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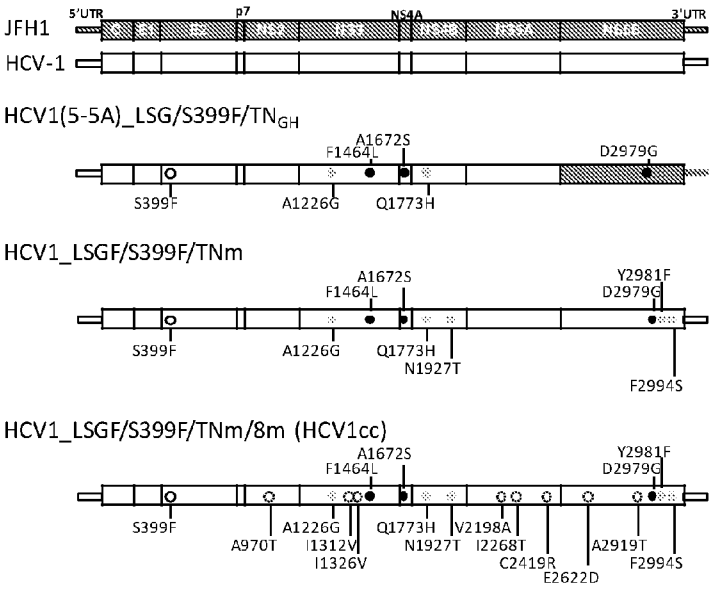

(57) Abstract: The present invention relates to nucleic acid sequences that encode hepatitis C viruses (HCV) that are useful in the fundamental research of HCV as well as in the search of a vaccine against HCV. In particular the present invention relates to nucleic acid sequences that comprises HCVs which are capable of expressing said virus when transfected into cells and are capable of infectivity in vivo.

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OPTIMIZED HCV FULL-LENGTH INFECTIOUS CELL CULTURE SYSTEMS AND APPLICATIONS THEREOF

Technical field of the invention

The present invention relates to nucleic acid sequences that encode hepatitis C viruses (HCV) that are useful in the fundamental research of HCV as well as in the search of drug candidates and a vaccine against HCV. In particular the present invention relates to nucleic acid sequences that comprises HCV, which are capable of expressing said virus when transfected into cells and/or are capable of infectivity *in vivo*.

Background of the invention

Hepatitis C is one of the most widespread infectious diseases in the world. About 180 million people are infected with hepatitis C virus (HCV) worldwide with a yearly incidence of 3-4 million.

While the acute phase of infection is mostly asymptomatic, the majority of acutely infected individuals develops chronic hepatitis and is at increased risk of developing liver cirrhosis and hepatocellular carcinoma.

Thus, HCV infection is a major contributor to end-stage liver disease and in developed countries to liver transplantation.

HCV is a small, enveloped virus classified as a member of the Flaviviridae family. Its genome consists of a 9.6 kb single stranded RNA of positive polarity composed of 5´ and 3´ untranslated regions (UTR) and one long open reading frame (ORF) encoding a polyprotein, which is co- and post-translationally cleaved and thus yields the structural (Core, E1, E2), p7 and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins.

HCV isolates from around the world exhibit significant genetic heterogeneity. At least 7 major HCV genotypes (genotypes 1-7) have been identified, which differ by 31-33% at the nucleotide level and deduced amino acid level.

In addition, there are numerous subtypes (a, b, c, etc.), which differ by 20-25% on the nucleotide and deduced amino acid level.

Since its discovery in 1989, research on HCV has been hampered by the lack of appropriate cell culture systems allowing for research on the complete viral life cycle as well as new therapeutics and vaccines.

In 2001, a genotype 2a isolate (JFH1) was described, which subsequently was found to yield high RNA titers in the replicon system without adaptive mutations.

A major breakthrough occurred in 2005, when formation of infectious viral particles was reported after transfection of RNA transcripts from the JFH1 full-length consensus cDNA clone into Huh7 cells.

At the same time, it was demonstrated that the intragenotypic 2a/2a recombinant genome (J6/JFH1), in which the structural genes (Core, E1, E2), p7 and NS2 of JFH1 were replaced by the respective genes of clone J6CF, produced infectious viral particles in Huh7.5 cells (a cell line derived from bulk Huh7 cells) with an accelerated kinetic.

Cell culture derived J6/JFH viruses were apparently fully viable *in vivo*.

Despite the importance of the described cell culture systems they represent only a single isolate (genotype 2a) of HCV.

It is important to develop cell culture systems for representative strains of other HCV isolates, subtypes and genotypes, since neutralizing antibodies are not expected to cross-neutralize all genotypes and new specific antiviral compounds have differential efficiencies against different isolates, subtypes and genotypes.

To date, only the JFH1 (genotype 2a) clone could autonomously replicate and release infectious virus in cultured human hepatoma cells, Huh7 and Huh7.5; its efficient growth depended on mutations.

A JFH1 chimera with the 5'UTR-NS2 region from another genotype 2a strain cDNA clone, J6CF, had enhanced infectivity.

Besides, an H77 (genotype 1a) clone containing replicon-derived mutations was shown to produce infectious virus particles.

To facilitate HCV research and obtain basic knowledge for better and individualized treatment, the present inventors have aimed at developing culture systems for other HCV patient isolates.

Hence, improved and alternative HCV genomes of all genotypes, which are capable of expressing said virus when transfected into cells and are capable of infectivity *in vivo,* would be advantageous.

Summary of the invention

Thus, an object of the present invention relates to nucleotide sequences that encode HCV that are useful in the fundamental research of HCV as well as in the search of drug candidates and a vaccine against HCV.

In particular, it is an object of the present invention to provide nucleotide sequences of HCV which are capable of expressing said virus when transfected into cells and are capable of infectivity *in vivo*.

Thus, one aspect of the invention relates to an isolated nucleic acid molecule which encodes a human hepatitis C virus wherein the hepatitis C virus is derived from genotype 1a, isolate HCV-1_LSGF/S399F/TNm/4m (TNm, A1226G/Q1773H/N1927T/F2994S; 4m, A970T/I1312V/C2419R/A2919T) (SEQ ID NO:2) and has a nucleic acid sequence with 90% sequence identity to HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:2).

The nucleotide and amino acid numbering throughout is according to the H77 (1a) reference sequence (GenBank accession number AF009606).

Another aspect of the invention relates to an isolated nucleic acid molecule which encodes a human hepatitis C virus wherein the hepatitis C virus is derived from

genotype 1a and is isolate HCV1cc (SEQ ID NO:1) and has a nucleic acid sequence with 90% sequence identity to isolate HCV1cc (SEQ ID NO:1).

Another aspect of the invention relates to an isolated nucleic acid molecule which encodes a human hepatitis C virus wherein the hepatitis C virus is derived from genotype 1a and is isolate H77Ccc (SEQ ID NO:3) and has a nucleic acid sequence with 90% sequence identity to isolate H77Ccc (SEQ ID NO:3).

Another aspect of the present invention relates to an isolated nucleic acid molecule which encodes a human hepatitis C virus, wherein said molecule is capable of expressing said virus when transfected into cells, is capable of infectivity in vivo, comprises at least one adaptive mutation in the amino acid sequence of NS3, which is F1464L, comprises at least one adaptive mutation in the amino acid sequence of NS4A which is A1672S, comprises at least one adaptive mutation in the amino acid sequence of NS5B which is D2979G, and at least one additional adaptive mutation in the amino acid sequence selected from the group consisting of S399F, A970T, A1226G, I1312V, I1326V, Q1773H, N1927T, V2198A, I2268T, C2419R, E2622D, A2919T, Y2981F, F2994S, M345T, A828V, L864R, K1052R, S1368P, V1663A, G1909S, M2105V, S2354G, V2417A, V2431I, and F2994R.

Yet another aspect of the present invention is to provide vectors, cells, compositions and viral particles that comprise the nucleic acids sequences of the present invention.

Still other aspects of the present invention are to provide methods for producing a hepatitis C virus particle, for *in vitro* producing a hepatitis C virus-infected cell, for screening an anti-hepatitis C virus substance and for producing a hepatitis C virus vaccine.

Brief description of the figures

Figure 1 shows 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. $J6^{5'UTR-NS2}$/JFH1 was used as a positive control. LSGF, F1464L/A1672S/D2979G/Y2981F. TN_{GH}, 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.

Figure 2 shows 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. $J6^{5'UTR-NS2}$/JFH1 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 $10^{2.4}$ 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.

Figure 3 shows identification of adaptive mutations sufficient for the viability of HCV-1 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. $J6^{5'UTR-NS2}$/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.

Figure 4 shows 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. 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-actin for detection of host-cell actin.

Figure 5 shows 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/TNmr/S1368P. (B) Transfection and passage of H77Ccc. J6 /JFH1 was used as a positive control.
H77Ccc, H77C_LSGF/TNmr/S1368P/10m.
LSGF, F1464L/A1672S/D2979G/Y2981F.
TNmr, A1226G/Q1773H/N1927T/F2994R.
10m,
M345T/A828V/L864R/K1052R/V1663A/G1909S/M2105V/S2354G/V2417A/V2431I.

Figure 6 [table 1 (figure 6)] shows characteristics of the HCV-1 5'UTR-NS5A (5-5A) recombinant and full-length viruses in Huh7.5 cell cultures. 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 (Figure 7).
b, the viruses collected at day 8, 10, and 12 were pooled and used for analysis.
c, the third-passage virus reached 4.2 log10 FFU/ml at day 6.
d, in two other independent transfections, HCV1cc produced 3.8-4.0 log10 FFU/ml at day 5.
TN_{GH}, A1226G/Q1773H.
LSG, F1464L/A1672S/D2979G.
LSGF, F1464L/A1672S/D2979G/Y2981F.
TNm, A1226G/Q1773H/N1927T/F2994S.
8m, A970T/I1312V/I1326V/V2198A/I2268T/C2419R/E2622D/A2919T.

4m, A970T/I1312V/C2419R/A2919T.
nd, not done.
-, not applicable

Figure 7 [table 2 (figure 7)] shows ORF sequence analysis of HCV-1 5'UTR-NS5A (5-5A) viruses. 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_{GH}, A1226G/Q1773H.

Figure 8 [table 3 (figure 8)] shows ORF sequence analysis of HCV-1 full-length viruses. The *in vivo* viable HCV-1/SF9_A (GenBank accession number AF271632) engineered with LSGF (F1464L/A1672S/D2979G/Y2981F), S399F identified from HCV-1 5-5A recombinant (figure 7), and TNm (A1226G/Q1773H/N1927T/F2994S) was adapted for growth in transfected Huh7.5 cells. Eight mutations (A970T/I1312V/I1326V/V2198A/I2268T/V2419R/E2622D/A2919T, designated "8m") were identified from passaged HCV1_LSGF/S399F/TNm viruses and were engineered back to the genome to make HCV1_LSGF/S399F/TNm/8m, designated "HCV1cc". The HCV1cc genome, containing seventeen mutations, showed efficient virus spread in tranfection cultures and released infectious virus particles with HCV infectivity titers of $10^{3.8}$ FFU/ml (Fig. 1). A HCV-1 full-length virus, HCV1_LSGF/S399F/TNm/4m ("4m", A970T/I1312V/C2419R/A2919T) replicated efficiently in the culture, with infectivity close to those of HCV1cc. The HCV-1 full-length viruses were passaged to naïve Huh7.5 cells, the viruses spread efficiently, and the culture supernatant were collected at indicated time-points for sequence analysis.

Shadings indicate the engineered mutations; LSGF mutations are indicated in white letters with black background, TNcc-derived mutations (TNm) are in dark shading, and mutations identified in this study are in light shading.
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.
b, no non-coding change was found in ORF sequence analysis.

Figure 9 [table 4 (figure 9)] shows ORF sequence analysis of H77C full-length viruses. The in vivo viable H77C genome 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 H77Ccc. The H77Ccc genome, which had a total of nineteen amino acid changes, showed efficient virus spread in tranfection- 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 H77Ccc 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 and HCV-1 (figure 7) 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.
H77Ccc, 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.

The present invention will now be described in more detail in the following.

Detailed description of the invention

The present invention advantageously provides hepatitis C virus (HCV) nucleotide sequences capable of replication, expression of functional HCV proteins, and infection in vivo and in vitro for development of antiviral therapeutics and diagnostics.

Nucleic acid molecules (cDNA clones and RNA transcripts)

The genomes of the different HCV genotypes have been standardized in a numbering system for HCV nucleotides, proteins and epitopes.

This work was done in an expert meeting and published in Kuiken et al., Hepatology, November 2006, page 1355-61.

This numbering system allows comparison of genes across genotypes with consistency and with an unambiguous method for referring to amino acid substitutions for specific positions in genes encoded by the HCV genome.

The numbering used in the present application uses this numbering system and with reference to the H77 reference sequence with GenBank accession number AF009606 used in Kuiken et al.

Thus, when no other statement is made will a specific number of a specific genotype refer to the H77 reference sequence with GenBank accession number AF009606.

In a broad aspect, the present invention is directed to an isolated nucleic acid molecule which encodes a human hepatitis C virus, wherein said molecule is capable of expressing said virus when transfected into cells, is capable of infectivity in vivo, comprises at least one adaptive mutation in the amino acid sequence of NS3, which is F1464L, comprises at least adaptive mutation in the amino acid sequence of NS4A which is A1672S, and comprises at least one adaptive mutation in the amino acid sequence of NS5B which is D2979G, and at least one additional adaptive mutation in the amino acid sequence selected from the group consisting of S399F, A970T, A1226G, I1312V, I1326V, Q1773H, N1927T, V2198A, I2268T, C2419R, E2622D, A2919T, Y2981F, F2994S, M345T, A828V, L864R, K1052R, S1368P, V1663A, G1909S, M2105V, S2354G, V2417A, V2431I, and F2994R according to the H77 reference sequence with GenBank accession number AF009606.

The adaptive mutations as shown above means that in the case of F1464L is phenylalanine at amino acid position 1464 changed to Leucine.

The original amino acids F1464, A1672, and D2979 (H77 reference numbers) at LSG positions are highly conserved across all HCV genotypes.

In one embodiment the present invention comprises the nucleic acid molecule with a shortened 3' UTR region.

A shortened 3' UTR region refers to any 3' UTR region wherein one or more nucleotides have been deleted. The present inventors have previously exemplified such shortened 3'UTR region by a 33 U deletion in the 3'UTR (Δ33U).

In another embodiment of the present invention the human hepatitis C virus is of a genotype selected from the group consisting of 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a, 6a and 7a.

The terms "isolate" and "strain" are used herein interchangeably.

In a preferred embodiment of the present invention the human hepatitis C virus is a strain of genotype 1a.

In a preferred embodiment of the present invention the human hepatitis C virus is a strain of genotype 2a or 2b.

In another preferred embodiment of the present invention the human hepatitis C virus is a strain of genotype 2a.

In another preferred embodiment of the present invention the human hepatitis C virus is a strain of genotype 2b.

In another preferred embodiment of the present invention the hepatitis C virus is of genotype 1a and is isolate HCV1cc (SEQ ID NO:1).

In another preferred embodiment of the present invention the hepatitis C virus is of genotype 1a and is isolate HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:2).

In another preferred embodiment of the present invention the hepatitis C virus is of genotype 1a and is H77Ccc (SEQ ID NO:3).

The hepatitis C virus can in some embodiments of the present invention comprise further adaptive mutations.

In one embodiment the present invention comprises the hepatitis C virus and at least six further adaptive mutations, such as five, such as four, such as three, such as two, such as one.

The adaptive LSG mutations F1464L/A1672S/D2979G are according to the H77 reference sequence with GenBank accession number AF009606. The work has been published as Li et al., Proc Natl Acad Sci U S A. 2012 May 1;109(18):E1101-10. Also see Author Summary in Proc Natl Acad Sci U S A on page 6806 (volume 109, number 18).

The present inventors have identified a wide variety of recombinants that generated different virus viability.

In an embodiment of the present invention are these sequences isolated nucleic acid sequences and amino acid sequence, respectively.

As commonly defined "identity" is here defined as sequence identity between genes or proteins at the nucleotide or amino acid level, respectively. Thus, in the present context "sequence identity" is a measure of identity between proteins at the amino acid level and a measure of identity between nucleic acids at nucleotide level. The protein sequence identity may be determined by comparing the amino acid sequence in a given position in each sequence when the sequences are aligned. Similarly, the nucleic acid sequence identity may be determined by comparing the nucleotide sequence in a given position in each sequence when the sequences are aligned.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps may be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions (e.g., overlapping positions) x 100).

In one embodiment the two sequences are the same length.

In another embodiment the two sequences are of different length and gaps are seen as different positions.

One may manually align the sequences and count the number of identical amino acids. Alternatively, alignment of two sequences for the determination of percent identity may be accomplished using a mathematical algorithm. Such an algorithm is incorporated into the NBLAST and XBLAST programs of (Altschul et al. 1990). BLAST nucleotide searches may be performed with the NBLAST program, score =

100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches may be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecule of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST may be utilised. Alternatively, PSI-Blast may be used to perform an iterated search which detects distant relationships between molecules. When utilising the NBLAST, XBLAST, and Gapped BLAST programs, the default parameters of the respective programs may be used. See http://www.ncbi.nlm.nih.gov. Alternatively, sequence identity may be calculated after the sequences have been aligned e.g. by the BLAST program in the EMBL database (www.ncbi.nlm.gov/cgi-bin/BLAST). Generally, the default settings with respect to e.g. "scoring matrix" and "gap penalty" may be used for alignment. In the context of the present invention, the BLASTN and PSI BLAST default settings may be advantageous.

The percent identity between two sequences may be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

An embodiment of the present invention thus relates to sequences of the present invention that has some degree of sequence variation.

One embodiment relates to HCV1cc (SEQ ID NO:1) in which the nucleic acid molecule comprises the nucleic acid sequence with a sequence identity of at least 80% to that of HCV1cc (SEQ ID NO:1).

In another embodiment, the nucleic acid comprises a sequence sharing at least 85 % identity with that set forth in HCV1cc (SEQ ID NO:1), such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

Another embodiment relates to HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:2) in which the nucleic acid molecule comprises the nucleic acid sequence with a sequence identity of at least 80% to that of HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:2).

In another embodiment, the nucleic acid comprises a sequence sharing at least 85 % identity with that set forth in HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:2), such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

Yet another embodiment relates to H77Ccc (SEQ ID NO:3) in which the nucleic acid molecule comprises the nucleic acid sequence with a sequence identity of at least 80% to that of H77Ccc (SEQ ID NO:3).

In another embodiment, the nucleic acid comprises a sequence sharing at least 85 % identity with that set forth in H77Ccc (SEQ ID NO:3), such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

It should be noted that while several of the sequences in the present application (SEQ ID NOs: 1-3) are DNA sequences (SEQ ID NOs: 4-6 are amino acid sequences), the present invention contemplates the corresponding RNA sequence, and DNA and RNA complementary sequences as well.

In another preferred embodiment of the present invention the hepatitis C virus is of genotype 1a and is isolate HCV1cc (SEQ ID NO:4).

In another preferred embodiment of the present invention the hepatitis C virus is of genotype 1a and is isolate HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:5).

In another preferred embodiment of the present invention the hepatitis C virus is of genotype 1a and is H77Ccc (SEQ ID NO:6).

One embodiment relates to HCV1cc (SEQ ID NO:4) in which the amino acid molecule comprises the amino acid sequence with a sequence identity of at least 80% to that of HCV1cc (SEQ ID NO:4).

In another embodiment, the amino acid comprises a sequence sharing at least 85 % identity with that set forth in HCV1cc (SEQ ID NO:4), such as 90 % identity, 91

% identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

Another embodiment relates to HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:5) in which the amino acid molecule comprises the amino acid sequence with a sequence identity of at least 80% to that of HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:5).

In another embodiment, the amino acid comprises a sequence sharing at least 85 % identity with that set forth in HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:5), such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

Yet another embodiment relates to H77Ccc (SEQ ID NO:6) in which the amino acid molecule comprises the amino acid sequence with a sequence identity of at least 80% to that of H77Ccc (SEQ ID NO:6).

In another embodiment, the amino acid comprises a sequence sharing at least 85 % identity with that set forth in H77Ccc (SEQ ID NO:6), such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

Thus, in cases where a DNA sequence is mentioned refers such DNA sequence also to the RNA equivalent i.e. with Ts exchanged with Us as well as their complimentary sequences.

In another embodiment, the HCV nucleic acid is a DNA that codes on expression or after in vitro transcription for a replication-competent HCV RNA genome, or is itself a replication-competent HCV RNA genome.

In one embodiment, the HCV nucleic acid of the invention has a full-length sequence as depicted in or corresponding to the sequences of the present invention.

Various modifications for example of the 5' and 3' UTR are also contemplated by the invention.

In another embodiment, the nucleic acid further comprises a reporter gene, which, in one embodiment, is a gene encoding neomycin phosphotransferase, Renilla luciferase, secreted alkaline phosphatase (SEAP), Gaussia luciferase or the green fluorescent protein.

Naturally, as noted above, the HCV nucleic acid sequence of the invention is selected from the group consisting of double stranded DNA, positive-sense cDNA, or negative-sense cDNA, or positive-sense RNA or negative-sense RNA or double stranded RNA.

Thus, where particular sequences of nucleic acids of the invention are set forth, both DNA and corresponding RNA are intended, including positive and negative strands thereof.

In a further embodiment, the nucleic acid sequences or the nucleic acid sequences with any mutation described in this document is obtained by any other means than what is described above.

Nucleic acid molecules according to the present invention may be inserted in a plasmid vector for translation of the corresponding HCV RNA. Thus, the HCV DNA may comprise a promoter 5' of the 5'UTR on positive-sense DNA, whereby transcription of template DNA from the promoter produces replication-competent RNA. The promoter can be selected from the group consisting of a eukaryotic promoter, yeast promoter, plant promoter, bacterial promoter, or viral promoter.

Thus, in one embodiment the present invention provides a cassette vector for cloning viral genomes, comprising, inserted therein, the nucleic acid sequence according to the invention and having an active promoter upstream thereof.

Adaptive Mutations

Adapted mutants of a HCV-cDNA construct or HCV-RNA full-length genome with improved abilities to generate infectious viral particles in cell culture compared to

the original HCV-cDNA construct or the original HCV-RNA full-length genome are characterized in that they are obtainable by a method in which the type and number of mutations in a cell culture adapted HCV-RNA genome are determined through sequence analysis and sequence comparison and these mutations are introduced into a HCV-cDNA construct, particularly a HCV-cDNA construct according to the present invention, or into an (isolated) HCV-RNA full-length genome, either by site-directed mutagenesis, or by exchange of DNA fragments containing the relevant mutations.

The present inventors here report adaptive mutations, which allow efficient formation and release of viral particles in cell culture, and thus the present invention relates to these adaptive mutations in the present use as well as use in other strains by changing equivalent positions of such genomes to the adapted nucleotide or amino acid described.

A group of preferred HCV-cDNA constructs, HCV-RNA full-length genomes with the ability to release viral particles in cell culture, which are consequently highly suitable for practical use, is characterized in that it contains one, several or all of the nucleic acid exchanges listed below and/or one or several or all of the following amino acid exchanges.

One embodiment of the present invention relates to adaptive mutations, wherein the adaptive mutation is a mutation that can be observed by clonal or direct sequencing of recovered replicating genomes of the sequences of the present invention.

Thus in a further embodiment, the present invention relates to nucleic acid molecules according to the present invention, wherein said molecule comprises one or more adaptive mutations in p7, NS2, NS3, NS4A, NS4B, NS5A or NS5B singly or in combination.

In the context of the present invention the term "adaptive mutation" is meant to cover mutations identified in passaged viruses that provide the original and any other HCV sequence the ability to grow efficiently in culture. Furthermore all introductions of mutations into the sequences described, whether or not yielding

better growth abilities, and the introduction of these mutations into any HCV sequence should be considered.

Thus the described mutations enable the HCV-RNA genome (e.g. derived from a HCV-cDNA clone) to form viral particles in and release these from suitable cell lines. In addition some of the described mutations might change the function of the concerned proteins in favourable ways, which might be exploited in other experimental systems employing these proteins.

This also includes other HCV genomes with adaptive mutations, all of them, combinations of them or individual mutations that grow in culture.

It should be understood that any feature and/or aspect discussed above in connection with the mutations according to the invention apply by analogy to both single mutations and any combination of the mutations.

In another embodiment all the amino acid changes observed herein are provided by the present application. The skilled addressee can easily obtain the same amino acid change by mutating another base of the codon and hence all means of obtaining the given amino acid sequence is intended.

Titer

To determine the efficiency of the developed system, HCV RNA titers are determined in IU/ml (international units/ml) with Taq-Man Real-Time-PCR and infectious titers are determined with a 50% tissue culture infectious dose method. This titer shows the dilution of the examined viral stock, at which 50% of the replicate cell cultures used in the essay become infected and is given in TCID50/ml.

Alternatively the infectious titers are determined as FFU/ml (focus forming unites/ml); in such method, infectivity titers are determined by infection of cell culture replicates with serial dilutions of virus containing supernatants and, following immuno-stainings for HCV antigens, counting of HCV-antigen positive cell foci.

HCV RNA titers and infectivity titers can be determined extracellularly, in cell culture supernatant (given as IU and TCID50 or FFU per ml, respectively) or intracellularly, in lysates of pelleted cells (given as IU and TCID50 or FFU related to a the given cell number or culture plate wells, which was lysed).

One embodiment of the present invention relates to a nucleic acid molecule of the present invention, wherein said molecule is capable of generating a HCV RNA titer of 10^4 IU/ml or above following transfection and/or subsequent viral passage, such as a titer of at least 10^5 IU/mL, such as a titer of at least 10^6 IU/mL, such as a titer of at least 10^7 IU/mL, such as a titer of at least 10^8 IU/mL, such as a titer of at least 10^9 IU/mL, such as a titer of at least 10^{10} IU/mL, such as a titer of at least 10^{11} IU/mL, or such as a titer of at least 10^{12} IU/mL.

In another embodiment, the present invention relates to a nucleic acid molecule according to the invention, wherein said molecule is capable of generating a HCV infectivity titer of at least 10^2 TCID50/ml or above following transfection and/or subsequent viral passage, such as a titer of at least 10^3 TCID50/ml, such as a titer of at least 10^4 TCID50/ml, such as a titer of at least 10^5 TCID50/ml, such as a titer of at least 10^6 TCID50/ml, such as a titer of at least 10^7 TCID50/ml, such as a titer of at least 10^8 TCID50/ml, such as a titer of at least 10^9 TCID50/ml or such as a titer of at least 10^{10} TCID50/ml.

It is of course evident to the skilled addressee that the titers described here are obtained using the assay described in this text. Any similar or equivalent titer determined by any method is thus evidently within the scope of the present invention.

Compositions

One embodiment of the present invention relates to a composition comprising a nucleic acid molecule according to the invention suspended in a suitable amount of a pharmaceutical acceptable diluent or excipient.

In another embodiment, this invention provides for compositions comprising an isolated nucleic acid, vector or cell of this invention, or an isolated nucleic acid obtained via the methods of this invention.

In one embodiment, the term "composition" refers to any such composition suitable for administration to a subject, and such compositions may comprise a pharmaceutically acceptable carrier or diluent, for any of the indications or modes of administration as described. The active materials in the compositions of this invention can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

It is to be understood that any applicable drug delivery system may be used with the compositions and/or agents/vectors/cells/nucleic acids of this invention, for administration to a subject, and is to be considered as part of this invention.

The compositions of the invention can be administered as conventional HCV therapeutics. The compositions of the invention may include more than one active ingredient which interrupts or otherwise alters groove formation, or occupancy by RNA or other cellular host factors, in one embodiment, or replicase components, in another embodiment, or zinc incorporation, in another embodiment.

The precise formulations and modes of administration of the compositions of the invention will depend on the nature of the anti-HCV agent, the condition of the subject, and the judgment of the practitioner. Design of such administration and formulation is routine optimization generally carried out without difficulty by the practitioner.

It is to be understood that any of the methods of this invention, whereby a nucleic acid, vector or cell of this invention is used, may also employ a composition comprising the same as herein described, and is to be considered as part of this invention.

"Pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state

government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans.

The term "excipient" refers to a diluent, adjuvant, carrier, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response. Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response.

Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvmm.

Preferably, the adjuvant is pharmaceutically acceptable.

Thus relates one embodiment of the present invention to a composition comprising a nucleic acid molecule according to the present invention suspended in a suitable amount of a pharmaceutical acceptable diluent or excipient.

Cells

The nucleotides of the present invention may be used to provide a method for identifying additional cell lines that are permissive for infection with HCV,

comprising contacting (e.g. transfecting) a cell line in tissue culture with an infectious amount of HCV RNA of the present invention, e.g., as produced from the plasmid clones, and detecting replication and formation and release of viral particles of HCV in cells of the cell line.

Naturally, the invention extends as well to a method for identifying an animal that is permissive for infection with HCV, comprising introducing an infectious amount of the HCV RNA, e.g., as produced by the plasmids, to the animal, and detecting replication and formation and release of viral particles of HCV in the animal. By providing infectious HCV, e.g. comprising a dominant selectable marker, the invention further provides a method for selecting for HCV with further adaptive mutations that permit higher levels of HCV replication in a permissive cell line or animal comprising contacting (e.g. transfecting) a cell line in culture, or introducing into an animal, an infectious amount of the HCV RNA, and detecting progressively increasing levels of HCV RNA and infectious HCV viral particles in the cell line or the animal.

In a specific embodiment, the adaptive mutation permits modification of HCV tropism. An immediate implication of this aspect of the invention is creation of new valid cell culture and animal models for HCV infection.

The permissive cell lines or animals that are identified using the nucleic acids of the invention are very useful, inter alia, for studying the natural history of HCV infection, isolating functional components of HCV, and for sensitive, fast diagnostic applications, in addition to producing authentic HCV virus or components thereof.

Because the HCV DNA, e.g., plasmid vectors, of the invention encode HCV components, expression of such vectors in a host cell line transfected, transformed, or transduced with the HCV DNA can be effected.

For example, a baculovirus or plant expression system can be used to express HCV virus particles or components thereof. Thus, a host cell line may be selected from the group consisting of a bacterial cell, a yeast cell, a plant cell, an insect cell, and a mammalian cell.

In one embodiment, the cell is a hepatocyte, or in another embodiment, the cell is the Huh-7 hepatoma cell line or a derived cell line such as Huh7.5, Huh7.5.1 cell line.

In one embodiment, the cell, or in another embodiment, cell systems of this invention comprise primary cultures or other, also non hepatic cell lines. "Primary cultures" refers, in one embodiment, to a culture of cells that is directly derived from cells or tissues from an individual, as well as cells derived by passage from these cells, or immortalized cells.

In one embodiment, "cell line" refers to a population of cells capable of continuous or prolonged growth and division in vitro. The term "cell lines" also includes immortalized cells. Often, cell lines are clonal populations derived from a single progenitor cell. Such cell lines are also termed "cell clones". It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell clones referred to may not be precisely identical to the ancestral cells or cultures. According to the present invention, such cell clones may be capable of supporting replication of a vector, virus, viral particle, etc., of this invention, without a significant decrease in their growth properties, and are to be considered as part of this invention.

It is to be understood that any cell of any organism that is susceptible to infection by or propagation of an HCV construct, virus or viral particle of this invention is to be considered as part of this invention, and may be used in any method of this invention, such as for screening or other assays, as described herein.

Thus relates one embodiment of the present invention to a cell comprising the nucleic acid according to the present invention, the composition of present invention or the cassette vector of the present invention.

Another embodiment of the present invention relates to a method for producing a cell, which replicates human hepatitis C virus and produces a virus particle comprising introducing a nucleic acid molecule of the present invention into a cell.

In a preferred embodiment is the cell is a Huh7.5 cell.

Another embodiment of the present invention relates to a cell obtainable by the methods of the present invention.

Also, a method for in vitro producing a hepatitis C virus-infected cell comprising culturing the cell which produces virus particles of the present invention and infecting other cells with the produced virus particle in the culture.

Naturally, the invention extends to any cell obtainable by such methods, for example any in vitro cell line infected with HCV, wherein the HCV has a genomic RNA sequence as described herein such as a hepatitis C virus infected cell obtainable by any of the methods described.

In one embodiment, the cell line is a hepatocyte cell line such as Huh7 or derived cell lines e.g. Huh7.5 or Huh7.5.1.

In another embodiment the cell is Huh7.5.

In another embodiment the cell is any cell expressing the genes necessary for HCV infection and replication, such as but not limited to CD81, SR-BI, Claudin-1, -4, -6 or –9 and the low-density lipid receptor.

The invention further provides various methods for producing HCV virus particles, including by isolating HCV virus particles from the HCV-infected non-human animal of invention; culturing a cell line of the invention under conditions that permit HCV replication and virus particle formation; or culturing a host expression cell line transfected with HCV DNA under conditions that permit expression of HCV particle proteins; and isolating HCV particles or particle proteins from the cell culture. The present invention extends to an HCV virus particle comprising a replication-competent HCV genome RNA, or a replication-defective HCV genome RNA, corresponding to an HCV nucleic acid of the invention as well.

Virus particle

The production of authentic virus proteins (antigens) may be used for the development and/or evaluation of diagnostics. The cell culture system according to the invention also allows the expression of HCV antigens in cell cultures. In principle these antigens can be used as the basis for diagnostic detection methods.

The production of HCV viruses and virus-like particles, in particular for the development or production of therapeutics and vaccines as well as for diagnostic purposes is an embodiment of the present invention. Especially cell culture adapted complete HCV genomes, which could be produced by using the cell culture system according to the invention, are able to replicate and form viral particles in cell culture with high efficiency. These genomes have the complete functions of HCV and in consequence they are able to produce infectious viruses.

Thus in one embodiment the present invention relates to a method for producing a hepatitis C virus particle of the present invention or parts thereof, comprising culturing a cell or an animal to allow either to produce the virus.

In another embodiment the inventions provides a hepatitis C virus particle obtainable by the method described.

Because the invention provides, inter alia, infectious HCV RNA, the invention provides a method for infecting an animal with HCV, which comprises administering an infectious dose of HCV RNA, such as the HCV RNA transcribed from the plasmids described above, to the animal. Naturally, the invention provides a non-human animal infected with HCV of the invention, which non-human animal can be prepared by the foregoing methods.

In one embodiment the introduced mutations attenuates the virus in vivo.

A further advantage of the present invention is that, by providing a complete functional HCV genome, authentic HCV viral particles or components thereof, which may be produced with native HCV proteins or RNA in a way that is not possible in subunit expression systems, can be prepared.

In addition, since each component of HCV of the invention is functional (thus yielding the authentic HCV), any specific HCV component is an authentic component, i.e., lacking any errors that may, at least in part, affect the clones of the prior art. Indeed, a further advantage of the invention is the ability to generate HCV virus particles or virus particle proteins that are structurally identical to or closely related to natural HCV virions or proteins. Thus, in a further embodiment, the invention provides a method for propagating HCV in vitro comprising culturing a cell line contacted with an infectious amount of HCV RNA of the invention, e.g., HCV RNA translated from the plasmids described above, under conditions that permit replication of the HCV RNA.

In one embodiment, the method further comprises isolating infectious HCV. In another embodiment, the method further comprises freezing aliquots of said infectious HCV.

According to this aspect of the invention, and in one embodiment, the HCV is infectious following thawing of said aliquots, and in another embodiment, the HCV is infectious following repeated freeze-thaw cycles of said aliquots.

A further embodiment of the present invention relates to a method for in vitro producing a hepatitis C virus-infected cell comprising culturing a cell according to the present invention and infecting other cells with the produced virus particle in the culture.

Screening for anti-viral drugs and the determination of drug resistance
It can be assumed that resistance to therapy occurs due to the high mutation rate of the HCV genome. This resistance, which is very important for the clinical approval of a substance, can be detected with the cell culture system according to the invention. Cell lines, in which the HCV-RNA construct or the HCV genome or subgenome replicates and produces infectious viral particles, are incubated with increasing concentrations of the relevant substance and the replication of the viral RNA is either determined by means of an introduced reporter gene or through the qualitative or quantitative detection of the viral nucleic acids or proteins. The release of viral particles is determined by measuring HCV RNA and infectivity titers in the cell culture supernatant. Alternatively, the number of antigen-

expressing cells is determined. Resistance is given if no or a reduced inhibition of the replication and release of viral particles can be observed with the normal concentration of the active substance. The nucleotide and amino acid replacements responsible for the therapy resistance can be determined by recloning the HCV-RNA (for example by the means of RT-PCR) and sequence analysis. By cloning the relevant replacement(s) into the original construct its causality for the resistance to therapy can be proven.

While the replicon systems facilitated testing of drugs interfering with replication such as NS3/4A protease and polymerase inhibitors, the variant genomes obtained in the present invention may prove useful for different research topics.

The systems developed in this invention are ideal candidates for specific testing of therapeutics in general and therapeutics targeting viral entry, assembly and release.

Genomes with the sequences of the present invention are valuable for testing of neutralizing antibodies and other drugs acting on entry level, such as fusion inhibitors.

In one embodiment the present invention relates to a method for identifying neutralizing antibodies.

In another one embodiment the present invention relates to a method for identifying cross-genotype neutralizing antibodies.

In one embodiment the present invention relates to a method of raising neutralizing antibodies.

In another embodiment the present invention relates to a method of raising cross neutralizing antibodies.

In one embodiment the present invention related to a method for screening new HCV genotype 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a, 6a and/or 7a inhibitors or neutralizing antibodies, comprising

a) culturing at least one selected from the group consisting of a cell according to the present invention, a hepatitis C virus infected cell according to the present invention and a hepatitis C virus particle obtainable by the present invention together with a hepatitis C virus permissive cell, and

b) subjecting said virus or virus infected cell culture to a blood sample or derivatives thereof from a HCV genotype 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a, 6a and/or 7a infected patient

c) detecting the amount of replicating RNA and/or the virus particles.

Inhibitors targeting the HCV non-structural proteins NS3/4A, NS5A and NS5B are currently being developed. The first directly-acting antiviral compounds targeting the NS3/4A protease were licensed in 2011 (Telaprevir and Boceprevir). Clinicial phase studies show promising results for inhibitors of NS5A and the NS5B polymerase. The present invention offers novel culture systems where additional HCV isolates can be tested to generate efficient cross-reactive inhibitors.

The p7 peptide features two transmembrane domains (TM1 and TM2), and p7 monomers multimerize to form a putative ion channel. Additionally p7 has been shown to contain genotype specific sequences required for genotype specific interactions between p7 and other HCV proteins. Hence, new compounds targeting the putative p7 ion-channel and autoprotease inhibitors interfering with NS2, and drugs targeting cellular proteins involved in the described processes can be tested.

Thus, one embodiment of the present invention relates to a method for screening an anti-hepatitis C virus substance, comprising

a) culturing at least one selected from the group consisting of a cell according to the present invention, a hepatitis C virus infected cell according to the present invention and a hepatitis C virus particle obtainable by the present invention together with a hepatitis C virus permissive cell,

b) subjecting said virus or virus infected cell culture to the anti-hepatitis C virus substance, and

c) detecting the replicating RNA and/or the virus particles in the resulting culture.

Another embodiment of the present invention relates to a method for screening an anti-hepatitis C virus substance, comprising

a) culturing at least one selected from the group consisting of a cell according to the present invention and the hepatitis C virus particle according to the present invention together with a hepatitis C virus permissive cell, and

b) detecting the replicating RNA or the virus particles in the resulting culture.

Yet another embodiment of the present invention relates to a hepatitis C vaccine comprising a hepatitis C virus particle of the present invention or a part thereof.

In another embodiment, the inhibition of HCV replication and/or infection and/or pathogenesis includes inhibition of downstream effects of HCV. In one embodiment, downstream effects include neoplastic disease, including, in one embodiment, the development of hepatocellular carcinoma.

In one embodiment, the invention provides a method of screening for anti-HCV therapeutics, the method comprising contacting a cell with an isolated nucleic acid molecule encoding an infectious recombinant HCV genome, comprising a chimeric HCV genome and contacting the cell with a candidate molecule, independently contacting the cell with a placebo and determining the effects of the candidate molecule on HCV infection, replication, or cell-to-cell spread, versus the effects of the placebo, wherein a decrease in the level of HCV infection, replication, or cell-to-cell spread indicates the candidate molecule is an anti-HCV therapeutic.

In one embodiment, the method may be conducted be in vitro or in vivo. In one embodiment, the cells as described may be in an animal model, or a human

subject, entered in a clinical trial to evaluate the efficacy of a candidate molecule. In one embodiment, the molecule is labelled for easier detection, including radio-labelled, antibody labelled for fluorescently labelled molecules, which may be detected by any means well known to one skilled in the art.

In one embodiment, the candidate molecule is an antibody.

Another embodiment of the present invention relates to an antibody against the hepatitis C virus particle of the present invention.

In one embodiment, the term "antibody" refers to intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, and Fv. In one embodiment, the term "Fab" refers to a fragment, which contains a monovalent antigen-binding fragment of an antibody molecule, and in one embodiment, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain, or in another embodiment can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain. In one embodiment, the term "F(ab')2", refers to the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction, F(ab')2 is a dimer of two Fab' fragments held together by two disulfide bonds. In another embodiment, the term "Fv" refers to a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains, and in another embodiment, the term "single chain antibody" or "SCA" refers to a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of producing these fragments are known in the art.

In another embodiment, the candidate molecule is a small molecule. In one embodiment, the phrase "small molecule" refers to, inter-alia, synthetic organic structures typical of pharmaceuticals, peptides, nucleic acids, peptide nucleic acids, carbohydrates, lipids, and others, as will be appreciated by one skilled in

the art. In another embodiment, small molecules, may refer to chemically synthesized peptidomimetics of the 6-mer to 9-mer peptides of the invention.

In another embodiment, the candidate molecule is a nucleic acid. Numerous nucleic acid molecules can be envisioned for use in such applications, including antisense, siRNA, ribozymes, etc., as will be appreciated by one skilled in the art.

It is to be understood that the candidate molecule identified and/or evaluated by the methods of this invention, may be any compound, including, inter-alia, a crystal, protein, peptide or nucleic acid, and may comprise an HCV viral product or derivative thereof, of a cellular product or derivative thereof. The candidate molecule in other embodiments may be isolated, generated synthetically, obtained via translation of sequences subjected to any mutagenesis technique, or obtained via protein evolution techniques, well known to those skilled in the art, each of which represents an embodiment of this invention, and may be used in the methods of this invention, as well.

In one embodiment, the compound identified in the screening methods as described, may be identified by computer modelling techniques, and others, as described herein. Verification of the activity of these compounds may be accomplished by the methods described herein, where, in one embodiment, the test compound demonstrably affects HCV infection, replication and/or pathogenesis in an assay, as described. In one embodiment, the assay is a cell-based assay, which, in one embodiment, makes use of primary isolates, or in another embodiment, cell lines, etc. In one embodiment, the cell is within a homogenate, or in another embodiment, a tissue slice, or in another embodiment, an organ culture. In one embodiment, the cell or tissue is hepatic in origin, or is a derivative thereof. In another embodiment, the cell is a commonly used mammalian cell line, which has been engineered to express key molecules known to be, or in another embodiment, thought to be involved in HCV infection, replication and/or pathogenesis.

In another embodiment, protein, or in another embodiment, peptide or in another embodiment, other inhibitors of the present invention cause inhibition of infection, replication, or pathogenesis of HCV in vitro or, in another embodiment, in vivo

when introduced into a host cell containing the virus, and may exhibit, in another embodiment, an IC50 in the range of from about 0.0001 nM to 100 μM in an in vitro assay for at least one step in infection, replication, or pathogenesis of HCV, more preferably from about 0.0001 nM to 75 μM, more preferably from about 0.0001 nM to 50 μM, more preferably from about 0.0001 nM to 25 μM, more preferably from about 0.0001 nM to 10 μM, and even more preferably from about 0.0001 nM to 1 μM.

In another embodiment, the inhibitors of HCV infection, or in another embodiment, replication, or in another embodiment, pathogenesis, may be used, in another embodiment, in ex vivo scenarios, such as, for example, in routine treatment of blood products wherein a possibility of HCV infection exists, when serology shows a lack of HCV infection.

In another embodiment, the anti-HCV therapeutic compounds identified via any of the methods of the present invention can be further characterized using secondary screens in cell cultures and/or susceptible animal models. In one embodiment, a small animal model may be used, such as, for example, a tree shrew Tupaia belangeri chinensis. In another embodiment, an animal model may make use of a chimpanzee. Test animals may be treated with the candidate compounds that produced the strongest inhibitory effects in any of the assays/methods of this invention. In another embodiment, the animal models provide a platform for pharmacokinetic and toxicology studies.

Vaccines

The construct according to the invention by itself can also be used for various purposes in all its embodiments. This includes the construction of hepatitis C viruses or HCV-like particles and their production in cell cultures as described.

These HCV or HCV-like particles can be used in particular as vaccine. Thus, one embodiment of the present invention relates to a hepatitis C vaccine comprising a hepatitis C virus particle according to the invention or a part thereof.

In another embodiment, the nucleic acids, vectors, viruses, or viral particles may be further engineered to express a heterologous protein, which, in another

embodiment, is mammalian or a derivative thereof, which is useful in combating HCV infection or disease progression. Such proteins may comprise cytokines, growth factors, tumor suppressors, or in one embodiment, may following infection, be expressed predominantly or exclusively on an infected cell surface. According to this aspect of the invention, and in one embodiment, such molecules may include costimulatory molecules, which may serve to enhance immune response to infected cells, or preneoplastic cells, or neoplastic cells, which may have become preneoplastic or neoplastic as a result of HCV infection. In one embodiment, the heterologous sequence encoded in the nucleic acids, vectors, viruses, or viral particles of this invention may be involved in enhanced uptake of a nucleic acids, vectors, viruses, or viral particles, and may specifically target receptors thought to mediate HCV infection.

Further, the present invention relates to a method for producing a hepatitis C virus vaccine comprising using a hepatitis C virus particle according to the invention as an antigen, and naturally any antibody against such hepatitis C virus particle.

Uses

The cell culture system developed of the present invention will be a valuable tool to address different research topics.

It will allow the isolate, subtype and genotype specific study of functions of all HCV genome regions and proteins using reverse genetics.

Accordingly the developed cell culture systems allow individual patient targeting. This means that when a new potential therapeutic candidate is discovered it is possible to test this particular candidate or combination of candidates on novel HCV isolates grown in culture.

Knowing which specific genotype the candidate is functioning towards, it allows an individual treatment of each patient dependent on which specific genotype the patient is infected with. Furthermore these cell culture systems allow the development of antibodies and vaccines targeting individual patients.

The replication level of a virus can be determined, in other embodiments, using techniques known in the art, and in other embodiments, as exemplified herein. For example, the genome level can be determined using RT-PCR, and northern blot. To determine the level of a viral protein, one can use techniques including ELISA, immunoprecipitation, immunofluorescence, EIA, RIA, and Western blotting analysis.

In one embodiment, the invention provides a method of identifying sequences in HCV associated with HCV pathogenicity, comprising contacting cells with an isolated nucleic acid molecule encoding an infectious recombinant HCV genome, comprising a chimeric HCV genome, contacting cells with an isolated nucleic acid molecule comprising at least one mutation of the chimeric HCV genome, independently culturing the cells and determining HCV infection, replication, or cell-to-cell spread, in cells contacted with the mutant, versus the chimeric HCV, whereby changes in HCV infection, replication, or cell-to-cell spread in cells contacted with the mutant virus shows the mutation is in an HCV sequence associated with HCV pathogenicity.

In one embodiment, the invention provides a method of identifying HCV variants with improved growth in cell culture, the method comprising contacting cells with an isolated nucleic acid molecule encoding an infectious recombinant HCV genome, comprising a chimeric HCV genome contacting cells with an isolated nucleic acid molecule comprising at least one mutation of the chimeric HCV genome, independently culturing the cells and determining HCV infection, replication, or cell-to-cell spread, in cells contacted with the chimeric HCV or the mutated virus, whereby enhanced HCV infection, replication, or cell-to-cell spread in cells contacted with the mutated virus shows that the HCV variant has improved growth in cell culture.

In some embodiments, HCV variants are selected for enhanced replication, over a long course of time, in vitro culture systems. According to this aspect of the invention, and in some embodiments, cells contacted with the variants are characterized by reduced infection, as compared to cells contacted with the chimeric HCV.

Kits

In a related aspect, the invention also provides a test kit for HCV comprising HCV virus components, and a diagnostic test kit for HCV comprising components derived from an HCV virus as described herein.

Furthermore the invention also provides test kits, for screening for new HCV inhibitors, neutralizing and cross neutralizing antibodies, comprising HCV virus components.

A further aspect of the present invention relates to a method for obtaining an isolated nucleic acid molecule encoding a human hepatitis C virus with adaptive mutations, comprising identification of one or more adaptive mutations as described in the above method, incorporation of said one or more adaptive mutations into a nucleic acid molecule encoding a full length human hepatitis C virus, and isolating the nucleic acid molecule encoding a human hepatitis C virus with adaptive mutations.

One embodiment of the present invention relates to an isolated nucleic acid molecule obtained from the above method.

Another embodiment of the present invention relates to an isolated nucleic acid molecule according to the present invention, wherein the human hepatitis C virus is of a genotype selected from the group consisting of 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a, 6a and 7a.

Examples

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 (H77Ccc) 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.

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). 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%. 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. Although incorporation of directly acting antivirals (DAA) improves the sustained virological response rate, emergence of drug resistance is a concern and may influence the outcome of these new therapies.

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, 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.

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), 2a (J6cc), and 2b (J8cc, DH8cc, and DH10cc) 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.

The HCV-1 strain was the first HCV genome to be cloned 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, identification of CD81 as an important viral receptor, and discovery of the frame-shifted F protein. An HCV-1 E1/E2-based vaccine was found to induce a neutralizing antibody responses with cross-reactivity against various HCV genotypes in rodents, chimpanzees, and humans, 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. The RNA transcripts of two H77 full-length cDNA clones, named H77C and H77, 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, and could be passaged to chimpanzees. Subsequently, studies using the sequences of H77 contributed greatly to HCV research, for example, to the study of viral entry using HCV-like particles and to the development of HCV pseudo-particles, the selection of highly permissive Huh7.5 cells, and the discovery of the importance of microRNA miR-122 in HCV replication. Efforts to propagate H77 in cell culture have been reported, using different cell lines, and H77C harboring mutations derived from its replicon (designated H77-S) could release infectious virus particles. 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 $\sim 10^{4.0}$ 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), was shown to be infectious in chimpanzees, and thus selected for this study. The HCV-1/SF9_A genome with the LSG (F1464L, A1672S, and D2979G) and TNcc-derived mutations 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. 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). 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. 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. 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 were used for immunostaining, as previously described. 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. Full-length adapted HCV-1 and H77C viruses showed slightly lower intensity in staining than the positive control virus, $J6^{5'UTR-NS2}$/JFH1. The number of FFU was automatically counted with an ImmunoSpot Series 5 UV Analyzer with customized software (CTL Europe GmbH). HCV RNA titers in the culture supernatant were determined using real time RT-PCR TaqMan method. 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.

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 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. 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 µg 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), 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), 2a (J6cc), and 2b (J8cc, DH8cc, and DH10cc), as well as 5-5A recombinants with JFH1 NS5B-3'UTR for genotypes 3a (S52), 4a(ED43), 5a(SA13), and 6a(HK6a).

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). The HCV-1/SF9_A shares nucleotide sequence identity of 96% and 95% to genotype 1a infectious clones H77C and TN, 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. 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, and they were both included in the TNcc recombinant.

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 (figure 6)].

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 (figure 6)]. We continued passaging one of the viruses and the second-passage recovered virus reached $10^{4.5}$ FFU/ml [Table 1 (figure 6), 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 (figure 7)].

Interestingly, F399 was also found in the originally published HCV-1 sequences [M62321 and AF387806]. 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 (figure 6)], 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 (figure 6)].

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 (figure 7)]. 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] were important for in vitro viability of the TNcc and full-length J6 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 (figure 6)].

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], 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 (figure 6)]. 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 (figure 8)].

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 (figure 6)].

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 (figure 6)]. 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, in vitro.

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. 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. However, the LSG and TN(5-5A)-adaptive mutations A1226G/Q1773H could efficiently adapt an H77C 5-5A recombinant [previously named "1a(H77)_LSG/A1226G/Q1773H" in Li et al. Gastroenterology. 2014 Mar;146(3):812-821]. After passage, the virus acquired a partial change in the NS3 helicase, S1368P, 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 (figure 7)].

We previously found that a TN genome containing only LSGF, designated TN_LSGF, was non-viable in a single transfection. However, when we repeated transfections of the TN_LGSF 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.

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 (figure 9)]. 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 (figure 9)]. 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".

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 H77Ccc, by using mutations and approaches previously developed for the J6cc (2a) and TNcc (1a) infectious clones. HCV1cc and H77Ccc 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 H77Ccc 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, 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. The LSG mutations were essential for the development of full-length in vitro infectious clones of HCV genotypes 1a (TNcc), 2a (J6cc), and 2b (J8cc, DH8cc, and DH10cc).

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. In this study, we further demonstrated that LSG combined with an NS5B mutation Y2981F (interestingly most genotype 2b isolates naturally have F2981) 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 (figure 7)]). 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, 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. This change may indicate that although an HCV-1 clone with S399 was found viable in vivo, 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. In addition, culture replication-enhancing mutations were found to prevent productive in vivo

replication of the Con1 genome. 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. In a recent study 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 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 H77Ccc 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. 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, which had been previously shown to adapt JFH1-based core-NS2 recombinants. Another HCV-1 change, C2419R, was identified in a DH8_LSG (2b) full-length genome and was observed for adaptation of a J6/JFH1-based recombinant expressing the NS5A from H77 and with p7 mutations.

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. 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 (figure 8)]. 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.

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. 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, but A2919V increased the replication of J6 by 10-fold. 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.

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.

The H77Ccc 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, H77Ccc 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 (figure 9)]. Therefore, the H77Ccc 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. Subsequently, this genome was improved to yield higher infectivity titers by introducing an additional mutation in E2 (H77S.3), and during the preparation of this manuscript a further adapted genome (H77D) that replicated and spread efficiently in cell culture was reported. Similarly to the adaptation process described in this study, H77D was generated by introducing our previously described adaptive TNcc mutations 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 H77Ccc 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. 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.

Claims

1. An isolated nucleic acid molecule which encodes a human hepatitis C virus, wherein said molecule:

(i) is capable of expressing said virus when transfected into cells,

(ii) is capable of infectivity *in vivo*,

(iii) comprises at least one adaptive mutation in the amino acid sequence of NS3, which is F1464L,

(iv) comprises at least one adaptive mutation in the amino acid sequence of NS4A which is A1672S, and

(v) comprises at least one adaptive mutation in the amino acid sequence of NS5B which is D2979G, and

(vi) at least one additional adaptive mutation in the amino acid sequence selected from the group consisting of S399F, A970T, A1226G, I1312V, I1326V, Q1773H, N1927T, V2198A, I2268T, C2419R, E2622D, A2919T, Y2981F, F2994S, M345T, A828V, L864R, K1052R, S1368P, V1663A, G1909S, M2105V, S2354G, V2417A, V2431I, and F2994R, and

wherein the positions are according to GenBank accession number AF009606.

2. The isolated nucleic acid molecule according to claim 1 comprising a shortened 3' UTR region.

3. The isolated nucleic acid molecule according to claims 1-2, wherein the human hepatitis C virus is of a genotype selected from the group consisting of 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a, 6a and 7a.

4. The isolated nucleic acid molecule according to claims 1 or 3, wherein the human hepatitis C virus is a strain of genotype 1a.

5. The isolated nucleic acid molecule according to claims 1, 3 or 4, wherein the hepatitis C virus is of genotype 1a and is isolate HCV1cc (SEQ ID NO:1).

6. The isolated nucleic acid molecule according to claims 1, 3 or 4, wherein the hepatitis C virus is of genotype 1a and is isolate HCV-1_LSGF/S399F/TNm/4m (SEQ ID NO:2).

7. The isolated nucleic acid molecule according to claims 1, 3 or 4, wherein the hepatitis C virus is of genotype 1a and is isolate H77Ccc (SEQ ID NO:3).

8. The isolated nucleic acid molecule according to claim 5 encoding the amino acid sequence according to SEQ ID NO:4.

9. The isolated nucleic acid molecule according to claim 6 encoding the amino acid sequence according to SEQ ID NO:5.

10. The isolated nucleic acid molecule according to claim 7 encoding the amino acid sequence according to SEQ ID NO:6.

11. A cassette vector for cloning viral genomes, comprising, inserted therein, the nucleic acid sequence according to any of claims 1-7 and having an active promoter upstream thereof.

12. A method for producing a cell, which replicates human hepatitis C virus and produces a virus particle comprising:

(i) introducing a nucleic acid molecule into a cell, wherein said nucleic acid molecule is selected from the nucleic acids of claims 1-7.

13. A cell obtainable by the method of claims 12.

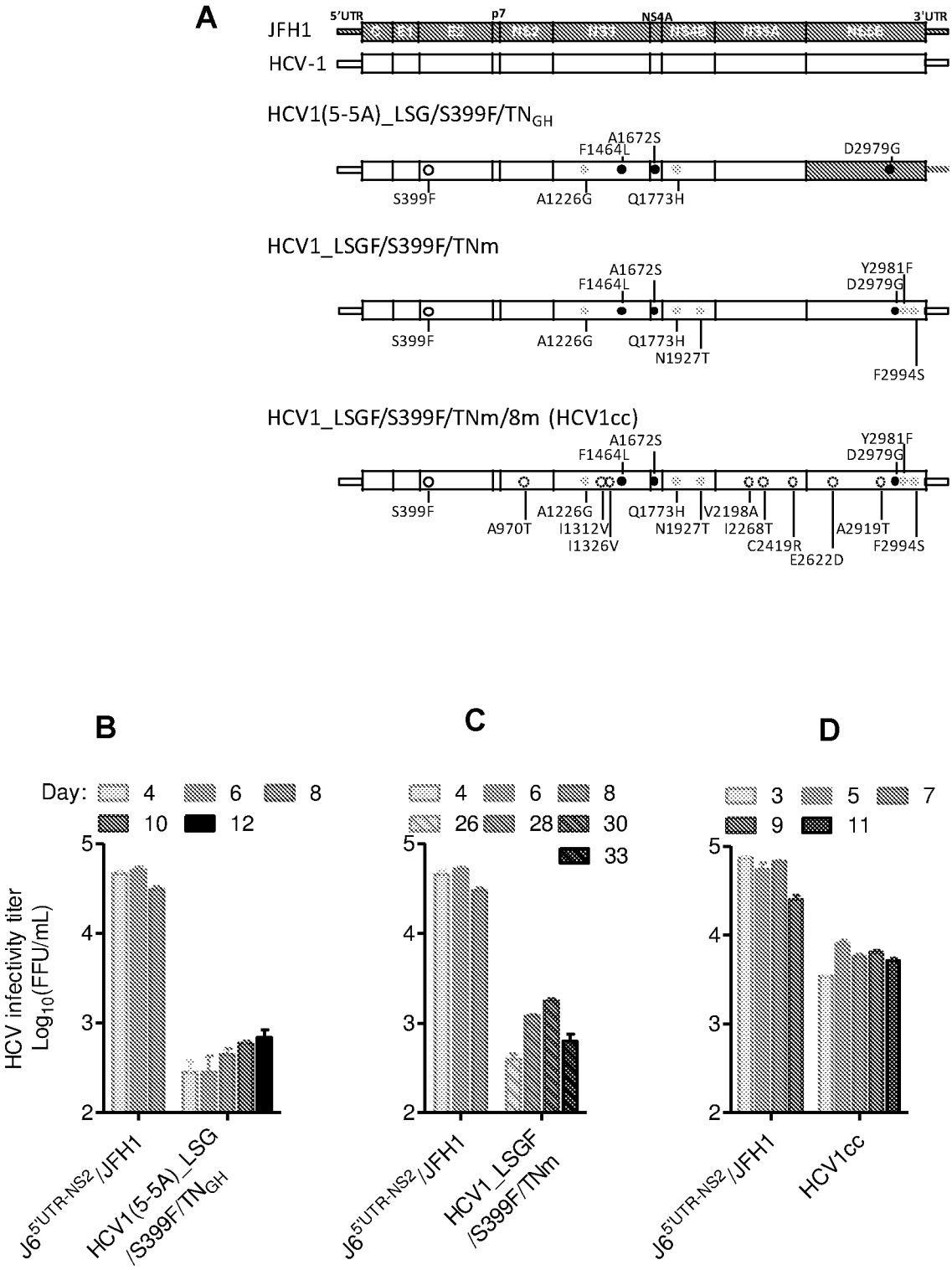
14. A method for producing a hepatitis C virus particle, comprising culturing a cell according to claim 13 to allow the cell to produce the virus.

15. A method for in vitro producing a hepatitis C virus-infected cell comprising culturing a cell according to claims 13 and infecting other cells with the produced virus particle in the culture.

16. A method for screening an anti-hepatitis C virus substance, comprising

a) culturing at least one selected from the group consisting of a cell comprising the nucleic acids of any of claims 1-7, a cell according to claim 13 and the hepatitis C virus particle obtainable from the method of claim 14 together with a hepatitis C virus permissive cell, and

b) detecting the replicating RNA or the virus particles in the resulting culture.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JFH1
5'UTR
p7
NS4A
3'UTR
HCV-1
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A1672S
F1464L
D2979G
S399F
A1226G
Q1773H
HCV1_LSGF/S399F/TNm
Y2981F
N1927T
F2994S
HCV1_LSGF/S399F/TNm/8m (HCV1cc)
A970T
I1312V
I1326V
V2198A
I2268T
C2419R
E2622D
A2919T
B
C
D
Day:
HCV infectivity titer
Log10(FFU/mL)
J6 5'UTR-NS2/JFH1
HCV1(5-5A)_LSG
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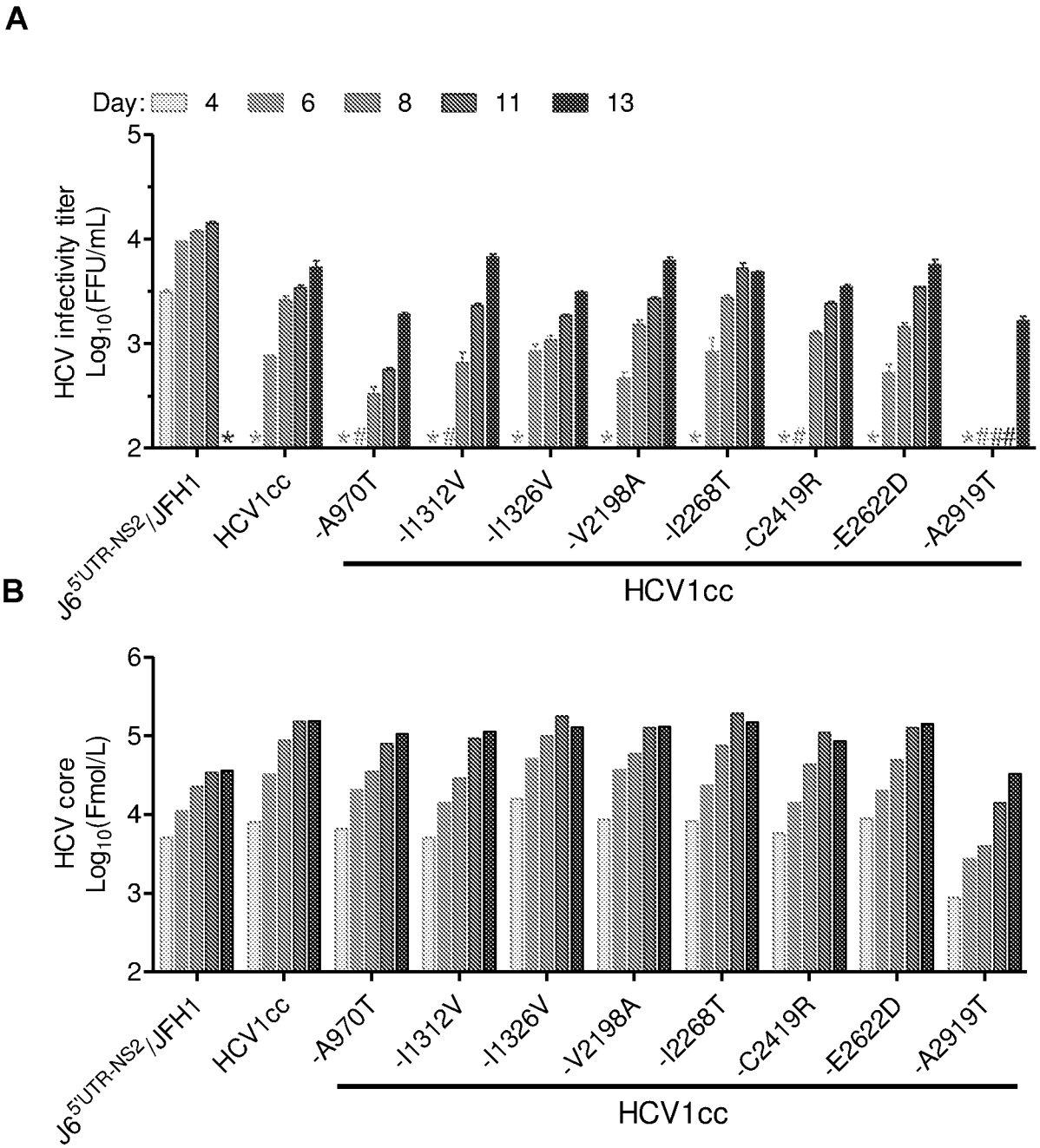
HCV1cc

Fig. 1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HCV infectivity titer
Log10(FFU/mL)
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4
3
2
J6 5'UTR-NS2/JFH1
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-I1312V
-I1326V
-V2198A
-I2268T
-C2419R
-E2622D
-A2919T
HCV1cc
B
HCV core
Log10(Fmol/L)
6
5
4
3
2
J6 5'UTR-NS2/JFH1
HCV1cc
-A970T
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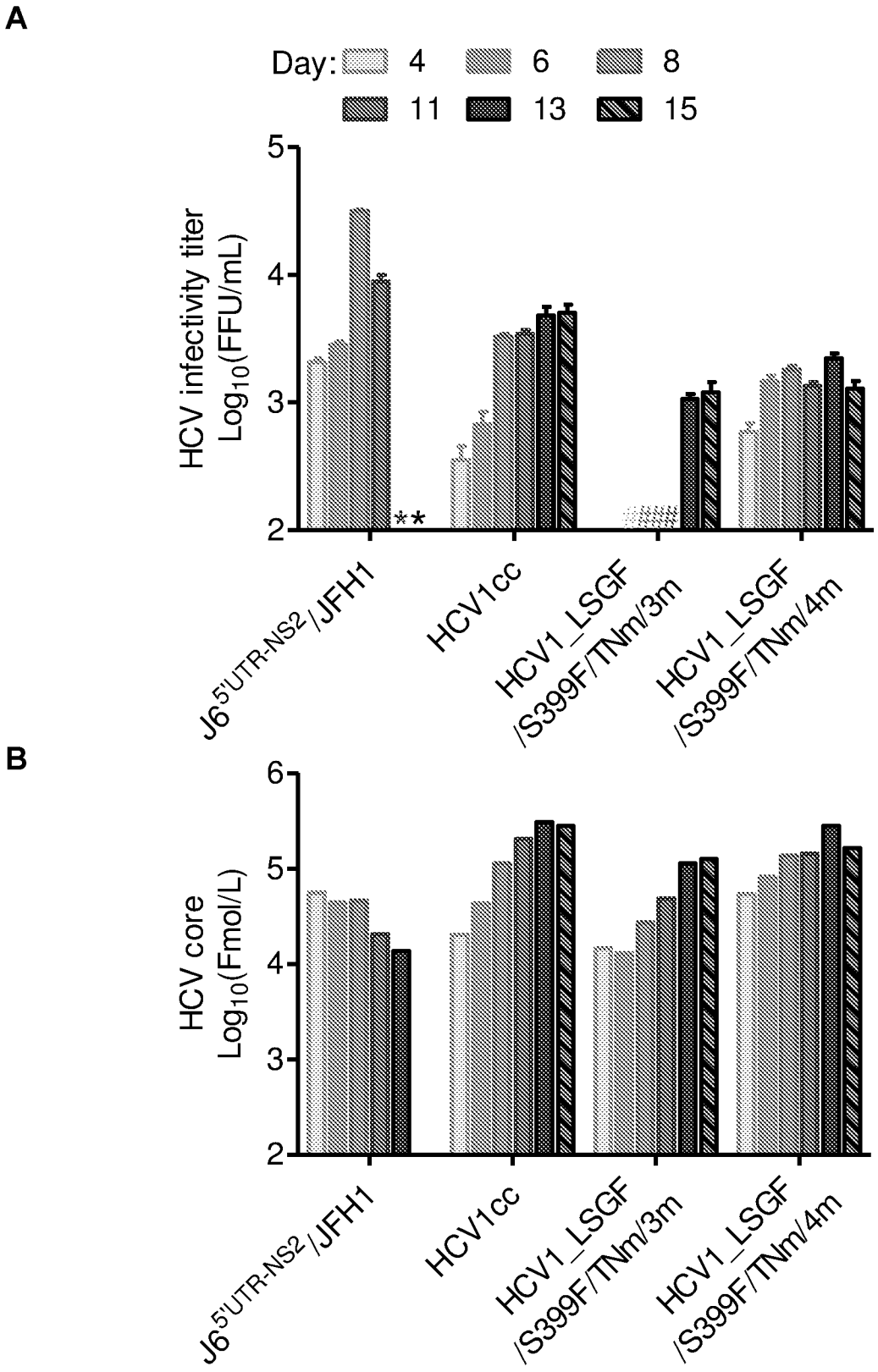
-E2622D
-A2919T
HCV1cc

Fig. 2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Day: 4 6 8
11 13 15
HCV infectivity titer
Log10(FFU/mL)
5
4
3
2
**
J6 5'UTR-NS2 /JFH1
HCV1cc
HCV1_LSGF /S399F/TNm/3m
HCV1_LSGF /S399F/TNm/4m
B
HCV core
Log10(Fmol/L)
6
5
4
3
2
J6 5'UTR-NS2 /JFH1
HCV1cc
HCV1_LSGF /S399F/TNm/3m
HCV1_LSGF /S399F/TNm/4m

Fig. 3

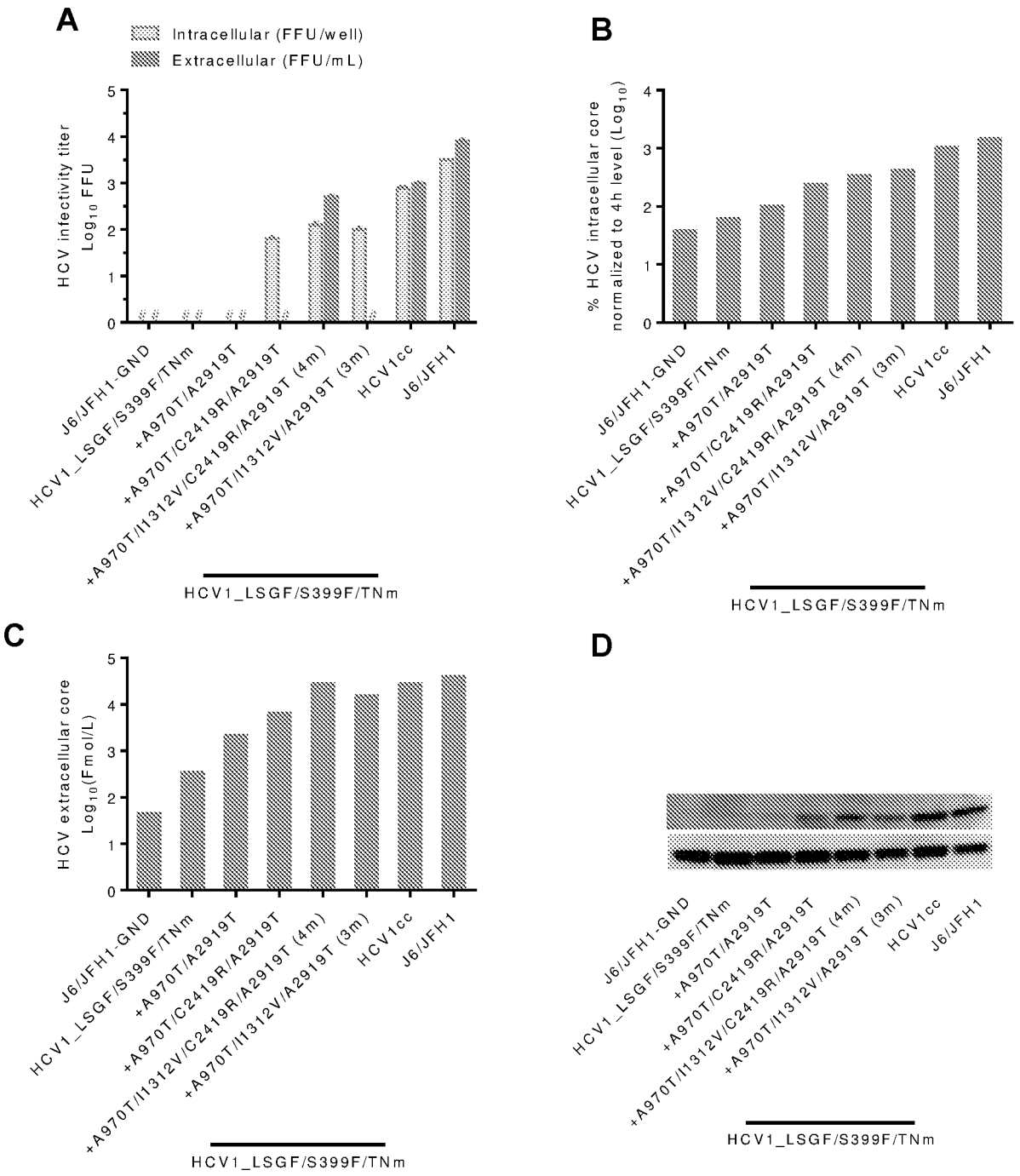
A
Intracellular (FFU/well)
Extracellular (FFU/mL)
HCV infectivity titer
Log10 FFU
J6/JFH1-GND
HCV1_LSGF/S399F/TNm
+A970T/A2919T
+A970T/C2419R/A2919T
+A970T/I1312V/C2419R/A2919T (4m)
+A970T/I1312V/A2919T (3m)
HCV1cc
J6/JFH1
HCV1_LSGF/S399F/TNm
B
% HCV intracellular core
normalized to 4h level (Log10)
HCV1_LSGF/S399F/TNm
C
HCV extracellular core
Log10(Fmol/L)
HCV1_LSGF/S399F/TNm
D
HCV1_LSGF/S399F/TNm

Fig. 4

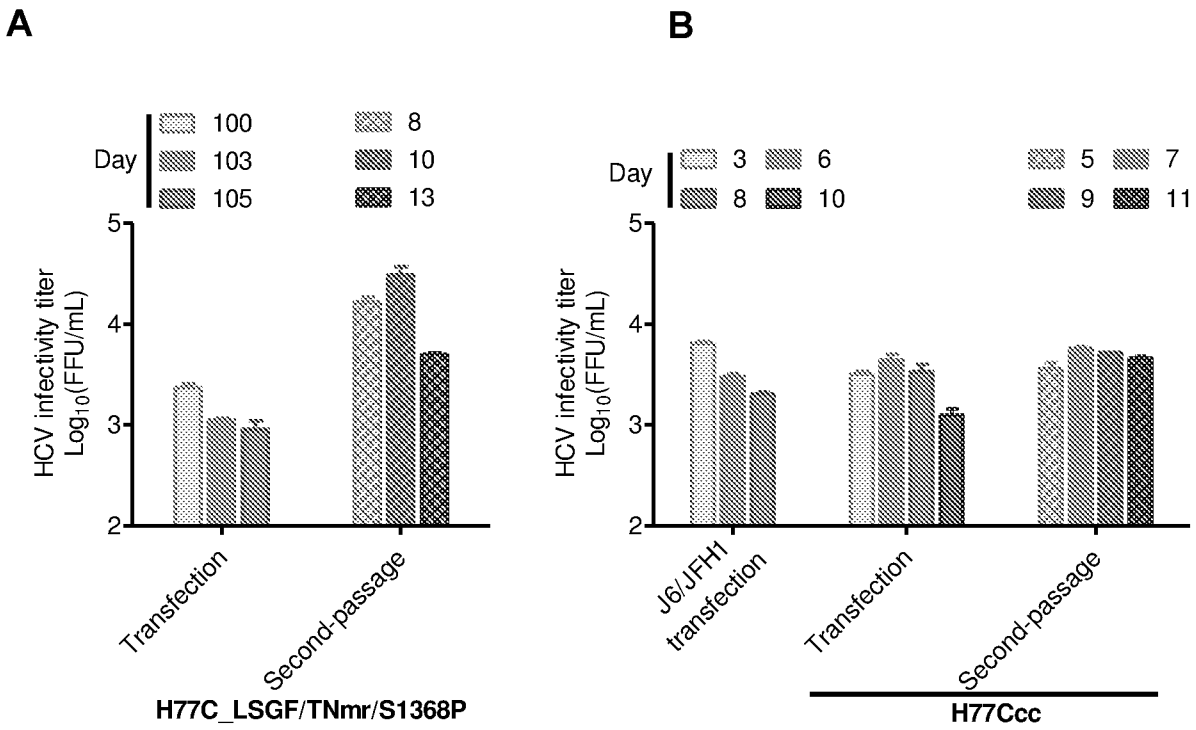
A
Day
100
103
105
8
10
13
HCV infectivity titer
Log10(FFU/mL)
5
4
3
2
Transfection
Second-passage
H77C_LSGF/TNmr/S1368P
B
Day
3
6
8
10
5
7
9
11
HCV infectivity titer
Log10(FFU/mL)
5
4
3
2
J6/JFH1 transfection
Transfection
Second-passage
H77Ccc

Fig. 5

Table 1. Characteristics of the HCV-1 5'UTR-NS5A (5-5A) recombinant and full-length viruses in Huh7.5 cell cultures.

	Transfection		First passage			Second passage			
	Day with ≥80% infected	Peak log_{10}FFU/ml (day)	Day with ≥80% infected	Peak log_{10}FFU/ml (day)		Day, ≥80% infected	Peak log_{10}FFU/ml (day)		Peak log_{10}IU/ml
HCV1(5-5A)									
+LSG/TN_{GA}, exp. 1	5	<2.4	38	4.0 (42)		nd	-		-
+LSG/TN_{GA}, exp. 2[a]	5	<2.4	27	4.0 (31)		8	4.5 (8/10/12)[b]		nd
+LSG/S399F/TN_{GH}, exp. 1	4	2.7 (8)	16	3.8 (18)		5	4.4 (7)		nd
+LSG/S399F/TN_{GH}, exp. 2	4	3.0 (12)	16	3.9 (16)		5	4.2 (7)		nd
HCV1 full-length									
+LSGF/S399F/TNm[c]	26	3.3 (28)	7	3.4 (11)		7	4.3 (9)		8.3
+LSGF/S399F/TNm/8m (HCV1cc)	3	3.8 (5)[d]	5	3.8 (7)		7	3.8 (9)		7.5
+LSGF/S399F/TNm/4m	4	3.4 (13)	11	3.5 (13)		11	3.9 (15)		nd

Fig. 6

Table 2. ORF sequence analysis of HCV-1 5'UTR-NS5A (5-5A) viruses.

	Passage (Day)	E2	NS3	NS3	NS3	NS3	NS3	NS3	NS3	NS4A	NS4B	NS5A	NS5A	NS5A	NS5B	NS5B
Nucleotide position		1537	3477	4018	4234	4443	4731	4989	5355	5660	6987	7172	7588	7899	9277	
Recombinant specific		1537	3477	4018	4234	4443	4731	4989	5355	5660	6987	7172	7588	7899	9277	
H77 reference (AF009606)		C	A	C	A	T	T	G	G	A	A	G	A	A	A	
Recombinant nucleotide																
HCV1(5-5A)																
+LSG/TN$_{GH}$, exp. 1	1st (31)^{c}	T	•	G	•	•	C	•	T	C	•	•	G	A/C	G	
	2nd (8/10/12)^{b}	T	G/a	G	T/a	C/t	C	•	T	C	A/C	G/C	G	C	G	
+LSG/S399F/TN$_{GH}$, exp. 1	2nd (9)^{c}	T	•	G	•	T/C	C	G/T	T	C	•	•	•	•	G	
Amino acid position																
Recombinant specific		399	1046	1226	1298	1368	1464	1550	1672	1773	2216	2277	2416	2520	2979	
H77 reference (AF009606)		399	1046	1226	1298	1368	1464	1550	1672	1773	2216	2277	2416	2520	2979	
Amino acid change		S-F	S-G	A-G	K-M	S-P	F-L	V-L	A-S	Q-H	T-P	K-N	D-G	K-Q	D-G	

Fig. 7

Table 3. ORF sequence analysis of HCV-1 full-length viruses.

Passage (day)	E2	NS2	NS3	NS3	NS3	NS3	NS4A	NS4B	NS4B	NS5A	NS5A	NS5A	NS5B	NS5B	NS5B	NS5B	NS5B
Nucleotide position																	
Recombinant specific	1537	3249	4018	4275	4317	4731	5355	5660	6121	6934	7144	7596	8207	9096	9277	9283	9322
H77 reference (AF009606)	1537	3249	4018	4275	4317	4731	5355	5660	6121	6934	7144	7596	8207	9096	9277	9283	9322
Recombinant nucleotide	C	G	C	A	A	T	G	A	A	T	T	T	A	G	A	A	T
HCV-1 full-length																	
+LSGF/S399F/TNm[a] 2nd (11)	T	A	G	G	G	C	T	C	C	C	C	C	C	A	G	T	C
3rd (11)	T	A	G	G	G	C	T	C	C	C	C	C	C	A	G	T	C
+LSGF/S399F/TNm/8m 2nd (9)	T	A	G	G	G	C	T	C	C	C	C	C	C	A	G	T	C
(HCV1cc)[b]																	
+LSGF/S399F/TNm/4m[c] 2nd (18)	T	A	G	G	•	C	T	C	C	•	•	C	•	A	G	T	C
Amino acid position																	
Recombinant specific	399	970	1226	1312	1326	1464	1672	1773	1927	2198	2268	2419	2622	2919	2979	2981	2994
H77 reference (AF009606)	399	970	1226	1312	1326	1464	1672	1773	1927	2198	2268	2419	2622	2919	2979	2981	2994
Amino acid change	S-F	A-T	A-G	I-V	I-V	F-L	A-S	Q-H	N-T	V-A	I-T	C-R	E-D	A-T	D-G	Y-F	F-S

Fig. 8

Table 4. ORF sequence analysis of H77C full-length viruses.

	Passage (Day)	E1	NS2	NS2	NS3	NS3	NS3	NS3	NS4A	NS4A	NS4B	NS4B	NS4B	NS5A	NS5A	NS5A	NS5B	NS5B	NS5B	NS5B	NS5B
Nucleotide position		1375	2824	2932	3496	4018	4443	4731	5329	5355	5660	6066	6121	6654	7401	7591	7632	9277	9283	9321[b]	9922[b]
Nucleotide (pCV-H77C)(24)		T	C	T	A	C	T	T	T	G	A	G	A	A	A	T	G	A	A	T	T
H77C_LSGF/TNnr/S1368P[a]	1[st] (13)	C	T	G	G	G	C	C	C	T	C	A	G	G	G	C	A	G	T	C	G
	2[nd] (13)	C	T	G	G	G	C	C	C	T	C	A	G	G	G	C	A	G	T	C	G
H77Ccc	2[nd] (9)	C	T	G	G	G	C	C	C	T	C	A	G	G	G	C	A	G	T	C	G
		345	828	864	1052	1226	1368	1464	1663	1672	1773	1909	1927	2105	2354	2417	2431	2979	2981	2994	2994
		M-T	A-V	L-R	K-R	A-G	S-P	F-L	V-A	A-S	Q-H	G-S	N-T	M-V	S-G	V-A	V-I	D-G	Y-F	F-R	F-R

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2015/050325

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/00 C12N15/40 C12N5/10 G01N33/50
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/139339 A1 (HVIDOVRE HOSPITAL [DK]; UNIV KOEBENHAVN [DK]) 26 September 2013 (2013-09-26) claim 13; figure 15B; sequences 111,153	1,3, 11-16
X	WO 2013/139340 A1 (HVIDOVRE HOSPITAL [DK]; UNIV KOEBENHAVN [DK]) 26 September 2013 (2013-09-26) figure 15B; sequences 92,107	1,3, 11-16
X,P	WO 2015/058772 A2 (HVIDOVRE HOSPITAL [DK]) 30 April 2015 (2015-04-30) claims 18-20,28-30; sequences 7,11	1,3, 11-16
E	WO 2015/179204 A1 (UNIV NORTH CAROLINA [US]) 26 November 2015 (2015-11-26) claims 1,35,36	1,3,4

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 January 2016	04/02/2016

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2015/050325

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2015/050325

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