCV-1 and H77 prototype sequences have generated important knowledge on HCV. Thus, the *in vitro* infectious clones developed here for these 1a strains will be of particular value in advancing HCV research. Moreover, our findings open new avenues for the culture adaptation of HCV isolates of different genotypes.
**Introduction**

Hepatitis C virus (HCV) has chronically infected over 130 million people worldwide and is a leading cause of liver fibrosis, cirrhosis, and hepatocellular carcinoma. More than 350,000 deaths annually are due to HCV-related liver diseases (World Health Organization website, 2014). HCV belongs to the *Hepacivirus* genus within the *Flaviviridae* family, and its genome is a positive-sense single-strand RNA of ~9.6-kb consisting of a single open reading frame (ORF) and 5’ and 3’ untranslated regions (UTRs). The ORF encodes viral structural proteins (Core and envelope glycoproteins E1 and E2), a small membrane protein (p7), and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (1). HCV has been classified in 7 major genotypes differing in nucleotide and amino acid sequences by ~30% and numerous subtypes with sequence heterogeneity of 15-20% (2-4). Genotype 1 accounts for the majority of HCV infections worldwide, and subtypes 1a and 1b are predominant. Furthermore, genotype 1 strains were found to be relatively resistant to interferon-α/ribavirin therapy (1). Although incorporation of directly acting antivirals (DAA) improves the sustained virologic response rate, emergence of drug resistance is a concern and may influence the outcome of these new therapies (5).

Robust infectious HCV cell culture systems from isolates of different genotypes represent valuable tools for the *in vitro* study of HCV genetic heterogeneity, which plays a major role in disease progression, response to antiviral therapy, and poses a significant challenge for vaccine development. Since the discovery of HCV-1 in 1989 (6), many attempts have been directed to adapt prototype strains of HCV to grow in cell culture. However, success did not come until 2005 when the cloned JFH1 (genotype 2a) full-length sequence was found to be able to spontaneously establish infection in hepatoma Huh7 cells and derivatives (7,8). To date, JFH1 remains the only cloned HCV sequence reported with spontaneous growth *in vitro*. Recently, we identified three mutations in NS3, NS4A, and NS5B (F1464L, A1672S, and D2979G, respectively – the LSG mutations) [nucleotide and amino acid numbering throughout is according to the H77 (1a) reference sequence, GenBank accession number AF009606] that enabled adaptation of HCV full-length genomes in cell culture. These mutations lead to the development of robust and highly infectious full-length cell culture systems for HCV genotype 1a (TNcc) (9), 2a (J6cc) (10), and 2b (J8cc, ...
DH8cc, and DH10cc) (10,11) strains; the TNcc represented the first effective cell culture system for genotype 1. Infectious culture systems were only reported for a few other genotype 1 and 2 strains (12-15).

The HCV-1 strain was the first HCV genome to be cloned (6) and has been a key tool commonly used by investigators in the HCV field. Studies using HCV-1 have led to important discoveries, such as elucidation of the genetic organization of the HCV genome (16), identification of CD81 as an important viral receptor (17), and discovery of the frame-shifted F protein (18). An HCV-1 E1/E2-based vaccine was found to induce a neutralizing antibody responses with cross-reactivity against various HCV genotypes in rodents (19), chimpanzees (20), and humans (21,22), thus making it a promising vaccine candidate for further development. H77 is another genotype 1a strain that has significantly contributed to HCV research. H77 is the reference sequence for HCV genome numbering (23). The RNA transcripts of two H77 full-length cDNA clones, named H77C (24) and H77 (25), were the first HCV genomes found to be infectious, as demonstrated by intrahepatic transfections in chimpanzees. Patient serum-derived H77 virus was reported to be able to replicate in lymphoblastoid cell lines at low levels (26), and could be passaged to chimpanzees (27). Subsequently, studies using the sequences of H77 contributed greatly to HCV research, for example, to the study of viral entry using HCV-like particles (28) and to the development of HCV pseudo-particles (29), the selection of highly permissive Huh7.5 cells (30), and the discovery of the importance of microRNA miR-122 in HCV replication (31). Efforts to propagate H77 in cell culture have been reported, using different cell lines (27,32-34), and H77C harboring mutations derived from its replicon (designated H77-S) could release infectious virus particles (12,35). Given the historical importance of HCV-1 and H77 isolates in HCV research, efficient infectious cell culture systems for these isolates would be very valuable tools for studies on HCV.

In this study, we developed robust and efficient infectious cell culture systems for HCV-1 and H77C by using the LSG mutations and approaches recently discovered for the TNcc and J6cc culture systems. An HCV-1 full-length genome with seventeen amino acid changes, named “HCV1cc”, produced infectious virus particles with titers of ~10^{4.0} focus-forming-units per milliliter (FFU/ml). By using novel mutations identified in TN full-length viruses and in HCV-1 and H77C recombinants expressing the NS5B-
3'UTR from JFH1 (5-5A recombinants), we finally succeeded in developing an infectious culture system for H77, designated “H77Ccc”. The H77Ccc, with nineteen amino acid changes, replicated efficiently and spread to most culture cells within 3-5 days after transfection and subsequent infection of Huh7.5 cells, and produced infectivity titers of ~10^{4.0} FFU/ml. The HCV1cc and H77Ccc represent robust infectious cell culture systems for these key prototype strains that will contribute to HCV basic research and the development of better antiviral therapies and vaccines.
Materials and Methods

Plasmids

The HCV-1 clone HCV-1/SF9_A (GenBank accession number AF271632), which has 12 amino acid (aa) differences from the first reported HCV-1 sequence (M62321) (6,36), was shown to be infectious in chimpanzees (36), and thus selected for this study. The HCV-1/SF9_A genome with the LSG (F1464L, A1676S, and D2979G) and TNcc-derived mutations (9) was synthesized (GenScript), and assembled into a pGEM-9zf(-) vector containing T7 promoter for initiation of in vitro-transcription immediately upstream of the 5'UTR and an XbaI cleavage site at the end of the HCV genome (Promega), which was previously used in pCV-H77C (24). Other mutations were introduced by fusion PCR or by site-directed mutagenesis using QuikChange II XL kit (Agilent Technologies). The junction of the HCV-1 NS5A and JFH1 NS5B-3'UTR was synthesized (GenScript). For strain H77, we used the in vivo infectious clone pCV-H77C (GenBank accession number AF011751) (24). The LSG and other mutations were introduced into H77C by fusion PCR or site-directed mutagenesis using the QuikChange II XL kit. All final plasmid preparations were confirmed by sequence analysis spanning the T7 promoter and the entire HCV genome (Macrogen).

Transfection and infection of Huh7.5 cells. The human hepatoma cell line Huh7.5 was maintained as described (37,38). Cells were plated in 6-well plates (~3.5×10^5 cells/well) ~24 hours before RNA transfection or viral infection, reaching 80-90% confluence at the time of inoculation. RNA transfection and viral infection were performed as previously described (10). The transfected or infected cultures were incubated for ~16 hours, and sub-cultured every 2-3 days; culture supernatant was collected, filtered (0.45 µm), and stored at -80°C until analysis.

Analysis of HCV in cultured cells. To monitor HCV infection in the transfected and infected cultures, combination of monoclonal anti-core antibody C7-50 (Enzo Life Sciences or Abcam) and the anti-NS5A antibody 9E10 (38) were used for immunostaining, as previously described (10,37). Percentage of HCV antigen positive cells in the culture was determined with fluorescence microscopy. Culture supernatants were collected when 80% of cells were HCV antigen positive (peak infection) and HCV infectivity titers
were determined by an FFU assay using a combination of C7-50 and 9E10, as previously described (9,10,37). Full-length adapted HCV-1 and H77C viruses showed slightly lower intensity in staining than the positive control virus, J6\(^{5'UTR-NS7}/\)JFH1 (39). The number of FFU was automatically counted with an ImmunoSpot Series 5 UV Analyzer with customized software (CTL Europe GmbH) (40,41). HCV RNA titers in the culture supernatant were determined using real time RT-PCR TaqMan method (37). Core antigen levels were determined by the Architect HCV Ag detection system (Abbott) following manufacturer’s instructions. Whole ORF sequences of passaged viruses were determined using procedures previously described for the sequencing of H77C and JFH1 genomes (37,42).

**Determination of intra- and extra- cellular HCV core levels and infectivity titers.**

For single- cycle production assays, an Huh7-derived CD-81 deficient cell line S29 (43) was transfected with HCV RNA and intracellular HCV core levels were measured 4 and 48 hours post transfection. Additionally, intra- and extra- cellular HCV infectivity titers, as well as extracellular HCV core concentration, were determined 48 hours post transfection, as described previously (44,45). Briefly, for this assay, S29 cells were seeded in 6 well plates (~3.5×10\(^5\) cells/well) 24 hours prior to transfection. Plasmids of the different full-length clones were digested with XbaI (New England Biolabs, NEB) for linearization and treated with Mung Bean nuclease (NEB). HCV RNA was generated by *in vitro* transcription using T7 RNA polymerase (Promega). *In vitro* transcripts were then digested with RNase-Free DNase Set (Qiagen) and purified with RNeasy MinElute Cleanup Kit (Qiagen). RNA was quantified using spectrophotometry (NanoDrop) and 10 \(\mu\)g of RNA was used for transfection with Lipofectamine 2000 (Invitrogen). Transfection was performed in duplicate, the transfection media was replaced by complete DMEM after 4 hours in one of the replicate wells, while cells from the other replicate well were harvested for determination of intracellular HCV core levels. Briefly, cells were rinsed with PBS and re-suspended in RIPA buffer (Pierce) supplemented with protease inhibitors (Calbiochem). Samples were stored at -80°C until analysis. Prior to analysis, cell lysates were cleared by centrifugation at 14,000 rpm for 15 min and supernatants were transferred to a new tube. The same procedure was used to harvest cells at 48 hours post transfection. At this time point, supernatants were collected and filtered, for determination of extracellular
core levels and infectivity titrations, as described above. Both, intra- and extra- cellular core antigen levels were determined by the Architect HCV Ag detection system (Abbott) following the manufacturer’s instructions. For intracellular infectivity titers, cells were harvested and washed with PBS, then re-
suspended in complete DMEM and subjected to three cycles of freeze-thaw to release intracellular virus particles. Specific dilutions were analyzed in triplicate for HCV infectivity titers, and the FFU was counted using an ImmunoSpot Series 5 UV Analyzer with customized software (CTL Europe GmbH) (40,41), and confirmed by manual count.

**Western blot**

Intracellular HCV core in transfected S29 cells was also visualized with western blot. Briefly, S29 transfected cell lysates (the same sample as used for determination of core with the Architect detection system) were subjected to protein denaturation at 70°C for 10 min in the presence of NuPAGE sample reducing agent (Invitrogen) and NuPAGE LDS sample loading buffer (Invitrogen). Samples were run through 10% bis-tris SDS-polyacrylamide pre casted gels (Invitrogen) for 1 hour and 30 minutes at 150 Volts. Afterwards, separated proteins were transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (GE Healthcare Amersham) by wet electroblotting (XCell SureLock minicell, Invitrogen), at constant current during 1 hour. Membranes were then washed with PBS plus 1% Tween-20 (PBS-T) and blocked with PBS plus 1% Tween-20 and 3% bovine serum albumin for 1 hour. Blocked membranes were incubated overnight at 4°C with anti-HCV core C7-50 or anti-β-actin (Santa Cruz Biotechnology) with gentle rocking. Immunoblotting was followed by washes with PBS-T and 1 hour incubation with ECL sheep anti-mouse IgG horseradish peroxidase-linked whole antibody (GE Healthcare Amersham). After washing, membranes were developed by chemiluminiscence using Signal West Femto maximum-sensitivity substrate (Pierce) and visualized with AutoChemi System (UVP Bio-Imaging Systems).
Results

Adaptation of an HCV-1 5'UTR-NS5A (5-5A) recombinant leads to identification of the S399F mutation.

We previously identified the LSG mutations (F1464L, A1672S, and D2979G), which permitted development of full-length HCV infectious culture systems for genotype 1a (TNcc) (9), 2a (J6cc) (10), and 2b (J8cc, DH8cc, and DH10cc) (10,11), as well as 5-5A recombinants with JFH1 NS5B-3'UTR for genotypes 3a (S52), 4a(ED43), 5a(SA13), and 6a(HK6a) (46). In this study, we initially attempted to use the LSG mutations and a similar approach previously applied to J6cc and TNcc cultures, to generate an HCV-1 infectious culture system. We selected the in vivo infectious clone HCV-1/SF9_A, a genome with 12 aa differences in comparison to the first reported HCV-1 sequence (M62321) (16,36). The HCV-1/SF9_A shares nucleotide sequence identity of 96% and 95% to genotype 1a infectious clones H77C (24) and TN (47), respectively. Additionally, in our previously reported infectious J6cc and TNcc cell culture systems, we demonstrated that culture adaptation of 5-5A recombinants can lead to the identification of mutations critical for replication of full-length HCV genomes (9,10). Based on these prior findings, we constructed an HCV-1 5-5A recombinant containing LSG substitutions, HCV1(5-5A)_LSG (Fig. 1A), and tested its viability by RNA transfection of Huh7.5 cells. In two independent transfections, HCV core and NS5A antigens were detected in <1% of cells at day 1, but spread of infection was not observed after 45 and 56 days of follow-up. Therefore we concluded that the genome was viable but highly attenuated. We previously showed that combination of A1226G (NS3 helicase, NS3 aa position 200) and Q1773H (NS4B aa 62) could efficiently enhance the viability of TN and H77C 5-5A recombinants (9,46), and they were both included in the TNcc recombinant (9). Thus, we added A1226G/Q1773H, designated as TN_{GH}, into HCV1(5-5A)_LSG (Fig. 1A). HCV1(5-5A)_LSG/TN_{GH} showed 25% HCV positive cells at day 1 in two transfection replicates and the infection spread to ≥80% of the cultured cells (peak infection) at day 5 post transfection (Table 1). However, titers of transfected cultures were below the detection limit (<10^{2.4} FFU/ml). Transfection supernatants could be passaged to naïve Huh7.5 cells and in first-passage the infectivity titers reached 10^{4.0} FFU/ml (Table 1). We continued passaging one of the viruses and the
second-passage recovered virus reached $10^{4.5}$ FFU/ml (Table 1, exp. 2). Sequence analysis of the ORF of first- and second-passage viruses revealed that the engineered mutations were maintained and that two additional complete changes had emerged, S399F in the hypervariable region 1 (HVR1) of E2 and D2416G in the NS5A domain III (Table 2). Interestingly, F399 was also found in the originally published HCV-1 sequences [M62321 (16) and AF387806 (48)]. To determine the effects of the adaptive S399F mutation, we engineered it into the HCV1(5-5A)_LSG/TN$_{GH}$ recombinant (Fig. 1A). HCV1(5-5A)_LSG/S399F/TN$_{GH}$ showed efficient viral replication with 60% of HCV positive cells at day 1, viral spread to most cultured cells at day 4, and peak infectivity titers of $10^{2.7}$ and $10^{3.0}$ FFU/ml at days 8 and 12 in two transfections (Fig. 1B and Table 1), indicating that S399F could enhance virus spread and infectivity. Collected culture supernatant from the two transfections was passaged to naïve Huh7.5 cells. In first-passage the peak infectivity titers increased to $10^{3.8}$ and $10^{3.9}$ FFU/ml and in second-passage to $10^{4.4}$ and $10^{4.2}$ FFU/ml (Table 1). Sequence analysis of one of the second-passage viruses revealed that the engineered mutations were maintained, and that two additional partial changes had emerged (Table 2). Taken together, these results indicate that combination of LSG, S399F, and TN-derived A1226G/Q1773H mutations permitted the HCV-1 5-5A recombinant to efficiently grow in Huh7.5 cells.

**Development of an efficient full-length infectious culture system for HCV-1.**

We previously demonstrated that LSG plus Y2981F [designated “F” mutation, NS5B aa 561 (49)] were important for *in vitro* viability of the TNcc (9) and full-length J6 (10) viruses. LSG plus S399F/TN$_{GH}$ could efficiently adapt the HCV-1 5-5A recombinant, in which S399F enhanced virus spread and infectivity (Fig. 1B and Table 1). Thus, here we attempted to combine these mutations and to test their adaptation potential in the full-length HCV-1 genome. For that purpose, we generated HCV-1 with LSGF/S399F/TN$_{GH}$, LSGF/S399F/TNm [“TNm” for four TNcc adaptive mutations A1226G/Q1773H/N1927T/F2994S (9)], or LSGF/TNm (Fig. 1A). Replication was not observed for HCV1_LSGF in transfected cultures for up to 20 days of follow-up. Cultures transfected with HCV1_LSGF/S399F/TN$_{GH}$ showed HCV positive cells beginning from day 4 but continued to have <1% of HCV positive cells for up to 20 days of follow-up with
no evidence of viral spread. The HCV1_LSGF/TNm culture showed 1% HCV positive cells at day 1, but no evidence of viral spread for up to 41 days. In contrast, the HCV1_LSGF/S399F/TNm culture showed 10% HCV infected cells at day 1, reached peak of infection after 26 days, and released HCV infectivity titers of $10^{3.3}$ FFU/ml (Fig. 1C), indicating that S399F mediated viral spread of full-length HCV1_LSGF/TNm and that N1927T and F2994S also contributed to a more efficient viral propagation. After passages to naïve Huh7.5 cells, the first-, second-, and third-passage HCV1_LSGF/S399F/TNm showed peak infectivity titers of $10^{3.4}$, $10^{4.3}$, and $10^{4.2}$ FFU/ml, respectively (Table 1). ORF sequence analysis of the second- and third-passage viruses revealed that the introduced mutations were all maintained and that eight additional amino acid changes [A970T, I1312V, I1326V, V2198A, I2268T, C2419R, E2622D, and A2919T (designated “8m”)] had emerged (Table 3).

In order to generate an efficient HCV-1 full-length virus, we tested the importance of the mutations identified from passaged HCV1_LSGF/S399F/TNm viruses (see above). We introduced individual mutations or a combination of “8m” into the HCV1_LSGF/S399F/TNm recombinant. At day 1 after transfection, the recombinants with single mutations A970T, I1312V, I1326V, V2198A, I2268T, C2419R, or E2622D showed low number of HCV positive cells, and in addition no evidence of viral spread was observed after one week of follow-up. In contrast, the recombinant with A2919T showed 40% HCV positive cells at day 1 and reached 80% at day 4, remaining at this percentage for two weeks, albeit with low HCV infectivity titers (<$10^{2.4}$ FFU/ml). These results indicated that A2919T may play a greater role than the remaining seven mutations in adaptation of HCV1_LSGF/S399F/TNm. The genome with all mutations combined, designated HCV1_LSGF/S399F/TNm/8m, showed 55% HCV positive cells at day 1 in two transfection replicates and released peak HCV infectivity titers of $10^{3.8}$-$10^{4.0}$ FFU/ml (Fig. 1D and Table 1). The transfection-derived virus showed efficient spread in first- and second-passage, and both passage-recovered viruses had peak infectivity titers of $10^{3.8}$ FFU/ml (Table 1). ORF sequence analysis of the second-passage virus demonstrated that all the engineered mutations were maintained, and that no additional mutations were present. Taken together, these results indicate that the combination of “8m” efficiently enhances the replication and viral production of HCV-1, resulting in an efficient full-length
HCV-1 infectious culture system. We therefore designated HCV1_LSGF/S399F/TNm/8m as “HCV1cc” (for “HCV-1 cell culture-derived”).

**Mutations important for the viability of HCV1cc**

As addition of the “8m” mutations led to a robust HCV1_LSGF/S399F/TNm virus (Fig. 1C and D), we next examined which of the mutations primarily contributed to efficient viral viability. For this purpose, we mutated each of the “8m” mutations individually back to the wild-type sequence, and tested the effect on the viability of the virus after transfection of Huh7.5 cells (Fig. 2A and B). Compared to HCV1cc, viruses with -A970T (adaptive mutation A970T reverted to wild-type), -I1312V, and -C2419R were attenuated and did not produce HCV infectivity titers detectable until day 8 after transfection, at which time point their titers were approximately 7.9, 1.7, and 2.0-fold lower than HCV1cc, respectively. Moreover, the virus with -A2919T was highly attenuated as HCV titers were first detected on day 13. Additionally, peak infectivity titers for both -A970T and -A2919T viruses were slightly lower than for the remaining viruses (Fig. 2A), specifically 2.8 and 3.2-fold lower than HCV1cc. When analyzing secreted core antigen levels in the supernatants of transfected cells, we observed that the mutant -A2919T, the most attenuated, had core levels that were 4.7-21 fold lower than those for HCV1cc at the same time points (Fig. 2B), whereas other viruses showed core levels 0.5-3 fold within those of HCV1cc. Together, these results indicate that the absence of A970T, I1312V, C2419R, or A2919T all affect the viability of HCV1cc, with the absence of A2919T having the greatest effect.

Next, we explored whether the four mutations A970T, I1312V, C2419R, and A2919T (designated “4m”), singly or combined, were sufficient to adapt HCV1_LSGF/S399F/TNm to comparable growth as HCV1cc. Since A2919T played a major role for the viability of HCV1cc (Fig. 2A), in addition to the “4m” we tested HCV1_LSGF/S399F/TNm with A2919T plus any combinations of the other three mutations, namely A970T/A2919T, I1312V/A2919T, C2419R/A2919T, A970T/I1312V/A2919T (designated “3m”), A970T/C2419R/A2919T, and I1312V/C2419R/A2919T. After transfection of Huh7.5 cells, only the viruses containing “3m” and “4m” spread to ≥80% of culture cells within 6 days (Fig. 3A);
the viruses with other combinations did not spread. HCV1_LSGF/S399F/TNm/4m produced detectable infectivity titers from day 4, though the HCV peak titers were slightly lower than those of HCV1cc (Fig. 3A), whereas HCV1_LSGF/S399F/TNm/3m did not produce detectable HCV infectivity titers until day 13. However, supernatant core levels of both “3m” and “4m” viruses were similar to those of HCV1cc at each time point (Fig. 3B). From these results, we conclude that “4m” mutations are the minimum required for efficient production of infectious viruses of the HCV1_LSGF/S399F/TNm genome, \textit{in vitro}.

\textbf{Effect of HCV-1 adaptive mutations on viral replication, assembly, and release.} To address the role of the identified adaptive mutations in replication, assembly and release of HCV1cc, we performed a single-cycle-production assay using Huh7 derived S29 cells, a cell line that is deficient for the HCV entry receptor CD81 (43). Since the A970T/I1312V/C2419R/A2919T (4m) mutations played a major role in the viability of HCV1cc, in which A970T and A2919T seemed to have a greater effect (Fig. 2A), we tested the effect of A970T/A2919T with I1312V, C2419R, or with I1312V/C2419R in the HCV1_LSGF/S399F/TNm backbone. After transfection of S29 cells, the intracellular and extracellular infectivity titers (Fig. 4A) and corresponding HCV core antigen levels (Fig. 4B and C) were determined. In addition, the intracellular HCV core levels were visualized by western blot (Fig. 4D), and the results agreed with the measurements obtained by using the Architect HCV Ag detection system (Fig. 4B and D).

As expected, in the absence of “4m” mutations, HCV1_LSGF/S399F/TNm failed to produce detectable intracellular and extracellular infectivity titers (Fig. 4A), and intracellular core levels were barely over those of the replication-deficient control, J6/JFH1-GND (Fig. 4B). In a separate experiment, we also tested HCV1_LSGF and HCV1_LSGF/TNm in parallel with HCV1cc. Likewise, HCV1_LSGF/S399F/TNm, HCV1_LSGF and HCV1_LSGF/TNm failed to produce detectable levels of both intracellular and extracellular infectivity titers and showed a low level of intracellular and extracellular core antigen 48 hours after transfection (data not shown). Addition of A970T/A2919T into the HCV1_LSGF/S399F/TNm genome had only a minor effect on core levels and no effect on infectivity titers. However, addition of I1312V or C2419R to A970T/A2919T mutations led to detectable intracellular
infectious titers and increase in extracellular core level, however, no extracellular infectious virus were detected (Fig. 4A-C). These results suggest that both I1312V and C2419R played an important role in assembly of infectious virus particles, but had no or insufficient effect in virus release. Interestingly, when both I1312V and C2419R were combined with A970T/A2919T, thus making the genome with the “4m” mutations, virus release of infectious viral particles was enhanced and the virus produced extracellular infectivity titers comparable to those of HCV1cc (Fig. 4A), with an increase in extracellular core levels (Fig. 4C). Based on the results of the single-cycle production assay, we concluded that the “4m” mutations (A970T/I1312V/C2419R/A2919T) when added into the HCV1_LSGF/S399F/TNm genome permit an efficient completion of the viral life cycle, and that the combination of I1312V/C2419R apparently is required for efficient virus release of infectious viral particles. It should also be noted that although “4m” mutations were essential for the viability of HCV1_LSGF/S399F/TNm, the combination of “8m” further increased intracellular core levels (Fig. 4B) and intracellular infectivity titers (Fig. 4A), thus suggesting that all “8m” mutations further increased replication and virus assembly of HCV-1.

Development of an efficient full-length infectious culture system for H77.

After our success in adapting genotype 1a strains TN and HCV-1 for efficient growth in vitro, we wanted to determine whether the key adaptive mutations we uncovered could be used to adapt H77C, another important HCV prototype strain. We recently demonstrated that the LSGF and LSGF/TNm mutations were not sufficient to adapt the H77C genome after transfection in Huh7.5 cells (46). However, the LSG and TN(5-5A)-adaptive mutations A1226G/Q1773H could efficiently adapt an H77C 5-5A recombinant [“1a(H77)_LSG/A1226G/Q1773H” in reference (46)]. After passage, the virus acquired a partial change in the NS3 helicase, S1368P (46), which became dominant after second-passage. Interestingly, this mutation was also identified here in the two cell culture adapted HCV-1 5-5A viruses (Table 2).

We previously found that a TN genome containing only LSGF, designated TN_LSGF, was non-viable in a single transfection (9). However, when we repeated transfections of the TN_LSGF for this study, we were able to obtain viral replication in one out of three transfections. In that unique transfection,
TN-LSGF showed a low number of HCV positive cells at day 4 with infection spreading to ≥80% of cultured cells after 28 days of follow-up. The transfection-derived TN_LSGF culture supernatant had infectivity titers of $10^{4.0}$ FFU/ml, and the titers reached $10^{4.7}$ and $10^{4.9}$ FFU/ml after first- and second-passage, respectively. Amongst other mutations, both passage-recovered viruses acquired the change F2994R, which may be of importance since F2994 was changed to serine (F2994S) in our previously reported TNcc system (9).

Based on this new information, we hypothesized that combining mutations S1368P and F2994R might permit replication of an H77C genome with LSGF/TNm, and therefore we generated H77C_LSGF/TNmr/S1368P (TNmr indicates that F2994S of TNm was replaced with F2994R). After transfection of Huh7.5 cells, the H77C_LSGF/TNmr/S1368P showed a few HCV positive cells for up to three months, but finally the infection spread to ≥80% of cells at day 96, producing HCV peak infectivity titers of $10^{3.5}$ FFU/ml (Fig. 5A). First- and second-passage viruses spread to ≥80% of cells within 8-10 days and reached peak HCV infectivity titers of $10^{3.5}$ and $10^{4.2}$ FFU/ml (Fig. 5A). ORF sequence analysis revealed that viruses had acquired 10 complete amino acid changes M345T, A828V, L864R, K1052R, V1663A, G1909S, M2105V, S2354G, V2417A, and V2431I, which we designated “10m” (Table 4). We therefore engineered the “10m” into the H77C_LSGF/TNmr/S1368P clone. After transfection of Huh7.5 cells, H77C_LSGF/TNmr/S1368P/10m spread to ≥80% of culture cells within 3 days. The culture supernatant had a peak infectivity titer of $10^{3.5}$ FFU/ml. The first- and second-passage viruses reached infectivity titers of $10^{4.4}$ and $10^{3.8}$ FFU/ml, respectively (Fig. 5B). In ORF sequencing analysis of the second-passage virus, all the engineered mutations were maintained, and no additional changes were found (Table 4). Hence, we had developed a robust infectious culture system for full-length H77C clone, with efficient virus spread after transfection and subsequent infection, and we named the adapted recombinant “H77Ccc”.

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Discussion

In this study, we developed highly efficient cell culture systems for full-length HCV prototype strains HCV-1 and H77 (both genotype 1a strains), named HCV1cc and H77Cc, by using mutations and approaches previously developed for the J6cc (2a) and TNcc (1a) infectious clones (9,10). HCV1cc and H77Cc replicated efficiently following RNA transfection of human hepatoma Huh7.5 cells and produced HCV infectivity titers of ~10^4 FFU/ml, showing no additional amino acid changes after second-round viral passage. Given the clinical significance of genotype 1 and the uniquely important role of these prototype strains in HCV research, the HCV1cc and H77Cc systems will be of particular value and will provide useful tools for future studies of HCV.

After development of the first HCV infectious culture system based on the genotype 2a JFH1 strain (7), tremendous efforts have been made to propagate HCV isolates of other genotypes in culture. Genotype 1 account for ~60% of HCV infections worldwide, thus culture systems for genotype 1 have great interest for HCV research. Recently, we identified three mutations, designated “LSG” (F1464L/A1672S/D2979G), initially through studies of genotype 2a J6-JFH1 recombinants (10). The LSG mutations were essential for the development of full-length in vitro infectious clones of HCV genotypes 1a (TNcc) (9), 2a (J6cc) (10), and 2b (J8cc, DH8cc, and DH10cc) (10,11). Replication of 2a and 2b recombinants could be initiated by LSG alone, thus permitting further adaptation that led to robust infectious culture systems. We also showed that the LSG mutations played an important role in the adaptation of JFH1-based 5-5A recombinants of genotypes 1-6 (46). In this study, we further demonstrated that LSG combined with an NS5B mutation Y2981F (49) (interestingly most genotype 2b isolates naturally have F2981 (11)) have the potential to initiate adaption of additional genotypes, such as the genotype 1a strain TN. It would be worth exploring in future studies, whether LSG or LSGF per se can promote cell culture viability for other HCV isolates, as a first step in the process of cell culture adaptation. Our experience with the TN_LSGF genome also suggests that multiple transfections may be required to start cell culture adaptation of isolates with low level of viral replication, and that negative results from a single experiment can be misleading.
Adaptation of HCV-1 and H77C required additional mutations other than LSGF, therefore strengthening the notion that cell culture adaptation is highly influenced by the nature of the genome sequence. In order to start replication of HCV-1 and H77C, we combined LSG or LSGF with other mutations, TNm or TNmr mutations from the efficient in vitro infectious full-length TN clones, S399F from HCV1(5-5A) recombinants, and S1368P from H77C(5-5A) or HCV1(5-5A) recombinants [Table 2 and reference (46)]. This broad set of adaptive mutations may represent a valid evolutionary path leading to the culture adaptation of several additional genotype 1a strains, including strains showing high genetic diversity to TN, H77 and HCV-1 (47), and thus permitting the generation of a wide panel of infectious clones, which could be relevant for studies of strain-related genetic variability in HCV genotype 1a.

Some positions in the HCV genome are under different selective pressure in vivo and in vitro. In this study, the HVR1 amino acid S399 was changed to F399, which matched the originally reported HCV-1 sequence (16,48). This change may indicate that although an HCV-1 clone with S399 was found viable in vivo (36), F399 may be a more optimal residue for in vitro viability. A recent study showed that cell culture adaptive mutations in H77-S with replicon adaptive mutations reverted to wild-type residues over time in persistently infected chimpanzees (50). In addition, culture replication-enhancing mutations were found to prevent productive in vivo replication of the Con1 genome (51). This discrepancy in requirements for in vitro and in vivo viability may explain the difficulties of propagating HCV recombinants in cell culture, even for those found viable in vivo, as was the case for the original genomes of HCV-1, H77, and TN (24,25,36,47). In a recent study by Yamane et al. (52) the natural resistance to lipid induced peroxidase stress of JFH1 was found to be correlated with its robust replication capacity in cell culture. Moreover, the authors demonstrated that our highly efficient cell culture adapted TNcc (9) similarly had resistance to lipid peroxidation. It would be of interest for future studies to explore if this interesting finding is also applicable to other cell culture adapted genomes, including genomes not depending on the specific TN-derived adaptive mutations.

In addition to the mutations used for promoting initial viral replication and adaptation of HCV-1 and H77C, most of which we had already described, the final HCV1cc and H77Cc clones
contained a number of additional mutations, for instance, HCV1cc contained a total of seventeen amino acid changes compared to the original HCV1/SF_A clone (36). We demonstrated that 4 of the additional HCV1cc amino acid changes were critical for efficient adaptation (A970T, I1312V, C2419R and A2919T). From those, A970T (aa 161 in NS2), is a highly conserved position (Los Alamos HCV Sequence Databases) and only 1 out of 4306 sequences has a different residue. C2419R is another highly conserved residue located carboxy-terminally in NS5A. On the other hand, amino acid 1312, in the NS3 helicase, is a polymorphic site. I1312 is present in genotypes 1a, 1b, 2a, and 6a, while V1312 is primarily found in genotypes 3 and 5. The fact that V1312 can be found in natural viral sequences shows that some cell culture adaptive changes can be found in circulating viruses, albeit from other genotypes. Likewise, amino acid 2919 in NS5B is a variable position, and A, V and T can be found in various genotypes. Among the remaining cell culture adaptive mutations present in HCV1cc, position 1326 (aa 300 in NS3, helicase domain) contain either I or V depending on the genotype. HCV isolates have different amino acids in NS5A position 2198 (aa 226 in NS5A), including L, V, E, M or Q, similarly to position 2268 (296 in NS5A, in the PKR binding domain) where V, I, P, L and M can be found. Finally, at NS5B position 2622 (aa 202 in NS5B), isolates from the HCV database contain only E or D.

In previous studies, it was assumed that certain cell culture adaptive mutations identified in JFH1-based inter-genotypic recombinants compensated for incompatibilities due to the chimeric nature of those genomes. However, since many of those changes also emerged in cell culture adapted full-length genomes, they are most likely related to the cell-culture adaptation process. One of these changes is the HCV-1 adaptive mutation I1312V, that was also identified in a passaged full-length DH10 (2b) virus (11), which had been previously shown to adapt JFH1-based core-NS2 recombinants (53). Another HCV-1 change, C2419R, was identified in a DH8_LSG (2b) full-length genome (11) and was observed for adaptation of a J6/JFH1-based recombinant expressing the NS5A from H77 and with p7 mutations (54).

To understand the role of adaptive mutations in various steps of the HCV1cc life cycle, we performed single-cycle production assays using S29 cells. The S29 cells were derived from Huh7 cells but are deficient in CD81, and thus only support HCV RNA replication, virus particle assembly and release,
but not infection (43). HCV-1 genomes containing only LSGF or LSGF/S399F/TNm failed to efficiently replicate or to produce detectable infectious particles in the S29 cell assays (Fig. 4A). These results agreed with observations after transfection of Huh7.5 cells, in which both viruses started to spread only after 26 days, and this culture spread was related to the emergence of additional mutations (Table 3). In line with this finding, we had previously shown that LSG alone did not lead to high HCV infectivity titers of the J6 full-length genome (10). In contrast, addition of the “8m” mutations in HCV-1 with LSGF/S399F/TNm resulted in efficient replication and virus production with augmented supernatant infectivity titers in S29 cells (Fig. 4). These results suggest that these mutations mediated efficient replication, virus assembly and release, which was also in agreement with the rapid virus spread of genomes containing “8m” mutations, after transfection of Huh7.5 cells (Fig. 1D). The “4m” (A970T/I1312V/C2419R/A2919T) mutations were sufficient to achieve efficient virus spread and release, although with slightly lower levels compared to HCV1cc. Therefore, “4m” apparently were the minimal set of mutations required to complete an efficient viral life cycle of the HCV-1 with LSGF/S399F/TNm. Of the “4m” mutations, the A970T/A2919T combination was not sufficient to confer efficient viral replication and assembly in S29 cells (Fig. 4), however, when combined with either C2419R or I1312V, replication and assembly were significantly augmented, and therefore we concluded that they played a major role in these processes (Fig. 4A). Additionally, for our experiments in Huh7.5 cells, changing T2919 back to wild-type A2919 significantly reduced viability of HCV1cc (Fig. 2A). Amino acid 2919 (NS5B 499) is in the thumb domain of the polymerase, and has been related to viral replication (55). This position shows a certain level of polymorphism, and amino acid changes at this site have different effects, depending on the strain. In genotype 2a replicon systems, the V2919A change had no effect on JFH1 (56), but A2919V increased the replication of J6 by 10-fold (57). In addition, in the context of full-length genotype 2a systems, A2919V was recently described as a cell culture adaptive mutation in PR63cc, having an effect in replication of this strain (15). Interestingly, when both I1312V and C2419R were combined with A970T/A2929T, extracellular infectivity titers were detectable (Fig. 4A), but increase in supernatant titers was not associated with an increase in replication, and therefore indicating that the combination of I1312V and
C2419R may have an important specific role in release of infectious viral particles. It will be of interest to elucidate, in future studies, whether the rescue of release is mediated uniquely through the interaction of these two positions, located in NS3 and NS5A, or by the recruitment of other viral or host proteins.

Given the historical importance of H77 in HCV research, much effort has been invested in propagating this strain in cell culture, in particular since infectious clones were developed in 1997 (24,25). The H77Cc developed in this study showed efficient replication after RNA transfection of Huh7.5 cells, with infectivity titers of $10^{3.6}$ FFU/ml (Fig. 5B). Importantly, H77Cc spread rapidly after passage to naïve Huh7.5 cells, and the first- and second-passage viruses reached peak of infection within 5-8 days and produced infectivity titers of $10^{4.4}$ and $10^{3.8}$ FFU/ml, respectively (Fig. 5B and Table 4). Therefore, the H77Cc represents a robust and efficient infectious cell culture system for HCV strain H77, with high replication levels after transfection and rapid spread in viral passage cultures.

It was initially found by Yi et al. that an H77C genome carrying mutations derived from its replicon system replicated at a low level in transfected Huh7.5 cells (12). Subsequently, this genome was improved to yield higher infectivity titers by introducing an additional mutation in E2 (H77S.3 (35)), and during the preparation of this manuscript a further adapted genome (H77D) that replicated and spread efficiently in cell culture was reported (52). Similarly to the adaptation process described in this study, H77D was generated by introducing our previously described adaptive TNcc mutations (9) into the H77S.3 backbone. Replication of H77S.3/LSGF/TNm was inhibited, but removal of an adaptive mutation from the original H77S.3 (S2204I) permitted replication and further adaptation of this genome, that was passaged until high titer viruses emerged. The cell culture adapted emerging viruses, which showed significant replication enhancement, contained 3 additional amino acid changes, G1909S (NS4B), D2416G (NS5A) and G2963D (NS5B). Interestingly, our adapted H77Cc also contains G1909S in NS4B, but with V2417A instead of D2416G in NS5A, and V2431I instead of G2963D in NS5B. Both independent approaches for efficient adaptation of H77C thus depended on adaptive mutations from TN cultures, which was the first efficient genotype 1 culture system (9). Thus, the TNcc adaptive mutations might be valuable for
adaptations of additional HCV strains to efficient growth in culture as was also found for the HCV-1 strain in the present study.

In conclusion, we have developed two efficient high-titer culture systems for the globally prevalent HCV genotype 1. The HCV1cc and H77Ccc represent efficient in vitro infectious systems for two historically important strains that have been the foundation for the development of diagnostic tests and key research material in the field, including the discovery of HCV. Both cell culture systems, together with other infectious full-length HCV genomes, will permit genotype- and isolate-specific functional studies of the viral life cycle and of specific viral proteins and their interactions with cellular components. This knowledge will then contribute to basic research on different aspects of HCV and help improving antiviral therapy and future vaccine development.
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Figure Legends

Fig. 1. Viability of HCV-1 5'UTR-NS5A (5-5A) and full-length recombinants in Huh7.5 cells.
(A) Schematic diagrams of HCV genomes. LSG mutations (F1464L/A1672S/D2979G) previously identified from J6 and JFH1 recombinants are highlighted by black dots, additional TNcc-adaptive mutations (TNm) are indicated by gray dots, S399F from HCV-1 5-5A recombinant is indicated by a circle, and eight mutations identified from passaged full-length HCV-1 viruses (8m) are indicated by broken circles. (B-D) RNA transcripts of HCV-1 5-5A and full-length recombinants with the indicated mutations were transfected into Huh7.5 cells, and cultures were monitored for HCV core/NS5A antigens by immunostaining. HCV infectivity titers in culture supernatant collected at the days indicated, after ≥80% of the cells were found HCV antigen positive, were determined by FFU assays, and shown as mean of triplicate infections ± SEM. J65'UTR-NS2/JFH1 (39) was used as a positive control.
LSGF, F1464L/A1672S/D2979G/Y2981F.
TNGH, combination of two TNcc-adaptive mutations A1226G/Q1773H
TNm, combination of four TNcc-adaptive mutations A1226G/Q1773H/N1927T/F2994S.
HCV1cc, HCV1_LSGF/S399F/TNm/8m, in which “8m” indicates the mutations A970T/I1312V/I1326V/V2198A/I2268T/V2419R/E2622D/A2919T.

Fig. 2. Effect of individual adaptive mutations on the viability of HCV1cc.
RNA transcripts of HCV1cc and HCV1cc with each of eight putative adaptive mutations (named “8m”) mutated back to the wild-type sequence were transfected into Huh7.5 cells. J65'UTR-NS2/JFH1 (39) was used as a positive control. Culture supernatants were collected at the days indicated. (A) HCV infectivity titers (FFU/ml) in supernatants from cultures with ≥80% of the cells found HCV antigen positive by immunostaining, and shown as the mean of triplicate infections ± SEM. *, not determined. #, the FFU titers were below the detection limit of 102.4 FFU/ml. (B) The HCV supernatant core antigen level as determined by the Architect HCV Ag detection system (Abbott).
HCV1cc, HCV1_LSGF/S399F/TNm/8m.
LSGF, F1464L/A1672S/D2979G/Y2981F.

TNm, A1226G/Q1773H/N1927T/F2994S.

8m, A970T/I1312V/I1326V/V2198A/I2268T/V2419R/E2622D/A2919T.

Fig. 3. Identification of adaptive mutations sufficient for the viability of HCV1 full-length genomes.

RNA transcripts from HCV1_LSGF/S399F/TNm recombinant with A970T/A2919T, I1312V/A2919T, C2419R/A2919T, A970T/I1312V/A2919T (designated “3m”), A970T/C2419R/A2919T, I1312V/C2419R/A2919T, and A970T/I1312V/C2419R/A2919T (“4m”) were transfected into Huh7.5 cells. The recombinants with A970T/A2919T, I1312V/A2919T, C2419R/A2919T, A970T/C2419R/A2919T, and I1312V/C2419R/A2919T did not spread in the transfection cultures. In contrast, recombinants with “3m” or “4m” spread to ≥80% of the cells during the first week. J65UTR

NS2/JFH1 and HCV1cc were used as controls. Culture supernatants were collected at the indicated days. (A) Supernatant HCV infectivity titers (FFU/ml), shown as mean of triplicate infections ± SEM. *, not determined. #, the FFU titers were below the detection limit of 10^{2.4} FFU/ml. (B) The HCV supernatant core antigen level as determined by the Architect HCV Ag detection system (Abbott).

LSGF, F1464L/A1672S/D2979G/Y2981F.

TNm, A1226G/Q1773H/N1927T/F2994S.

3m, A970T/I1312V/A2919T.

4m, A970T/I1312V/C2419R/A2919T.

Fig. 4. Functional analysis of the role of HCV1cc adaptive mutations in the HCV life cycle.

RNA transcripts from the indicated recombinants were transfected into HCV entry-deficient S29 cells (43). All clones contained LSGF/S399F/TNm plus specific mutations as indicated under each bar graph. Cell lysates were collected at 4 and 48 hours, and culture supernatants were collected at 48 hours. Both intra- and extra- cellular HCV infectivity titers and core levels were determined at 48 hours after transfection. Intracellular core at 4 hours was also determined as a measure of replication independent genome translation following transfection, and used to normalize the 48 hour values. HCV1_LSGF and
HCV1_LSGF/TNm were analyzed in a separate experiment (not shown); no intra- and extracellular infectivity titers were detected, and intra- and extracellular core levels were lower than for HCV1_LSGF/S399F/TNm. A replication incompetent form of J6/JFH1 was included in each experiment (J6/JFH1-GND). A) Intracellular and extracellular infectivity titers. #, no FFU detected by manual count. Values are expressed as log_{10}(FFU/mL) for extracellular titers and as log_{10}(FFU/well) for intracellular infectivity titers. B) Intracellular core levels; the HCV core level at 48 hours was normalized in percentage to the level at 4 hours. C) Extracellular core levels, expressed as log_{10}(Fmol/mL). D) Western blots. Cell lysates harvested 48 hours post-transfection were separated through acrylamide gels and proteins were transferred to PVDF membranes (see Materials and Methods). Immunoblot was performed with anti-HCV core C7-50 for detection of HCV core and anti-β-actin for detection of host-cell actin.

Fig. 5. Viability of adapted H77C in Huh7.5 cells.

RNA transcripts of H77C full-length recombinants with the indicated mutations were transfected into Huh7.5 cells, and cultures were monitored for HCV core/NS5A antigens by immunostaining. Cell-free transfection supernatants collected from peak infection were passaged to naïve Huh7.5 cells (first-passage) and after viral spread, the culture supernatant of first-passage was subsequently used to infect naïve Huh7.5 cells (second-passage). HCV infectivity titers in culture supernatant are shown as mean FFU/ml of triplicate infections ± SEM. (A) Transfection and passage of H77 full-length virus H77C_LSGF/TNm/S1368P. (B) Transfection and passage of H77Ccc. J6/JFH1 was used as a positive control (38).

H77Ccc, H77C_LSGF/TNm/S1368P/10m.
LSGF, F1464L/A1672S/D2979G/Y2981F.
TNmr, A1226G/Q1773H/N1927T/F2994R.
10m, M345T/A828V/L864R/K1052R/V1663A/G1909S/M2105V/S2354G/V2417A/V2431I.
Table 1. Characteristics of the HCV-1 5'UTR-NS5A (5-5A) recombinant and full-length viruses in Huh7.5 cell cultures.

<table>
<thead>
<tr>
<th>Transfection</th>
<th>First passage</th>
<th>Second passage</th>
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<tbody>
<tr>
<td></td>
<td>Transfection</td>
<td>Day with ≥80% infected (day)</td>
</tr>
<tr>
<td>HCV1(5-5A)</td>
<td>+LSG/TNexp. 1</td>
<td>5 ≤2.4</td>
</tr>
<tr>
<td></td>
<td>+LSG/TNexp. 2</td>
<td>5 ≤2.4</td>
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<tr>
<td></td>
<td>+LSG/S399F/TNexp. 1</td>
<td>4 2.7 (8)</td>
</tr>
<tr>
<td></td>
<td>+LSG/S399F/TNexp. 2</td>
<td>4 3.0 (12)</td>
</tr>
<tr>
<td>HCV1 full-length</td>
<td>+LSGF/TNm</td>
<td>26 3.3 (28)</td>
</tr>
<tr>
<td></td>
<td>+LSGF/TNm/8m (HCV1cc)</td>
<td>3 3.8 (5)</td>
</tr>
<tr>
<td></td>
<td>+LSGF/TNm/4m</td>
<td>4 3.4 (13)</td>
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One milliliter of transfection- or first passage-recovered supernatant was used for subsequent infection of cells grown in 6-well plates.

a, the first- and second-passage viruses were sequenced (Table 2).
b, the viruses collected at day 8, 10, and 12 were pooled and used for analysis.
c, the third-passage virus reached 4.2 log_{10} FFU/ml at day 6.
d, in two other independent transfections, HCV1cc produced 3.8-4.0 log_{10} FFU/ml at day 5.

TNexp, A1226G/Q1773H.
LSG, F1464L/A1672S/D2979G.
LSGF, F1464L/A1672S/D2979G/V2981F.
TNm, A1226G/Q1773H/N1927T/F2994S.
8m, A970T/I1312V/I1326V/V2198A/E2208T/C2419R/E2622D/A2919T.
4m, A970T/I1312V/C2419R/A2919T.
nd, not done.
-, not applicable
Table 2. ORF sequence analysis of HCV-1 5’UTR-NS5A (5-5A) viruses.

<table>
<thead>
<tr>
<th>Passage (Day)</th>
<th>E2</th>
<th>NS3</th>
<th>NS3</th>
<th>NS3</th>
<th>NS3</th>
<th>NS3</th>
<th>NS4A</th>
<th>NS4B</th>
<th>NS5A</th>
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<tr>
<td>HCV1(5-5A)</td>
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<tr>
<td>+LSG/TN(TN, exp. 2)</td>
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<tr>
<td>1st (31)</td>
<td>T</td>
<td>G</td>
<td>•</td>
<td>C</td>
<td>•</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>•</td>
<td>G</td>
<td>A/C</td>
<td>G</td>
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<tr>
<td>2nd (8/10/12)</td>
<td>T</td>
<td>G/a</td>
<td>T/a</td>
<td>C/t</td>
<td>C</td>
<td>•</td>
<td>T</td>
<td>C</td>
<td>A/C</td>
<td>G/C</td>
<td>G</td>
<td>C</td>
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<tr>
<td>+LSG/S399F/TN(TN, exp. 1)</td>
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<tr>
<td>2nd (9)</td>
<td>T</td>
<td>G</td>
<td>•</td>
<td>C</td>
<td>G/L</td>
<td></td>
<td>T</td>
<td>C</td>
<td></td>
<td>G</td>
<td>A/C</td>
<td>G</td>
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<tr>
<td>Amino acid position</td>
<td>Recombinant specific</td>
<td>399</td>
<td>1046</td>
<td>1226</td>
<td>1298</td>
<td>1368</td>
<td>1464</td>
<td>1550</td>
<td>1672</td>
<td>1773</td>
<td>2216</td>
<td>2277</td>
</tr>
<tr>
<td>H77 reference (AF009606)</td>
<td>399</td>
<td>1046</td>
<td>1226</td>
<td>1298</td>
<td>1368</td>
<td>1464</td>
<td>1550</td>
<td>1672</td>
<td>1773</td>
<td>2216</td>
<td>2277</td>
<td>2416</td>
</tr>
</tbody>
</table>

Shadings indicate the engineered mutations; LSG mutations (F1464L/A1672S/D2979G) are indicated in white letters with black background, TN-derived mutations are in dark shading, and S399F identified in this study is in light shading. Coding changes are shown; the capital/capital letters indicate a 50/50 nucleotide quasispecies, while the capital/lowercase letters indicates a dominant/minor ratio. Dots indicate identity with the original plasmid sequence.

a, the virus had a non-coding nucleotide change T5750C.
b, the viruses collected at day 8, 10, and 12 were pooled and used for analysis; the recovered sequence contained non-coding nucleotide changes.
T4868C/T, T5750C, and T6251T/C.
c, the virus acquired a non-coding nucleotide change C6131C/T.
TN(TN, A1226G/Q1773H.  810
811
812
813
Table 3. ORF sequence analysis of HCV-1 full-length viruses.

<table>
<thead>
<tr>
<th>Passage (day)</th>
<th>E2</th>
<th>NS2</th>
<th>NS3</th>
<th>NS3</th>
<th>NS3</th>
<th>NS3</th>
<th>NS4A</th>
<th>NS4B</th>
<th>NS5A</th>
<th>NS5B</th>
<th>NS5B</th>
<th>NS5B</th>
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<tr>
<td>HCV1 full-length</td>
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<tr>
<td>+LSGF/S399F/TNm&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>2&lt;sup&gt;nd&lt;/sup&gt; (11)</td>
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<td>A</td>
<td>G</td>
<td>G</td>
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<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; (11)</td>
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<td>A</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
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<td>C</td>
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<tr>
<td>+LSGF/S399F/TNm&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; (9)</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>+LSGF/S399F/TNm&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; (18)</td>
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<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

Amino acid position

| Recombinant specific |     |     |     |     |     |     |      |      |      |      |      |      |
| H77 reference (AF009606) |     |     |     |     |     |     |      |      |      |      |      |      |
| Recombinant nucleotide |     |     |     |     |     |     |      |      |      |      |      |      |
| C | G | C | A | A | T | G | A | A | T | T | T | A | G | A | A | T |

Nucleotide position

| Recombinant specific |     |     |     |     |     |     |      |      |      |      |      |      |
| H77 reference (AF009606) |     |     |     |     |     |     |      |      |      |      |      |      |
| Recombinant nucleotide |     |     |     |     |     |     |      |      |      |      |      |      |
| C | G | C | A | A | T | G | A | A | T | T | T | A | G | A | A | T |

Amino acid position

| Recombinant specific |     |     |     |     |     |     |      |      |      |      |      |      |
| H77 reference (AF009606) |     |     |     |     |     |     |      |      |      |      |      |      |
| Recombinant nucleotide |     |     |     |     |     |     |      |      |      |      |      |      |
| C | G | C | A | A | T | G | A | A | T | T | T | A | G | A | A | T |

<sup>a</sup> The in vivo viable HCV-1/SF9_A (GenBank accession number AF271632) (36) engineered with LSGF (F1464L/A1672S/D2979G/Y2981F), S399F identified from HCV-1 5-5A recombinant (Table 2), and TNm (A1226G/Q1773H/N1927T/F2994S) was adapted for growth in transfected Huh7.5 cells. Eight mutations (A970T/I1312V/I1326V/V2198A/I2268T/V2419R/E2994S/A2919T) were identified from passaged HCV1cc/S399F/TNm viruses and were engineered back to the genome to make HCV1LSGF/S399F/TNm viruses, designated “HCV1cc”. The HCV1cc genome, containing seventeen mutations, showed efficient virus spread in transfection cultures and released infectious virus particles with infectivity titers of 10<sup>6.3</sup> FFU/ml (Fig. 1). A HCV-1 full-length virus, HCV1LSGF/S399F/TNm<sup>d</sup> (“4m”, A970T/I1312V/C2419R/A2919T) and third passage viruses acquired non-coding nucleotide changes T2408C and C2765T.

<sup>b</sup> a, the constructed plasmid contained a non-coding nucleotide change C3210A, which was maintained in the passage-recovered viruses. The second- and third-passage viruses acquired non-coding nucleotide changes T2408C and C2765T.

<sup>c</sup> b, no non-coding change was found in ORF sequence analysis.
Table 4. ORF sequence analysis of H77C full-length viruses.

<table>
<thead>
<tr>
<th>Passage (Day)</th>
<th>E1</th>
<th>NS2</th>
<th>NS3</th>
<th>NS4A</th>
<th>NS4B</th>
<th>NS5A</th>
<th>NS5B</th>
<th>NS5B</th>
<th>NS5B</th>
</tr>
</thead>
<tbody>
<tr>
<td>H77C_LSGF/TNmr/S1368P</td>
<td>135</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>G</td>
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<tr>
<td>H77Cc</td>
<td>203</td>
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<td>203</td>
<td>203</td>
<td>203</td>
<td>203</td>
<td>203</td>
<td>203</td>
<td>203</td>
</tr>
</tbody>
</table>

The in vivo viable H77C genome (24) engineered with ten nucleotides changes (resulting in nine amino acid changes, as nucleotides 9321 and 9322 are in the same codon, see “b” below), named H77C_LSGF/TNmr/S1368P (see below), had low level replication after transfection of Huh7.5 cells and spread to most culture cells at day 96. Ten mutations (“10m”) was identified from H77C_LSGF/TNmr/S1368P, and engineered into the genome to make H77Cc. The H77Cc genome, which had a total of nineteen amino acid changes, showed efficient virus spread in transfection- and infection-cultures, and released infectious virus particles with HCV infectivity titers of 10^3.5-10^4.4 FFU/ml (Fig. 5). The H77C full-length viruses were passaged to naïve Huh7.5 cells, the viruses spread to ≥80% of culture cells, within three days for H77Cc and within 8 days for H77C_LSGF/TNmr/S1368P, and then the culture supernatants were collected for sequence analysis. Shadings indicate the engineered mutations; LSGF mutations (LSG, F1464L/A1672S/D2979G; F, Y2981F) are indicated in white letters with a black background, TNcc-adaptive mutations (TNm) are in dark shading, and mutations identified in this study are in light shading.

a, mutation S1368P was previously identified from H77C (46) and HCV-1 (Table 2) 5-5A recombinant viruses; the first- and second-passage H77C_LSGF/TNmr/S1368P viruses both acquired non-coding nucleotide changes A2558G, A3089G, G3860A, C4403T, T4904C, A6437G, A6713G, A7727G, A8804G, and T9227C.

b, nucleotides 9321 and 9322 are in the same codon for F2994R change.

H77Cc, H77C_LSGF/TNmr/S1368P/10m.

LSGF, F1464L/A1672S/D2979G/Y2981F.

TNmr, A1226G/Q1773H/N1927T/F2994R (the F2994R was identified from a LSGF-adapted TN full-length virus, see Results for details).

“10m”, M345T/A828V/L864R/K1052R/V1663A/G1909S/M2105V/S2354G/V2417A/V2431I.