The present inventors have developed a robust and genetically stable cell culture system for HCV genotype 3a, which has a high prevalence worldwide. Since intergenotypic recombinant genomes exploiting the replication characteristics of JFH1 will be a valuable tool for the genotype specific study of the replaced genes and related therapeutics, the present inventors constructed a genotype 3a/2a (S52/JFH1) recombinant containing the structural genes (Core, E1, E2), p7 and NS2 of strain S52 and characterized it in Huh7.5 cells. S52/JFH1 and J6/JFH viruses passaged in cell culture had comparable growth kinetics and yielded similar peak HCV RNA titers and infectivity titers. Direct genome sequencing of cell culture derived S52/JFH1 viruses identified putative adaptive mutations in Core, E2, p7, NS3 and NS5A; clonal analysis revealed, that all genomes analyzed exhibited different combinations of these mutations. Finally, viruses resulting from transfection with RNA transcripts of five S52/JFH1 recombinant containing these combinations of putative adaptive mutations performed as efficiently as J6/JFH viruses in Huh7.5 15 cells and were all genetically stable after viral passage.

In conclusion, the present inventors have developed a robust and genetically stable cell culture system for HCV genotype 3a.
CELL CULTURE SYSTEM OF A HEPATITIS C GENOTYPE 3A AND 2A CHIMERA

Field of the invention

This invention provides infectious recombinant hepatitis C viruses (HCV), and vectors, cells and animals comprising the same. The present invention provides methods of producing the infectious recombinant HCV, and their use in identifying anti-HCV therapeutic and including for use in vaccines and diagnostics and, as well as sequences of HCV associated with HCV pathogenesis.

Background

Hepatitis C virus (HCV) is one of the most widespread infectious diseases in the world. About 170 million people are infected with 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 posttranslationally 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 6 major HCV genotypes (genotypes 1-6) have been identified, which differ by 31-33% at the nucleotide level. In addition, there are numerous subtypes (a, b, c, etc.). In general different subtypes and isolates differ respectively by 20-25% and 2-8% at the nucleotide level. In the U.S., the majority of HCV infected individuals has genotype 1 (Ia or Ib), while most others are infected with genotype 2 (2a or 2b) or 3a. Genotype 3a is more prevalent in Europe infecting up to 50% of patients in several countries with a high prevalence in specific risk
groups, such as intravenous drug users and its prevalence in Europe is expected to rise.

Furthermore, genotype 3a is very prevalent in many highly populated countries in Asia such as India and Pakistan, as well as the former USSR, Australia and Brazil. In HCV infected patients, genotype 3 was found to be associated with more pronounced hepatic steatosis compared to other genotypes. The only approved therapy for HCV, combination therapy with interferon and ribavirin, is expensive and associated with severe side effects and contraindications. Sustained viral response can be achieved in only about 55% of treated patients in general, in 85-90% of patients infected with genotypes 2 and 3 and only in 40-50% of patients infected with genotype 1. There is no vaccine against HCV.

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. Full-length consensus cDNA clones of HCV strain H77 (genotype 1a) and J6 (genotype 2a) shown to be infectious in the chimpanzee model, were apparently not infectious in \textit{vitro}. Replicon systems permitted the study of HCV RNA replication in cell culture using the human liver hepatoma cell line Huh7 but were dependent on adaptive mutations that were deleterious for infectivity \textit{in vivo}.

In 2001, a genotype 2a isolate (JFHI) was described (Kato et al., 2001), which yielded high RNA titers in the replicon system without adaptive mutations (Kato et al., 2003).

A major breakthrough occurred in 2005, when formation of infectious viral particles was reported after transfection of RNA transcripts from the JFHI full-length consensus cDNA clone into Huh7 cells and the derived Huh7.5.1 (Wakita et al., 2005) (Zhong et al., 2005).

At the same time, Lindenbach et al. demonstrated that the intragenotypic 2a/2a recombinant genome (J6/JFH1), in which the structural genes (C, E1, E2), p7 and NS2 of JFHI were replaced by the corresponding genes of the infectious cDNA clone J6CF, produced infectious viral particles in Huh7.5 cells (a cell line derived
from bulk Huh7 cells) with an accelerated kinetic (Lindenbach et al., 2005). Cell culture derived J6/JFH viruses were apparently fully viable in vivo (Lindenbach et al., 2006). Intragenotypic and intergenotypic recombinant HCV genomes are naturally occurring. Interestingly, in several of these isolates the recombination breakpoint apparently maps in close proximity to the NS2/NS3 junction, the site of recombination in the J6/JFH genomes.

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

Pietschmann et al. 2006 disclose the construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus recombinants. The authors created a series of recombinant genomes allowing production of infectious viral particles containing Core through NS2 of genotype 1a, 1b, 2a and 3a by constructing intra- and intergenotypic recombinant genomes between the JFH1 isolate and the HCV isolates: H77 (genotype 1a), Conl (genotype 1b), J6 (genotype 2a) and 452 (genotype 3a) respectively. Thus, disclosing a genotype 3a isolate completely different from the isolate disclosed in the present application.

The infectious titers of the 1a, 1b and 3a genotypes disclosed in Pietschmann et al. 2006 are not at a level sufficiently high for practical utilization in functional analysis, drug and vaccine development or most other applications. For such applications, including screening of potential drugs and development of potential vaccine candidates the skilled person will know that infectivity titers below $10^3$ TCID$_{50}$/mL contain insufficient amounts of infectious virus. Besides from disclosing a genotype 3a isolate different from the genotype 3a isolate disclosed in the present application Pietschmann et al. 2006 provides no sequence data of the virus produced in the cell culture. Accordingly, the study does not attempt cell culture adaptation of the genotype recombinants, e.g. by serial passage of cell culture derived viruses to naïve cells and it is not investigated whether adaptive mutations developed after transfection in cell culture.

In fact, Pietschmann et al does not even provide any sequence data of the virus produced in the cell culture.
Summary of the invention

It is important to develop cell culture systems for representative strains of other HCV genotypes with infectious titers enabling in vitro studies, since neutralizing antibodies are not expected to cross-neutralize all genotypes and new specific antiviral compounds might have differential efficiencies against different genotypes. For the genotype specific study of the function of the structural proteins, p7 and NS2 as well as related therapeutics such as neutralizing antibodies, fusion inhibitors, ion-channel blockers and protease inhibitors, it would be sufficient to construct intergenotypic recombinant viruses in analogy to J6/JFH.

In this study, the present inventors used the S52 reference isolate (genotype 3a) to construct such a viable, JFH1-based genome. The present inventors serially passaged S52/JFH1 virus in cell culture, obtained HCV RNA titers and infectivity titers as high as those observed in the J6/JFH cell culture system, and identified adaptive mutations required for efficient growth. Finally the present inventors generated different adapted genetically stable genomes suitable for efficient and sustainable growth of S52/JFH1 in cell culture.

The present inventors have developed a robust cell culture system for HCV genotype 3a. This is an important advance for the study of HCV, since genotype 3a is highly prevalent worldwide. Because most full-length consensus HCV cDNA clones were not or only poorly infectious in vitro, the present inventors exploited the exceptional replication characteristics of the HCV genotype 2a isolate JFHI, which recently permitted the development of cell culture systems to study the viral life cycle of HCV.

In this study the inventors describe for the first time the near complete genome sequence of J6/JFH virus recovered from cell culture and show that its efficient growth does not depend on the acquisition of adaptive mutations.

Thus, in one embodiment the present invention relates to a replicating RNA comprising the structural genes (Core, E1, E2), p7 and non-structural genes NS2 of genotype 3a and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B from the JFHI strain and an isolated nucleic acid molecule which encodes human hepatitis C virus of genotype 3a/JFHI (e.g. S52/JFH1).
In another embodiment the present invention pertains to a composition comprising a nucleic acid molecule according to the present invention, a cassette vector for cloning viral genomes, a method for producing a cell which replicates HCV 3a/JFH1 RNA and cells obtainable by this method.

In yet another embodiment the present invention relates to a method for producing a hepatitis C virus particle, a hepatitis C virus particle obtainable by said method and a method for in vitro producing a hepatitis C virus-infected cell and a hepatitis C virus infected cell obtainable by said the method.

In a further embodiment the invention relates to a method for screening an anti-hepatitis C virus substance, a hepatitis C vaccine, a method for producing a hepatitis C virus vaccine and an antibody against the hepatitis C.

**Detailed Description**

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.

Nucleotide acid molecules (cDNA clones and RNA transcripts)

In a broad aspect, the present invention is directed to a genetically engineered hepatitis C virus (HCV) encoded by nucleic acid sequences such as a complementary DNA (cDNA) sequence and replicating RNA comprising the structural genes (Core, E1, E2), p7 and non-structural gene NS2 of genotype 3a (e.g. strain S52) and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B from the JFH1 strain.

The invention provides an isolated nucleic acid molecule encoding an infectious recombinant HCV genome, which nucleic acid comprises an intergenotypic HCV genome. In one embodiment, the intergenotypic HCV genome comprises sequences encoding structural genes (Core, E1, E2), p7 and nonstructural gene (NS2) from a first HCV strain, and sequences encoding the 5’ untranslated region
(UTR), nonstructural genes NS3, NS4A, NS4B, NS5A, NS5B, and the 3' UTR from a second HCV strain.

In one embodiment, the first HCV strain and the second HCV strain are from different genotypes.

In one embodiment, the first HCV strain is strain S52, and in another embodiment, the second HCV strain is strain JFH1.

Importantly, the present inventors demonstrated that J6/JFH viruses recovered from Huh7.5 cells had the original J6/JFH open reading frame sequence after one cell free passage, thus confirming the robustness of this system. This is in contrast to the JFH1 system described by Zhong et al., in which the establishment of sustained infection depends on the acquisition of adaptive mutations.

As observed for JFH1, the present inventors found that J6/JFH viruses at the peak of infection exerted a cytopathic effect on Huh7.5 cells. This was subsequently demonstrated also for the present S52/JFH1 intergenotypic viruses.

In one embodiment, the HCV nucleic acid molecule of the present invention comprises the nucleic acid sequence (cDNA) of SEQ ID NO: 1. In another embodiment the nucleic acid molecule has at least a functional portion of a sequence as shown in SEQ ID NO: 1, which represents a specific embodiment of the present invention exemplified herein.

In another embodiment, the nucleic acid comprises a sequence sharing at least 90% identity with that set forth in 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.

In another embodiment the nucleic acid comprises at least 90% sequence identity to that of SEQ ID NO: 1.

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.

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.

It should be noted that while SEQ ID NO: 1 is a DNA sequence, the present invention contemplates the corresponding RNA sequence, and DNA and RNA complementary sequences as well.

In a further embodiment, a region from an HCV isolate is substituted for a corresponding region, e.g., of an HCV nucleic acid having a sequence of SEQ ID NO: 1.

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 SEQ ID NO: 1. Various modifications for example of the 5' and 3' LTR 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 sequence of SEQ ID NO: 1 or the said nucleic acid sequence with any mutation described in this document is obtained by any other means than what is described above. Thus, one aspect of the present invention relates to any of the nucleic acid sequence disclosed herein, such as but not limited to SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21.

In another embodiment, the complementary DNA (cDNA) clone, which encodes human hepatitis C virus of genotype 3a/JFH1 (e.g. S52/JFH1), wherein said molecule is capable of expressing said virus when transfected into cells and further capable of infectivity in vivo and wherein said molecule encodes the amino acid sequence of SEQ ID NO: 2.

In yet another embodiment, the complementary DNA (cDNA) clone, which encodes human hepatitis C virus of genotype 3a/JFH1 (e.g. S52/JFH1), wherein said molecule is capable of expressing said virus when transfected into cells and further capable of infectivity in vivo and wherein said molecule encodes the amino acid sequence with at least 90% sequence identity to that of SEQ ID NO: 2.

In another embodiment, the amino acid sequence comprises a sequence sharing at least 90% identity with that set forth in 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.

It is to be understood that a sequence identity of at least 90%, such as 90% identity, 91% identity, 92% identity, 93% identity, 94% identity, 95% identity, 96% identity, 97% identity, 98% identity, or 99% identity applies to all sequences disclosed in the present application.

According to an aspect of the invention, HCV nucleic acid, including the polyprotein coding region, may be mutated or engineered to produce variants or derivatives with, e.g., silent mutations, conservative mutations, etc. In a further
embodiment, silent nucleotide changes in the polyprotein coding regions (i.e., variations of the first, second or third base of a codon leading to a new codon that encodes the same amino acid) are incorporated as markers of specific HCV clones.

5 In another embodiment, the nucleic acid molecule in any form with any changes from SEQ ID NO: 1 encodes the amino acid sequence of SEQ ID NO: 2 or the said amino acid with any mutation described in this document including conservative amino acid mutations as described.

10 In a further embodiment, the amino acid sequence of SEQ ID NO: 2 or the said amino acid with any mutation described in this document is obtained by any other means than what is described above. Thus, one aspect of the present invention relates to any of the amino acid sequences disclosed herein, such as but not limited to SEQ ID NO: 2, 4, 6, 8, 10,12, 14, 16, 18, 20 and 22.

15 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'-L)TR 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.

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 inventor here reports adaptive mutations, which allow efficient formation and release of viral particles in cell culture, and thus the present invention relates to these adaptive mutation in the present use as well as use in other strains.

After transfection of S52/JFH1 RNA transcripts into Huh7.5 cells, the present inventors observed in six independent experiments (FIG. IA, FIG. 2A, FIG. 5A, FIG. 7) a significant delay in virus spread compared to the J6/JFH positive control culture, which indicates dependence on the acquisition of adaptive mutations.

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, several or all of the following amino acid exchanges.

Direct genome sequencing of recovered S52/JFH1 viruses indicated the occurrence of different adaptive mutations during the first transfection experiment and consecutive viral passages (TABLE 5 and below).

Thus in one embodiment, the present invention relates to nucleic acid molecules according to the present invention, wherein said molecule comprises adaptive mutations in Core, E2, p7, NS3 and NS5A singly or in combination, such as but not limited to sequences with SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21.

In the context of the present invention the term "adaptive mutation" is meant to cover mutations identified in passaged S52/JFH1 viruses that provide the original S52/JFH1 genome and any other HCV sequence the ability to grow efficiently in culture. Furthermore all introductions of mutations into the S52/JFH1 sequence 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. For example mutations in Core could increase Core stability and target Core more efficiently to lipid droplets, which could be exploited in in vitro Core expression systems. Mutations in NS3 and NS5A could increase replication capacity, which could be exploited in studies using the replicon system.

Thus in one embodiment, the present invention relates to other HCV genomes with adaptive mutations, all of them, combinations of them or individual mutations, that grow in culture. In this case the titers might be lower than those listed.

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 mutation and any combination of the mutations.

Clonal analysis confirmed the presence of several different adapted genomes in 2nd passage cell culture supernatant and supported the hypothesis, that a primary viable genome (with mutations in E2, p7, and/or N3) might have been outnumbered by a set of even fitter genomes with key mutations in Core, p7, and NS5A (TABLE 5).

A hallmark in this clonal analysis was the occurrence of a p7 mutation in all genomes, either T2718G (7/9 clones) or A2721G (2/9 clones). The mutations in p7 were always combined with one mutation in the JFH1 part of the genome, either in NS3 (A4845T; 1/9 clones) or NS5A (T7160C; 8/9 clones). T7160C in NS5A always occurred together with T728C in Core.

Five recombinant S52/JFH1 genomes with the identified combinations of adaptive mutations ( SEQ ID NO: 3, 5, 7, 9, 11) performed as efficiently in Huh7.5 cells as J6/JFH (FIG. 5, FIG. 6), and in contrast to the original S52/JFH1 the resulting
S52/JFH1 viruses could be passaged without acquiring additional mutations. Genomes with just two mutations, in p7 and NS3 (SEQ ID NO: 11), or with three mutations, in Core, p7, and NS5A (SEQ ID NO: 7), were fully viable. It did not appear to make a difference which of the two p7 mutations identified was combined with the Core and NS5A mutations. The identified mutations in E2 did apparently not improve growth of these recombinants.

Furthermore the following mutations were identified in passaged S52/JFH1 viruses and are therefore considered as adaptive mutations in the context of this invention:

1. identified in the 1st passage of the 2nd transfection experiment by direct sequencing: T2718C and A4550C

2. identified in the 2nd passage of the 1st transfection experiment by direct sequencing: mutations in E1 and NS5A (T1191C and G7182A) as well as noncoding mutations in Core and NS5A (C688A and T6685C).

3. identified only by clonal analysis. The following nucleotide changes occurred in more than one clone and are therefore more likely to contribute to cell culture adaptation, even though they were not prominent in direct sequencing: A824G (occurred in 3/9 clones), A1937G (2/9 clones), G2916A (2/9 clones), C6328T (3/9 clones).

4. identified in transfection or 1st passage of viruses containing single adaptive mutations (Table 5A): C1527T, A2297T, T2720A, A3023C, A3748G, G4464T, G4464A, A4552C, A4552T, C5407T, A7154C.

The present inventors constructed up to seven recombinant S52/JFH1 genomes with combinations of adaptive mutations; four with the combinations suggested by clonal analysis (SEQ ID NO: 3, 5, 7, 9) and three with one p7 mutation combined with another mutation either in NS3 or NS5A (SEQ ID NO: 11, 19, 21) (Table 5A). These recombinants performed as efficiently as J6/JFH in Huh7.5 cells (Figure 5, 6, 7) and the resulting S52/JFH1 viruses could be passaged without acquiring additional mutations. In contrast 7/9 S52/JFH1 recombinants with single
mutations acquired additional nucleotide changes in cell culture, in most instances at positions at which adaptive mutations had been detected previously and with a preference for the combination of one mutation in p7 with a second mutation in NS3 or NS5A (Table 5A). Only two S52/JFH1 recombinants with single mutations did not require additional nucleotide changes: T2718G (I793S) in p7 (SEQ ID 15) and A4550C (K1404Q) in NS3 (SEQ ID 17), respectively. Interestingly, these were the two amino acid changes conferring viability to S52/JFH1(T728C) 25 days after transfection (Table 5B). It is of note, that neither of these two mutated nucleotides / corresponding amino acids is found in other HCV isolates (156 and 204 isolates deposited in a ready-made alignment of NS3 and p7, respectively, as provided by the American HCV database website by June 14, 2007).

T2718G in p7 and A4550C in NS3 were the only adaptive mutations able to individually confer cell culture adaptation of S52/JFH1. S52/JFH1(T2718G; A4550C) (SEQ ID 13) was constructed in order to test if combination of T2718G and A4550C on one S52/JFH1 genome was possible. After transfection and passage in Huh7.5 cells S52/JFH1(T2718G; A4550C) viruses yielded infectivity titers between 10^4 and 10^5 TCID_{50}/mL; additionally, direct sequencing of the complete ORF of S52/JFH1(T2718G; A4550C) genomes revealed that these viruses were genetically stable after passage in Huh7.5 cells. Thus, it was shown that the S52/JFH1(T2718G; A4550C) genome (SEQ ID 13), combining the two adaptive mutations, which were able to individually provide adaptation of the S52/JFH1 genome, is viable and efficient in Huh7.5 cell culture.


One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said nucleotide at the said position of SEQ ID NO: 1 by the following said nucleotide
selected from the group consisting of T728C, A1553G, A1907C, T2718C, T2718G, A2721G, A4550C, A4845T and T7160C.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said nucleotide at the said position of SEQ ID NO: 1 by the following said nucleotide selected from the group consisting of T728C, A1553G, A1907C, T2718G and T7160C. The resulting sequence is shown in SEQ ID NO: 3.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said nucleotide at the said position of SEQ ID NO: 1 by the following said nucleotide selected from the group consisting of T728C, A1553G, A1907C, T2721G and T7160C. The resulting sequence is shown in SEQ ID NO: 5.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said nucleotide at the said position of SEQ ID NO: 1 by the following said nucleotide selected from the group consisting of T728C, T2718G, and T7160C. The resulting sequence is shown in SEQ ID NO: 7.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said nucleotide at the said position of SEQ ID NO: 1 by the following said nucleotide selected from the group consisting of A1553G, A2721G and A4845T. The resulting sequence is shown in SEQ ID NO: 9.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said nucleotide at the said position of SEQ ID NO: 1 by the following said nucleotide selected from the group consisting of A2721G and A4845T. The resulting sequence is shown in SEQ ID NO: 11. One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutations is a replacement of T in position 2718 of SEQ ID NO: 1 with G and a replacement of T in position 7160 of SEQ ID NO: 1 with C. The resulting sequence is shown in SEQ ID NO: 19.
One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutations is a replacement of T in position 2718 of SEQ ID NO: 1 with C and a replacement of A in position 4550 of SEQ ID NO: 1 with C. The resulting sequence is shown in SEQ ID NO: 21.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutations is a replacement of T in position 2718 of SEQ ID NO: 1 with G and a replacement of A in position 4550 of SEQ ID NO: 1 with C. The resulting sequence is shown in SEQ ID NO: 13.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of T in position 728 of SEQ ID NO: 1 with C.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of C in position 1527 of SEQ ID NO: 1 with T.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 1553 of SEQ ID NO: 1 with G.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 1907 of SEQ ID NO: 1 with C.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 2297 of SEQ ID NO: 1 with T.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of T in position 2718 of SEQ ID NO: 1 with G. The resulting sequence is shown in SEQ ID NO: 15.
One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of T in position 2718 of SEQ ID NO: 1 with C.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of T in position 2720 of SEQ ID NO: 1 with A.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 2721 of SEQ ID NO: 1 with G.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 3023 of SEQ ID NO: 1 with C.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 3748 of SEQ ID NO: 1 with G.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of G in position 4464 of SEQ ID NO: 1 with T.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of G in position 4464 of SEQ ID NO: 1 with A.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 4550 of SEQ ID NO: 1 with C. The resulting sequence is shown in SEQ ID NO: 17.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 4552 of SEQ ID NO: 1 with C.
One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 4552 of SEQ ID NO: 1 with T.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 4845 of SEQ ID NO: 1 with T.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of C in position 5407 of SEQ ID NO: 1 with T.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of A in position 7154 of SEQ ID NO: 1 with C.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is a replacement of T in position 7160 of SEQ ID NO: 1 with C.

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.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said amino acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of F130L, I162V, V284A, A396V, M405V, K523Q, N533D, T653S, I793S, I793T, Y794C, Y794N, C859Y, T895P, R1375L, R1375Q, K1404N, K1404Q, Q1502L, I2272L, S2274P and S2281N.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said amino
acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of F130L, M405V, K523Q, I793S, I793T, Y794C, K1404Q, Q1502L and S2274P.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said amino acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of F130L, M405V, K523Q, I793S, and S2274P. The resulting sequence is shown in SEQ ID NO: 4

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said amino acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of F130L, I793S, and S2274P. The resulting sequence is shown in SEQ ID NO: 8

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said amino acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of F130L, M405V, K523Q, Y794C and S2274P. The resulting sequence is shown in SEQ ID NO: 6

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said amino acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of M405V, Y794C, and Q1502L The resulting sequence is shown in SEQ ID NO: 10

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutation is at least one of the replacements of the first said amino acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of Y794C, and Q1502L The resulting sequence is shown in SEQ ID NO: 12
One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutations is a replacement of I in position 793 of SEQ ID NO: 2 with S and a replacement of S in position 2274 of SEQ ID NO: 2 with P. The resulting sequence is shown in SEQ ID NO: 20.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutations is a replacement of I in position 793 of SEQ ID NO: 2 with T and a replacement of K in position 1404 of SEQ ID NO: 2 with Q. The resulting sequence is shown in SEQ ID NO: 22.

One embodiment of the present invention relates to adaptive mutations, wherein said adaptive mutations is a replacement of I in position 793 of SEQ ID NO: 2 with S and a replacement of K in position 1404 of SEQ ID NO: 1 with Q. The resulting sequence is shown in SEQ ID NO: 14.

Another embodiment of the present invention relates said adaptive mutation is a replacement of F in position 130 of SEQ ID NO: 2 with L.

Another embodiment of the present invention relates said adaptive mutation is a replacement of A in position 396 of SEQ ID NO: 2 with V.

Another embodiment of the present invention relates said adaptive mutation is a replacement of M in position 405 of SEQ ID NO: 2 with V.

Another embodiment of the present invention relates said adaptive mutation is a replacement of K in position 523 of SEQ ID NO: 2 with Q.

Another embodiment of the present invention relates said adaptive mutation is a replacement of T in position 653 of SEQ ID NO: 2 with S.

Another embodiment of the present invention relates said adaptive mutation is a replacement of I in position 793 of SEQ ID NO: 2 with S. The resulting sequence is shown as SEQ ID NO: 16.
Another embodiment of the present invention relates said adaptive mutation is a replacement of I in position 793 of SEQ ID NO: 2 with T.

Another embodiment of the present invention relates said adaptive mutation is a replacement of Y in position 794 of SEQ ID NO: 2 with C.

Another embodiment of the present invention relates said adaptive mutation is a replacement of Y in position 794 of SEQ ID NO: 2 with N.

Another embodiment of the present invention relates said adaptive mutation is a replacement of T in position 895 of SEQ ID NO: 2 with P.

Another embodiment of the present invention relates said adaptive mutation is a replacement of R in position 1375 of SEQ ID NO: 2 with L.

Another embodiment of the present invention relates said adaptive mutation is a replacement of R in position 1375 of SEQ ID NO: 2 with O.

Another embodiment of the present invention relates said adaptive mutation is a replacement of K in position 1404 of SEQ ID NO: 2 with Q. The resulting sequence is shown as SEQ ID NO: 18.

Another embodiment of the present invention relates said adaptive mutation is a replacement of K in position 1404 of SEQ ID NO: 2 with N.

Another embodiment of the present invention relates said adaptive mutation is a replacement of Q in position 1502 of SEQ ID NO: 2 with L.

Another embodiment of the present invention relates said adaptive mutation is a replacement of I in position 2272 of SEQ ID NO: 2 with L.

Another embodiment of the present invention relates said adaptive mutation is a replacement of S in position 2274 of SEQ ID NO: 2 with P.
Possible Mechanisms of identified adaptive mutations
Q1502L in the JFH1 NS3 portion of our S52/JFH1 viruses might facilitate interaction with genotype 3a proteins or optimize the JFH1 NS3 protein, since 6/6 genotype 3a and 43/45 genotype 2 isolates (published as confirmed in euHCVdb18) also encode L at this amino acid position, respectively. However, Q1502L was not selected in J6/JFH cultures. NS3 and NS5A proteins have been hot spots for cell culture adaptation in the replicon system. A leucine substitution at amino acid 470 (referring to H77 and S52/JFH1 NS3 protein and corresponding to position 1502 on the S52/JFH1 polyprotein) has been implied in cell culture adaptation of the H77 replicon. Concerning NS5A it is intriguing that S2274P, observed in S52/JFH1 viruses, localizes immediately downstream of a cluster of conserved serine residues involved in NS5A hyperphosphorylation and thus regulation of replication. Even though not conserved for all genotypes, S is conserved for all 45 genotype 2 isolates.

F130L is located in domain 2 of Core, which is supposed to be critical for Core stability and localization to lipid droplets. At position 130, F is conserved in most isolates, while L is only rarely occurring. In future studies it would be interesting to test, if F130L can enhance Core stability and thus lead to greater amounts of intracellular Core, which is thought to be of importance for production of infectious viral particles.

The crucial role of adaptive mutations for the viability of intergenotypic recombinant viruses has recently been found also by others. After transfection of intergenotypic 1a/2a (H77/JFH1) recombinants, a lag phase was observed following transfection before infectious viruses were produced yielding infectivity titers of $10^4$-$10^5$ FFU/ml. It is difficult to evaluate the performance of the 1a/2a recombinants, since the original non-adapted JFH1 genome was used as reference system, which has been shown to perform sub-optimally in the absence of adaptive mutations.

Further the efficiency of the 1/2a recombinants cannot be directly compared with that of the recombinants used in this study, because it has not been clarified, how different measures of infectivity (FFL versus TCID$_{50}$) compare. The viability of the
la/2a recombinant featuring the same junction (NS2/NS3) as our S52/JFH1 virus appeared to depend on a single mutation in NS3.

However, this conclusion was drawn by studying a time-frame of only 72 hours post-transfection, and the sequence of viruses present in this culture was not determined. Thus, it is possible that additional mutations, accounting for viability, had been acquired. In the present study, we found that a mutation in NS3, probably in combination with one of the mutations observed in p7, is sufficient but not required to confer viability to an intergenotypic (3a/2a) recombinant genome featuring the NS2/NS3 junction.

Polyclonal 2nd passage viruses, as well as recombinant adapted S52/JFH1 viruses, performed as efficiently as the J6/JFH reference viruses with respect to peak genome and infectivity titers as well as growth kinetics (TABLE 4, FIG. 3, FIG. 5, FIG. 6). This is in contrast to a recently described intergenotypic recombinant 3a/JFH1 virus based on genotype 3a strain HCV-452, which performs with poor efficiency in cell culture leading to low infectivity titers (< $10^2$ TCID$_{50}$/ml) when compared to J6/JFH recombinant genomes (Pietschmann et al. 2006). These differences might be due to differential assembly capabilities of the structural proteins of different isolates. However, since a timeframe of only 96 hours post-transfection was studied, this could also indicate a low infectivity of the original 3a/2a viruses in the absence of adaptive mutations.

Our study also points to a low infectivity of the original S52/JFH1 virus. First, the present inventors found comparable to the previously described study low infectivity titers shortly after transfection with pS52/JFH1 in vitro transcripts (FIG. 5B), which eventually became undeterminable. Second, the original pS52/JFH1 sequence could not be detected in clonal analysis of virus genomes derived from a 2nd viral passage. Thus, the original S52/JFH1 viruses seem to be viable but unable to establish sustained infection in cell culture.

In summary, the present inventors have provided the first efficient HCV genotype 3a cell culture system leading to sustained infection in Huh7.5 cell culture. The present inventors have developed an intergenotypic recombinant, containing the Core through NS2 sequence of the S52 reference genome on a JFH1 backbone.
The present inventors have identified and characterized combinations of adaptive mutations, which allow efficient growth of S52/JFH1 viruses. The recombinant adapted S52/JFH1 genomes will be valuable tools for genotype 3a specific in vitro studies of Core through NS2 involved in viral entry, assembly and release as well as related therapeutics. Furthermore they will permit studies of genotype specific interactions and functions and might further the development of a genotype 3a full-length cell culture system.

The skilled addressee may use the present invention to determine whether the identified sets of mutations can confer viability to other JFH1 based intergenotypic genotype 3a recombinants, which would allow in vitro studies of any patient 3a isolate of interest, or to other JFH1 based intergenotypic genotype recombinants or to any other HCV isolate.

Finally, it would be interesting to elucidate the mechanism of action of the identified mutations. In principle they might enable efficient intergenotypic protein interaction and/or lead to improvement of protein function independent of these intergenotypic interactions, for example by influencing interactions with host cell proteins.

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 tissue culture infectious dose -50 method. This titer indicates 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 TCIDso/ml.

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 $10^2$ TCID$_{50}$/ml or above following transfection and/or subsequent viral passage, such as a titer of at least $10^3$ TCID$_{50}$/ml, such as a titer of at least $10^4$ TCID$_{50}$/ml, such as a titer of at least $10^5$ TCID$_{50}$/ml, such as a titer of at least $10^6$ TCID$_{50}$/ml, such as a titer of at least $10^7$ TCID$_{50}$/ml, such as a titer of at least $10^8$ TCID$_{50}$/ml, such as a titer of at least $10^9$ TCID$_{50}$/ml or such as a titer of at least $10^{10}$ TCID$_{50}$/ml.

Thus such molecules which following transfection and/or subsequent viral passage, are capable of generating a HCV infectivity titer of $10^2$ TCID$_{50}$/ml (50% tissue culture infectious doses)/ml or above using the assay described in this text or what is equivalent to this titer determined by any method.

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.

"Pharmacologically 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 "pharmacologically 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 parvum. Preferably, the adjuvant is pharmaceutically acceptable.

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 in one embodiment the present invention relates to a method for producing a cell which replicates HCV 3a/JFH1 RNA and produces a virus particle comprising introducing the said RNA according to the invention into a cell.

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.

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) 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. 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 or parts thereof, comprising culturing a cell or an animal to allow either to produce the virus.

In another embodiment the invention 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.
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.

Further it will be important to determine the viability of the developed viruses in vivo, either in SCID-uPA mice engrafted with human liver tissue or in chimpanzees as shown in Lindenbach et al. 2006.

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.

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. 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 genomes obtained in the present study may prove useful for different research topics. Genomes with the original S52 Core could be applied to examine genotype 3a specific features of Core, such as its presumed role in the development of hepatocellular steatosis.

The systems developed in this study are ideal candidates for the genotype 3a specific testing of therapeutics targeting viral entry, assembly and release.

Genomes with the original S52 El and E2 are valuable for testing of neutralizing antibodies and other drugs acting on entry level, such as fusion inhibitors.

In one embodiment the present invention related to a method for screening new HCV genotype 1a/lb, 2a, 3a, 4a, 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,

b. subjecting said virus or virus infected cell culture to a blood sample or derivatives thereof or synthetically produced
equivalents from a HCV genotype la/lb, 2a, 3a, 4a, 5a, 6a and/or 7a infected patient, and
detecting the amount of replicating RNA and/or the virus particles.

As proof of principle of using the present invention in testing of anti-hepatitis C virus substances, the effects of interferon alfa, currently used in combination therapy for HCV, were tested on the infected cell culture. Previously, replication of different HCV replicons as well as of the J6/JFH1 virus have been shown to be sensitive to treatment with interferon. Thus we treated Huh7.5 cell cultures infected with J6/JFH1, S52/JFH1(T2718G; A4550C) and S52/JFH1(A4550C) with cell culture medium containing 500IU/ml interferon alfa 2b (Figure 10). After initiation of treatment, we observed for all cultures a fast decline of HCV antigen positive cells. Thus, for J6/JFH1 and S52/JFH1(T2718G; A4550C) the percentage of NS5A positive cells was 90 and 60 the day prior to treatment, whereas only 20% and 10% NS5A positive cells were detected in the respective cultures on day 3 of treatment (Figure 5). Interestingly, prolonged treatment intervals were followed by an increase in NS5A positive cells; thus, on day 6, when the cultures had not been treated for 48 hrs, 40% of cells in the J6/JFH1 and S52/JFH1(T2718G; A4550C) cultures were NS5A positive.

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. The p7 mutations observed in this study map to TM2, and enables interactions between the S52 p7 and p7-associated JFH1 derived proteins. Hence, new compounds targeting the putative p7 ion-channel, protease 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 claim 17, a hepatitis C virus infected cell according to claim
21 and a hepatitis C virus particle obtainable by claim 18 together with a hepatitis C virus permissive cell, and

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

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

In another embodiment 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 claim 17, a hepatitis C virus infected cell according to claim 21 and a hepatitis C virus particle obtainable by claim 18 together with a hepatitis C virus permissive cell, and

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.

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 labeled for easier detection, including radiolabeled, antibody labeled for fluorescently labeled 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.

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 modeling 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 indicates 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, the SCID-uPA mouse model or 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 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. To determine the level of a viral protein, one can use techniques including ELISA, immunoprecipitation, immunofluorescence, EIA, RIA, and Western blotting analysis. To determine the replication rate of a virus, one can use the method described in, e.g., Billaud et al., Virology 266 (2000) 180-188.

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 indicates 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 indicates 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.

General

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.
As will be apparent, preferred features and characteristics of one aspect of the invention may be applicable to other aspects of the invention. The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced by reference therein.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In addition, singular reference does not exclude a plurality. Thus, references to "a", "an", "first", "second" etc. do not preclude a plurality.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced by reference therein.

The invention will hereinafter be described by way of the following non-limiting Figures and Examples.
Sequences

SEQ ID NO: 1: S52-JFH1 - DNA
SEQ ID NO: 2: S52-JFH1 - Amino Acid Sequence
SEQ ID NO: 3: S52-JFH1-Core(728)E2(1553,1907)p7(T2718G)NS5A(7160) - DNA
SEQ ID NO: 4: S52-JFH1-Core(728)E2(1553,1907)p7(T2718G)NS5A(7160) - Amino Acid Sequence
SEQ ID NO: 5: S52-JFH1-Core(728)E2(1553,1907)p7(2721)NS5A(7160) - DNA
SEQ ID NO: 6: S52-JFH1-Core(728)E2(1553,1907)p7(2721)NS5A(7160) - Amino Acid Sequence
SEQ ID NO: 7: S52-JFH1-Core(728)p7(T2718G)NS5A(7160) - DNA
SEQ ID NO: 8: S52-JFH1-Core(728)p7(T2718G)NS5A(7160) - Amino Acid Sequence
SEQ ID NO: 9: S52-JFH1-E2(1553)p7(2721)NS3(4845) - DNA
SEQ ID NO: 10: S52-JFH1-E2(1553)p7(2721)NS3(4845) - Amino Acid Sequence
SEQ ID NO: 11: S52-JFH1-p7(2721)NS3(4845) - DNA
SEQ ID NO: 12: S52-JFH1-p7(2721)NS3(4845) - Amino Acid Sequence
SEQ ID NO: 13: S52-JFH1-p7(T2718G)NS3(4550) - DNA
SEQ ID NO: 14: S52-JFH1-p7(T2718G)NS3(4550) - Amino Acid Sequence
SEQ ID NO: 15: S52-JFH1- p7(T2718G) - DNA
SEQ ID NO: 16: S52-JFH1- p7(T2718G) - Amino Acid Sequence
SEQ ID NO: 17: S52-JFH1-NS3(4550) - DNA
SEQ ID NO: 18: S52-JFH1-NS3(4550) - Amino Acid Sequence
SEQ ID NO: 19: S52-JFH1- p7(T2718G)NS5A(7160) - DNA
SEQ ID NO: 20: S52-JFH1- p7(T2718G)NS5A(7160) - Amino Acid Sequence
SEQ ID NO: 21: S52-JFH1-p7(T2718C)NS3(4550) - DNA
SEQ ID NO: 22: S52-JFH1-p7(T2718C)NS3(4550) - Amino Acid Sequence
SEQ ID NO: 23: HCV3aFIT7NotI-G
SEQ ID NO: 24: HCV3aR3420BsaBIAscI
SEQ ID NO: 25: HCV3aF3336NotI-BsaBI
SEQ ID NO: 26: HCV3aR5209AflIAscI
SEQ ID NO: 27: 947OR(24)_JFH1
SEQ ID NO: 28: HCV3aCoSeR1072
SEQ ID NO: 29: H39X58R
SEQ ID NO: 30: 7848R_JFH1
SEQ ID NO: 31: -285SJHV-CV-MOD
SEQ ID NO: 32: HCV3aSeqR831
SEQ ID NO: 33: Chim seq JFH Fl
SEQ ID NO: 34: HCV2aF9251
SEQ ID NO: 35: 7234R_JFH1
SEQ ID NO: 36: HCVconsR337
SEQ ID NO: 37: HCVconsR312
SEQ ID NO: 38: HCVconsR169
SEQ ID NO: 39: -84SJHV-CV-MOD
SEQ ID NO: 40: HCV3aCoSeF918
SEQ ID NO: 41: HCV3aCoSeR1819
SEQ ID NO: 42: HCV3aCoSeF1288
Examples

MATERIALS AND METHODS

Source of HCV genotype 3a.
A plasma pool of strain S52 was prepared from acute-phase plasmapheresis units collected from a chimpanzee experimentally infected with serum of an Italian patient chronically infected with hepatitis C virus genotype 3a (strain S52). This HCV pool has an HCV RNA titer of approximately $10^4$ IU/ml and an infectivity titer of approximately $10^3$ chimpanzee infectious doses/ml.

Sequencing of Core through NS2 of strain S52.
Total RNA from 200 µl of the S52 plasma was extracted using High Pure Viral Nucleic Acid Kit (Roche) and eluted in 20 µl of elution buffer. RT-PCR was performed on RNA equivalent to 100 µl plasma using random hexamers (TAG Copenhagen) and Superscript II (Invitrogen) for one hour at 42°C followed by inactivation of the enzyme for 10 min at 70°C. Remaining RNA templates were digested by incubation with RNAseH (4L, Invitrogen) and RNAseTI (I00OU, Ambion) for 20 min at 37°C. The region of interest was PCR amplified from cDNA in two overlapping fragments using BD Advantage 2 Polymerase Mix (Clontech). The first fragment spanning nucleotides (nts) 1-3404 (positions referring to S52 sequences correspond to the full-length genome sequence of reference isolate H77, accession number AF009606, as proposed by Kuiken et al.41) was amplified with primers HCV3aF3336NotIBsaBI (SEQ ID NO: 23) and HCV3aR3420BsaBIAscI (SEQ ID NO: 24) (TABLE 1) and the following cycling parameters: initial denaturation of 1 min at 95°C, 40 cycles with 30 sec at 95°C, 30 sec at 62°C, 8 min at 68°C, and final extension of 8 min at 68°C. The second fragment spanning nucleotide 3320 to 5193 was amplified with primers HCV3aF3336NotIBsaBI (SEQ ID NO: 25) and HCV3aR5209AfllIAstcl (SEQ ID NO: 26) (TABLE 1) using cycling parameters as above with an annealing temperature of 62°C. Both amplicons were subcloned in pCR2.1 Topo (Invitrogen) and 5 clones were sequenced of each fragment to determine the consensus sequence of core through NS2 of strain S52 (nts 342-3419).
Construction of pS52/JFH1 and recombinant adapted genomes.
For construction of pS52/JFH1 the S52 Core-NS2 fused to JFH1 5’ UTR and NS3 was assembled in pGEM-9Zf(-) (Promega) using fusion PCR with Pfu DNA polymerase (Stratagene) and standard cloning procedures with appropriate restriction sites. S52 fragments were derived from the clones described above. The two JFH1 fragments used for fusion of JFH1-5’ UTR/S52-Core and S52-NS2/JFH1-NS3, were amplified from plasmid pFL-J6/JFH (generous gift from Charles M. Rice, Rockefeller University) including the EcoRI (vector sequence upstream of JFH1 5’ UTR) and AvrII (in NS3 of JFH1) sites, respectively. The S52 consensus Xbal site was removed by site directed mutagenesis (A916T) in order to permit Xbal linearization of pS52/JFH1 at the very 3’ end of the 3’ UTR prior to in vitro transcription. The EcoRI/AvrII fragment of the resulting pGEM-9Zf(-) clone was finally inserted into pFL-J6/JFH. pS52/JFH1 contains the 5’ UTR of the JFH1 isolate (nts 1-340), which differs from the sequence provided for JFH1 (accession number AB047639) at one position (C301T), Core through NS2 of S52 (nts 341-3436), and NS3 through 3’ UTR of JFH1 (nts 3437-9684).

pS52/JFH1(GND) has a single point mutation in NS5B (G8624A), which abolishes replication; it was created by transferring the EcoRI/AvrII fragment of pS52/JFH1 into pFL-J6/JFH(GND) (gift from Charles M. Rice, Rockefeller University). For construction of five recombinant adapted S52/JFH1 genomes mutations were introduced in pS52/JFH1 by fusion PCR (Pfu DNA polymerase, Stratagene) and standard cloning techniques using appropriate restriction sites. The HCV sequence of the described plasmids was verified by sequencing of the final DNA preparation (EndoFree Plasmid Maxi Kit, Qiagen). All sequencing reactions were carried out at Macrogen Inc., Seoul, South Korea.

In vitro transcription from full-length HCV cDNA genomes.
Plasmid DNA was linearized with XbaI (New England BioLabs), gel purified (Wizard SV Gel and PCR Clean-Up System, Promega), and subjected to in vitro transcription with T7 RNA Polymerase (Promega) for 2 hours at 37°C according to the manufacturers protocol. The amount of RNA transcripts was estimated by standard agarose gel electrophoresis.
Culture of Huh7.5 cells.
The human hepatoma cell line Huh7.5 has been described previously (gift from Charles M. Rice, Rockefeller University). Cells were cultured in D-MEM + 4500 mg/L Glucose + GlutaMAX-I + Pyruvate (Gibco / Invitrogen Corporation) containing 10% heat inactivated fetal bovine serum (FBS) (Sigma), penicillin at 100 units/ml and streptomycin at 100 mg/ml (Gibco / Invitrogen Corporation) at 5% CO2 and 37°C. Cells were split every 2nd to 3rd day at a ratio of 1:2 to 1:3.

Transfection of RNA transcripts into Huh7.5 cells.
24 hrs before transfection cells were washed with PBS (Dulbecco’s Phosphate Buffered Saline; Sigma) and trypsinized (Trypsin/EDTA; Gibco / Invitrogen Corporation). 4x10^5 cells were plated per well of a 6 well plate in D-MEM supplemented with 10% FBS without antibiotics. Transfection was carried out by lipofection using 2.5 µg in vitro RNA transcripts and 5µl Lipofectamine 2000 Transfection reagent (Invitrogen) for formation of lipofection complexes in serum free medium (Opti-MEM, Invitrogen). Cell cultures were incubated with lipofection complexes for ~12 hrs at 5% CO2, at 37°C.

Infection of Huh7.5 cells with cell culture supernatants.
24 hrs before infection cells were washed with PBS and trypsinized. 4x10^5 cells were plated per well of a 6 well plate. On the day of infection growth medium was removed, cells were washed with PBS and incubated with the desired dilution of virus containing cell culture supernatants or negative control supernatants in complete growth medium. The incubation times for the respective experiments are given in the figure legends.

Collection of viral stock of supernatants.
Cell culture supernatants of virus infected cell cultures or controls were rescued every 2-3 days. After filtration, cell free supernatants were aliquoted immediately and stored at -80° C.

Immuno-histochemistry staining for HCV NS5A.
Analyses were carried out as previously described with modifications. Huh7.5 cells were washed with PBS, trypsinized and plated on chamber slides (Nunc). After ~24 hours cells were washed twice with PBS and fixed 5min with ice-cold...
methanol. After washing 2x with PBS and 1x with PBS/Tween (0.1% Tween-20), slides were blocked with PBS containing bovine serum albumin (BSA, 1%) and skim milk (0.2%) for 1 hour at room temperature. Endogenous peroxidase staining was reduced by adding 3% H2O2 in PBS for 5min. After washing as above the 1st antibody (anti-NS5A, 9E10, a generous gift from Charles M. Rice, used at 1:200 in PBS/Tween) was added and incubated at 4°C overnight. After washing slides were incubated with secondary antibody (ECLTM Anti-mouse IgG, horseradish peroxidase linked whole antibody, Amersham Biosciences, used at 1:300 in PBS/Tween) for 30min at room temperature. After washing horseradish peroxidase substrate (DAB substrate kit, DAKO) was added. After incubation for 30min, slides were washed with H2O, mounted with Fluoromount-G (Southern Biotech) and cover slipped. Percentage of infected cells was evaluated by light microscopy assigning values of 0% (no cells infected), 1%, 5%, 10-90% (in steps of 10%), 95% and 100% (all cells infected).

Immuno-histochemistry staining for HCV Core.

Huh7.5 cells were washed with PBS, trypsinized and plated on chamber slides. After ~24 hours cells were washed twice with PBS and fixed with acetone for 5 minutes. After washing 2x with PBS and 1x with PBS/Tween (0.1%), slides were incubated with 1st antibody (MAB Murine Anti-Human HCV (Core Protein) Clone B2, Anogen, used at 1:200 in PBS containing 5% BSA) for 20min at room temperature. After washing as above secondary antibody (Alexa Fluor 594 goat anti-mouse IgG (H+L), Invitrogen, used at 1:500 in PBS/Tween) was added for 5min. Cell nuclei were counterstained with Hoechst for 5min. Finally slides were washed with PBS, mounted with Fluoromount-G and cover slipped. Percentage of infected cells was evaluated by fluorescence confocal microscopy as described above.

Staining and quantification of intracellular lipid droplets

Cells were fixed as described previously and incubated with anti-Core (as above) followed by incubation with 2nd antibody Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen) at 1:300 in PBS containing 5% BSA. Lipid droplets were stained with oil red O (Fisher Scientific) as described previously. Cell nuclei were counterstained with Hoechst 33342. Images were obtained by confocal
microscopy. For quantification of intracellular lipid contents pixel saturation of the oil red O signal was avoided and images were analyzed using MetaMorph (Universal Imaging). The relative amount of intracellular lipid per cell was determined as the intensity of oil red O signal per nucleus. Eight randomized areas were analyzed per infected / mock infected culture, each area representing 50 cells in average.

Determination of 50% tissue culture infectious doses (TCID\textsubscript{50}).

In general, the TCID\textsubscript{50} of culture supernatants was determined as previously described. Huh7.5 cells were washed with PBS, trypsinized and plated at 6x10\textsuperscript{3} cells per well of a poly-D-lysine coated 96 well plate (Nunc). After ~24 hrs cells were incubated with 10-fold serial dilutions of viral stock cell culture supernatant in complete growth medium. For each dilution, six replicate wells were inoculated. After incubation for 48 hrs the plate was subjected to immuno staining for NS5A as described above. Wells were evaluated by light microscopy and scored positive if at least one positive cell could be detected. TCID\textsubscript{50} values were calculated as described by Reed and Muench.

CD81 blocking assay

6x10\textsuperscript{3} Huh7.5 cells were plated per well of a poly-D-lysine coated 96-well plate. After 24 hrs cells were incubated with anti-CD81 (JS-81, BD Pharmingen) or isotype matched control antibody (anti-HIV, p24, clone Kal-I, DAKO) for 1 hr. Subsequently cells were infected with 100 TCID\textsubscript{50} of S52/JFH1(T728C;T2718G;T7160C) for 3 hrs and washed with PBS. Supernatants were collected after 1, 2 and 3 days. Cells were stained for HCV NS5A after 3 days to determine the number of focus forming units (FFU) per well.

Real-time PCR (TaqMan) assay for determination of HCV RNA titers.

RNA was purified from 200 µl of heat inactivated (56°C for 30 min) cell culture supernatant and eluted in a final volume of 50 µl using the Total Nucleic Acid Isolation Kit (Roche) in combination with the Total NA Variable Elution Volume protocol on a MagNA Pure LC Instrument (Roche). As an internal control, Phocine Distemper Virus (PDV) was added to the lysis buffer in a concentration titrated to yield a Ct of ~32 upon real-time PCR analysis. In parallel to RNA purified from cell
culture supernatants, a quantitative HCV standard panel covering RNA concentrations of 0 to 5 x 10^6 IU/ml in one log increments (OptiQuant HCV Panel, AcroMetrix) was analysed. Real-time PCR analyses of HCV and PDV RNA were carried out in two separate reactions using the TaqMan EZ RT-PCR Kit (Applied Biosystems). For HCV, primers and a FAM-labelled MGB-probe were directed against the 5' LTR and were previously shown to perform equivalently against a panel of the six major HCV genotypes in a different TaqMan assay (Engle RE, Bukh J, and Purcell RH, 2007) For PDV, a ready-to-use primer/probe mix was used (Dr. H.G.M. Niesters, Department of Virology, Erasmus Medical Centre, Rotterdam, The Netherlands). The PCR analysis was performed on a 7500 Real-Time PCR System (Applied Biosystems) using 50°C for 2 min, 60°C for 30 min and 95°C for 5 min followed by 45 cycles of 94°C for 20 sec and 62°C for 1 min. HCV RNA titers (IU/ml) were calculated using a standard curve created from the known concentrations of the standard panel and their corresponding Ct values (cycle number, at which the normalised fluorescence signal rises above a fixed threshold of 0.2 being directly proportional to the amount of template in the PCR reaction). The reproducible detection limit of the assay was 500 IU/ml. In order to confirm successful purification, amplification and the absence of PCR inhibitors, the Ct value of the PDV reaction was compared to the expected Ct value (based on a mean of all previous runs; n>9) using the MedLab QC freeware programme. The results of samples with an actual Ct value within ±2SD of the expected Ct value were accepted.

Direct sequencing of the HCV ORF of recovered virus genomes.

In order to determine the consensus sequence of the complete ORF, RNA was extracted from virus containing cell culture supernatant as described above. RT-PCR was performed using the gene specific primer 947OR(24)_JFH1 (SEQ ID NO: 27) (TABLE 2) and Superscript III (Invitrogen) for one hour at 50°C followed by 10 min incubation at 70°C and treatment with RNase H and RNase T1 as described above. First round PCR was carried out with primers -285S_HCV-MOD (SEQ ID NO: 31) and 947OR(24)_JFH1 (SEQ ID NO: 27) (TABLE 2) using BD Advantage 2 Polymerase Mix and the following cycling parameters: 35 cycles with denaturation at 99°C for 35 sec, primer annealing at 67°C for 30 sec and amplification at 68°C for 10 min (5 cycles), 11 min (10 cycles), 12 min (10 cycles), 13 min (10 cycles). In a 2nd round PCR, 12 overlapping fragments spanning the
complete ORF were generated with the primer combinations shown in TABLE 2 for S52/JFH1 and in TABLE 3 for J6/JFH with the following cycling parameters: initial denaturation of 35sec at 99°C and 35 cycles with 35sec at 99°C, 30sec at 67°C and 6min at 68°C. PCR products were agarose gel purified and directly sequenced in both directions.

Sequence analysis of 5’ and 3’ UTR of recovered S52/JFH1 viruses. RNA was extracted from cell culture supernatant as described above. RT was performed using primer HCV3aCoSeR1072 ( SEQ ID NO: 28) (TABLE 1) for the 5’ UTR and H39X58R ( SEQ ID NO: 29) (TABLE 1) for the 3’ UTR with Superscript III for one hour at 50°C followed by one hour at 55°C. For 2nd passage S52/JFH1 viruses a fragment spanning nts 56-832 was amplified with the primers -285SJHCV-MOD ( SEQ ID NO: 31) (TABLE 2) and HCV3aSeqR831 ( SEQ ID NO: 32) (TABLE 1) and for 1st passage recombinant adapted viruses a fragment from nts 1-832 was generated with the primers Chim seq JFH FI ( SEQ ID NO: 33) (TABLE 1) and HCV3aSeqR831 ( SEQ ID NO: 32) (TABLE 1). PCR amplification was performed with BD Advantage 2 Polymerase Mix with the following cycling parameters: 3min at 95°C followed by 35 cycles with 40sec at 95°C, 40sec at 52°C, 3min at 68°C, and final extension of 6min at 68°C. For 2nd passage S52/JFH1 viruses a fragment spanning the variable region and the poly U tract (nts 9332-9644) of the 3’ UTR was amplified with the primers HCV2aF9251 ( SEQ ID NO: 34) and H39X58R ( SEQ ID NO: 29) (TABLE 1) using the following cycling parameters: 10min at 94°C, 45 cycles with 1min at 94°C, 1min at 60°C, 2min at 68°C, and final extension of 2min at 68°C. PCR products were agarose gel purified and directly sequenced.

Clonal sequence analysis of 2nd viral passage S52/JFH1 viruses. RNA was extracted as described above. RT was performed with the primer 7848R_JFH1 ( SEQ ID NO: 30) (TABLE 2) and Superscript III for one hour at 50°C. A long PCR product was generated with primers -285SJHCV-MOD ( SEQ ID NO: 31) and 7234R_JFH1 ( SEQ ID NO: 35) (TABLE 2) using BD Advantage 2 Polymerase Mix with the following cycling parameters: 1min at 95°C, 40 cycles with 35sec at 95°C, 35sec at 67°C, 18min at 68°C, and final extension of 10min
at 68°C. The resulting PCR product was gel purified, subcloned into pCR-XL-TOPO (Invitrogen), and the resulting clones were sequenced.

5′ RACE (rapid amplification of cDNA ends)

The extreme 5′ end of 2nd passage S52/JFH1 viruses was determined using the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) with modifications. RNA was extracted using the Trizol (Invitrogen) protocol. First strand synthesis was carried out with primer HCVconsR337 (SEQ ID NO: 36) (TABLE 1) using SuperScriptIII for 40min at 50°C and 30min at 55°C. To optimize binding on S.N.A.P. cDNA purification columns samples were reloaded twice, and 16,5 µl of the eluate were used for TdT-tailing according to the protocol.

1st round PCR was done according to the manufacturer’s protocol using primer HCVconsR312 (SEQ ID NO: 37) (TABLE 1) and AmpliTaq Gold DNA Polymerase (Applied Biosystems). Cycle parameters were: initial denaturation of 10min at 94°C and 40 cycles of 45sec at 94°C, 45sec at 55°C and 1min 30sec at 72°C followed by a final extension of 10min at 72°C. 2nd round PCR was done using primer HCVconsR169 (SEQ ID NO: 38) (TABLE 1) and cycle parameters as above. The consensus sequence of the amplified fragment was determined by direct sequencing and by sequencing of 4 clones obtained by subcloning into pCR2.1 Topo.

Sequence analysis software and databases.

Sequence analysis was performed using Sequencher 4.6, Gene Codes Corporation and freeware BioEdit v. 7.0.5. HCV sequences used for alignments were retrieved from The European HCV database website (euHCVdb; http://euhcVdb.ibcp.fr/euHCVdb/) and the American HCV database website (LANL; http://hcv.lanl.gov/content/hcv-db/index42).

Example 1

The J6/JFH cell culture system yields high viral titers and does not require adaptive mutations.

The intragenotypic 2a/2a recombinant J6/JFH, in which the structural genes (Core, E1 and E2), p7 and NS2 of JFH1 were replaced by the corresponding
sequence of the infectious clone pJ6CF, produced infectious viral particles in the human hepatoma cell line Huh7.5. In our hands, transfection of J6/JFH RNA transcripts into Huh7.5 cells resulted in infection of most cells within 5 days as determined by NSSA immuno-staining (FIG. 1A), and J6/JFH virus from supernatant collected on day 6 readily infected naive Huh7.5 cells (data not shown). In supernatant from day 8 of this 1st passage the present inventors recorded an HCV infectivity titer of $10^{4.6}$ TCID$_{50}$/ml and an HCV RNA titer of $10^{7.2}$ IU/ml with a specific infectivity (infectious dose per genomes measured in IU) of 1:398 (TABLE 4). These results are comparable to those obtained by Lindenbach et al.. The present inventors demonstrated that J6/JFH did not require mutations for efficient growth since the present inventors did not detect a single mutation compared to the original J6/JFH plasmid in the ORF consensus sequence of 1st passage virus recovered on day 8 post-infection.

After a subsequent transfection of Huh7.5 cells with J6/JFH, the culture was followed for 40 days (FIG. 2A). After rapid viral spread, massive cell death occurred, followed by recovery and decrease in percentage of infected cells, presumably caused by a lower susceptibility or permissiveness of the remaining cells.

Example 2

The intergenotypic 3a/2a recombinant S52/JFH1 is viable in Huh7.5 cells. To generate pS52/JFH1, the present inventors first determined the consensus sequence of Core through NS2 of HCV strain S52 (genotype 3a) by amplifying two overlapping fragments from the chimpanzee challenge pool (see Material and Methods), subcloning and analyses of 5 clones of each fragment. The final S52/JFH1 clone contains Core, E1, E2, p7 and NS2 genes of S52, and the 5' and 3' UTR, as well as the NS3, NS4A, NS4B, NS5A, and NS5B genes of JFH1. Compared to the S52 consensus sequence, pS52/JFH1 contains only noncoding nucleotide changes, which are present at 10 of 3096 (0.3%) nucleotide positions: A641G, A916T (introduced to eliminate an XbaI site), G1039A, C1490T, G1577A, C1709T, G1912A, C2639T, C2792T, C3053T (corresponding to H77 reference genome).
The delayed viral spread of S52/JFH1 compared to J6/JFH indicated selection of adaptive mutations. To further characterize the cell culture derived S52/JFH1 viruses the present inventors performed 1st and 2nd passages by inoculation of naïve Huh7.5 cells with filtered cell free supernatant derived from the transfection and the following transfection of Huh7.5 cells with RNA transcripts of pS52/JFH1, the present inventors found evidence of replication with ~10% NS5A antigen positive cells on day 1 (FIG. IA). However, while infection with J6/JFH viruses spread to most Huh7.5 cells within 5 days, the percentage of NS5A positive cells decreased in the S52/JFH1 culture. Evidence of spread of S52/JFH1 virus was detected from day 8 post-transfection (FIG. IA) and the present inventors recorded an HCV infectivity titer of 10^{2.5} TCID_{50}/ml in day 11 supernatant (TABLE 4). The present inventors did not detect NSSA positive cells in the negative control culture, transfected with RNA transcripts of the replication defective pFL-J6/JFH(GND).

The 1st passage, respectively (FIG. IB and C). On day 11, the 2nd passage S52/JFH1 culture had an infectivity titer of 10^{4.2} TCID_{50}/ml and an HCV RNA titer of 10^{6.9} IU/ml (specific infectivity 1:501) (TABLE 4). These values are comparable to those the present inventors (see above) and others found in the J6/JFH culture.

The delayed growth kinetic of S52/JFH1 initially observed was confirmed in a second transfection experiment (FIG. 2A). Spread of S52/JFH1 viruses was not observed until day 26 and reached its peak on day 33, yielding an infectivity titer of 10^{3.2} TCID_{50}/ml (TABLE 4). In contrast, the J6/JFH control virus had spread to most cells on day 5. In the S52/JFH1 culture the present inventors observed, similar to the J6/JFH cell culture, progressing cell death and decrease in percentage of NS5A positive cells after peak infection. In contrast to the delayed viral spread after transfection, S52/JFH1 viruses rescued on day 33 post-transfection infected a high percentage of naïve Huh7.5 cells within 6 to 8 days (FIG. 2B), with an infectivity titer of 10^{4.5} TCID_{50}/ml and an HCV RNA titer of 10^{7.2} IU/ml (TABLE 4).
Example 3

Similar Growth Kinetics of S52/JFH1 and J6/JFH viruses. Naïve Huh7.5 cells were inoculated with \(~10^4\) TCID$_{50}$ of the S52/JFH1 3a/2a (2nd passage virus) and J6/JFH 2a/2a (1st passage virus), respectively (TABLE 4), and in both cultures >90% of cells became infected after 6 days (FIG. 3). Furthermore, HCV RNA titers and infectivity titers of S52/JFH1 and J6/JFH were similar at all time points analyzed. HCV RNA titers peaked at close to \(10^7\) copies/ml at day 6 and 9 and peak infectivity titers were detected on day 3 (\(10^{4.6}\) TCID$_{50}$/ml for S52/JFH1 and \(10^{4.7}\) TCID$_{50}$/ml for J6/JFH) (TABLE 4). When comparing specific infectivity, the present inventors recorded the highest values on day 3 post-infection (1:25 for S52/JFH1 and 1:20 for J6/JFH) and the lowest values on day 1 post-infection (1:794 for S52/JFH1 and 1:398 for J6/JFH). Growth kinetics of S52/JFH1 and J6/JFH viruses after infection with \(~10^3\) TCID$_{50}$ of the respective viruses were also comparable (data not shown).

The kinetics of S52/JFH1 infection was dependent on the virus dose. After infection of naïve Huh7.5 cells with approximately \(10^4\), \(10^3\) and \(10^{2.5}\) TCID50, the viruses spread to the entire culture after 3, 9, and 12 days, respectively, with differences reflected also in the HCV RNA titers (FIG. 4). Specific infectivities of S52/JFH1 viruses derived from these cell cultures at the peak of infection were comparable (TABLE 4).

Example 4

Identification of several adaptive mutations in serially passaged S52/JFH1 viruses. The present inventors determined the consensus sequence of viral genomes recovered from cell culture supernatants derived from the first transfection experiment and consecutive 1st, 2nd and 3rd viral passages by direct sequencing of overlapping PCR fragments spanning the entire open reading frame (TABLE 5A). Analysis of S52/JFH1 genomes recovered on day 11 post-transfection (sample used for 1st viral passage; FIG. IA) revealed coding mutations in p7 (A2721G) and NS3 (A4845T). Both occurred as quasispecies with the original pS52/JFH1 sequence. Sequence analysis of the day 21 1st viral passage viruses (used for the 2nd viral passage, FIG. IB) demonstrated additional coding
mutations in Core (T728C), E2 (A1553G, A1907C), p7 (T2718G) and NS5A (T7160C), again occurring as quasispecies with the pS52/JFH1 sequence. Analysis of day 11 2nd passage viruses (used for the 3rd viral passage/kinetic experiment) and 3rd passage viruses (FIG. 4) revealed mutations at all seven positions identified above, with the ones arisen during the 1st passage now showing a clear dominance over the original pS52/JFH1 sequences. For the 2nd viral passage virus the potential for mutations outside the ORF was examined by sequencing of the entire 5' LTR and partial 3' LTR (see Material and Methods); no mutations were found. Interestingly, the nucleotide changes at all seven positions with clear evidence of mutations resulted also in changes of the deduced amino acid sequence (TABLE 5B).

Another 2nd passage of S52/JFH1 conducted by inoculation with day 18 1st passage supernatant (FIG. IB), showed in addition to the mutations identified above evidence of coding mutations in E1 and NS5A (T1191C/V284A and G7182A/S2281N) as well as noncoding mutations in Core and NS5A (C688A and T6685C).

To confirm the requirement for adaptive mutations, the present inventors determined the consensus sequence of S52/JFH1 viruses in day 8 supernatant from the 1st passage of the second transfection experiment (FIG. 2B). As for the passages derived from the 1st transfection experiment the present inventors found evidence of a mutation at position 2718 in p7 (T2718C coding for I793T) occurring as a 50/50 quasispecies with the pS52/JFH1 sequence. Additionally the present inventors detected a mutation in NS3 (A4550C coding for K1404Q), showing strong predominance of the mutated over the original pS52/JFH1 sequence. There was no evidence of mutation at this position in the consensus sequence data obtained from viruses recovered from the first transfection and consecutive passage experiments.

Example 5

Clonal analysis of adaptive mutations of 2nd viral passage S52/JFH1 viruses. The absence of an unambiguous consensus sequence in cell culture derived S52/JFH1 genomes could be explained in two ways: Either the original sequence
was still present and/or different viral genomes coexisted, which had the detected mutations in various combinations. Thus, the present inventors performed clonal analysis of a long PCR fragment amplified from 2nd passage viral genomes, which contained all nucleotide positions, at which clear evidence of mutations had been observed. Interestingly, the clones represented several different viral genomes with specific combinations of the mutations identified by direct sequencing (TABLE 5). However, the present inventors could not identify any genomes with the original pS52/JFH1 sequence.

Overall this clonal analysis reflected the results of direct sequencing with the combination of mutations in Core (T728C), p7 (T2718G) and NS5A (T7160C), which dominated the direct sequencing of viruses derived from the 2nd and 3rd viral passage, being present in 7 of 9 clones analyzed. In one additional clone the mutations in Core and NS5A were combined with another p7 mutation (A2721G).

Finally, one clone had a combination of A2721G in p7 and A4845T in NS3, which were present as a minor species in direct sequencing of the cloned 2nd passage virus. A1553G and A1907G in E2 occurred in about half of the clones analyzed, reflecting a 50/50 distribution in direct sequencing. In addition to the described mutations all 9 clones analyzed had nucleotide changes at other positions, which could at least partly have resulted from errors in the PCR amplification process. The following nucleotide changes occurred in more than one clone and are therefore more likely to contribute to cell culture adaptation, even though they were not prominent in direct sequencing: A824G in Core (I162V; 3/9 clones), A1937G in E2 (N533D; 2/9 clones), G2916A in NS2 (C859Y; 2/9 clones), C6328T in NS5A (no amino acid change; 3/9 clones). In summary the present inventors identified different adapted S52/JFH1 genomes coexisting in the 2nd viral passage cell culture supernatant.

Example 6

Recombinant adapted S52/JFH1 viruses have significantly improved growth potential in Huh7.5 cells.

In order to determine the influence of the described mutations on viability and efficiency of the S52/JFH1 genome, the present inventors constructed six S52/JFH1 cDNA clones with different combinations of the putative adaptive
mutations: Four with three or more mutations in combinations identified in the clonal analysis (SEQ ID NO: 3, 5, 7, 9) and two with two mutations each, S52/JFH1(T2718G;T7160C) (SEQ ID NO: 19), and S52/JFH1(A2721G;A4845T) (SEQ ID NO: H) (TABLE 5). Equal amounts of RNA transcripts of five of these constructs and the original pS52/JFH1 and pFL-J6/JFH were transfected into Huh7.5 cells (FIG. 5A). Following transfection, all five recombinant S52/JFH1 viruses infected, like J6/JFH viruses, 50% and 90% of the cells on day 3 and day 7, respectively, as measured by anti-Core staining. However, following transfection with RNA transcripts of the original pS52/JFH1, the percentage of infected cells decreased during the period of observation as previously observed. Peak infectivity titers of the different recombinant S52/JFH1 viruses were comparable to that of J6/JFH viruses, whereas the original S52/JFH1 viruses showed significantly lower titers, which decreased during the observation period and became undeterminable on day 9 post-transfection (FIG. 5B).

To examine, whether recombinant adapted S52/JFH1 viruses were genetically stable, the present inventors performed a 1st viral passage by transferring 1ml of filtered supernatant (~10^4 TCID_{50}; rescued at day 9 post-transfection; FIG. 5A) onto naïve Huh7.5 cells. Although spread appeared to be somewhat slower for the recombinant adapted S52/JFH1 and the J6/JFH viruses (FIG. 6A) compared to the polyclonal 2nd passage S52/JFH1 and control J6/JFH viruses (FIG. 3), as measured with staining of Core and NS5A, respectively, the present inventors did not observe differences in the HCV RNA titers (FIG. 3 and FIG 6B). For each of the adapted S52/JFH1 viruses, the present inventors demonstrated that the consensus sequence of nts 27-9445 including the entire ORF of the viral genomes present in 1st passage supernatants (derived on day 12; FIG. 6A) was identical to that of the parental plasmid used to generate the in vitro RNA transcripts for the transfection experiment. Thus, as was the case for J6/JFH cell culture derived viruses (see above), adapted S52/JFH1 viruses were genetically stable during transfection and 1st viral passage and did not require additional mutations for efficient growth in Huh7.5 cells.

To determine whether all mutations of each combination tested were required for S52/JFH1 viability we generated two S52/JFH1 recombinants with only two
mutations: S52/JFH1(T2718G;T7160C) (SEQ ID NO: 19) (Figure 7) and S52/JFH1(A2721G;A4845T) (SEQ ID NO: 11) (Figure 5, 6, 7).

We further developed seven S52/JFH1 recombinants containing the mutations identified during the first transfection experiment individually (Table 5A,B). On day 7 most cells of the cultures transfected with the two recombinants with two mutations each were HCV Core positive (data not shown) and showed relatively high infectivity titers (Figure 7). In contrast, for recombinants with single mutations we observed significant variations in viral spread (data not shown) and infectivity titers (Figure 7). To investigate the genetic stability of these genomes, we infected naïve Huh7.5 cells with day 7 supernatant and directly sequenced nts 297-9445 of 1st passage viral genomes, when most cells in these cultures had become HCV Core positive and yielded an HCV RNA titer of 10^7 IIL/ml. For recombinants containing single mutations in Core (T728C) or E2 (A1553G and A1907C), sequence analysis was performed on genomes derived from the transfection culture because of the long eclipse phase preceding viral spread (25, 13 and 13 days post-transfection, respectively) reducing the risk of obtaining input DNA/RNA sequences. Sequence analysis showed that in contrast to the two recombinants with combinations of two mutations (and the four recombinants with three or more mutations) only 1/7 recombinants with single mutations (T2718G in p7) (SEQ ID NO: 15) was genetically stable, although a minor quasispecies was detected at nt position 4552, which would lead to an aa change at the same position as A4550C, observed in the 2nd transfection experiment (Table 5A,B). In contrast, 6/7 recombinants with single mutations acquired additional nucleotide changes (present at least as a 50/50 quasispecies), notably frequently at positions, which also had acquired changes during the original experiments (Table 5A,B). Analysis of these mutation patterns showed a preference for the combination of mutations in p7 with a second mutation in either NS3 or NS5A. For example, S52/JFH1(T728C) acquired T2718G in p7 and A4550C in NS3, and two independent transfections with S52/JFH1(T716OC) resulted in the additional changes T2718G and T2718C, respectively.

We finally analyzed mutations identified in the 2nd transfection experiment. S52/JFH1 recombinants with A4550C in NS3, singly (SEQ ID NO: 17) or in combination with T2718C in p7, yielded infectivity titers of >10^4 TCID_{50}/ml on day
7 post-transfection (Figure 7). These two viruses were also genetically stable after 1st passage, whereas the recombinant with the single p7 mutation T2718C had a lower infectivity titer at day 7 (Figure 7) and acquired additional mutations (Table 5A,B).

Overall, 7/7 recombinants with combinations of the nine mutations identified in S52/JFH1, but only 2/9 recombinants with single mutations yielded relatively high infectivity titers 7 days post-transfection, and were genetically stable after a 1st viral passage. Single mutations able to confer viability to S52/JFH1 without an apparent requirement for additional mutations were T2718G (I793S) in p7 (SEQ ID NO: 15) and A4550C (K1404Q) in NS3 (SEQ ID NO: 17).

Because T2718G in p7 and A4550C in NS3 were the only adaptive mutations able to individually confer cell culture adaptation of S52/JFH1. S52/JFH1(T2718G; A4550C) (SEQ ID NO: 13) was constructed in order to test if combination of T2718G and A4550C on one S52/JFH1 genome was possible. After transfection and passage in Huh7.5 cells S52/JFH1(T2718G; A4550C) viruses yielded infectivity titers between 10^4 and 10^5 TCID_{50}/mL; additionally, direct sequencing of the complete ORF of S52/JFH1(T2718G; A4550C) genomes revealed that these viruses were genetically stable after passage in Huh7.5 cells.

Thus, it was shown that the S52/JFH1(T2718G; A4550C) genome, combining the two adaptive mutations, which were able to individually provide adaptation of the S52/JFH1 genome, is viable and efficient in Huh7.5 cell culture.

Example 7

HCV genotype 3a infection depends on CD81

It has previously been shown that HCV genotype 1a and 2a infection can be inhibited by blocking the tetraspanin and putative HCV co-receptor CD81 with specific antibodies. We examined whether genotype 3a entry also depended on CD81 and whether recombinant S52/JFH1 containing the authentic E1/E2 proteins could be used as a model system to study HCV entry events and interfering agents in a genotype 3a specific manner. Pretreatment of Huh7.5 cells with anti-CD81 could prevent infection with 100 TCID_{50} of
S52/JFH1(T728C;T2718G;T7160C) as determined by the number of FFU (Figure 8A) and HCV RNA titers (Figure 8B).

Example 8

Infection with S52/JFH1 recombinants leads to redistribution of intracellular lipid droplets and co-localization with HCV Core

HCV genotype 3a infection is thought to be associated with increased hepatic steatosis. HCV Core co-localized with lipid droplets and recombinantly expressed genotype 3a Core was implicated in increased cellular lipid accumulation in vitro. However, these effects have not been studied on cells infected with HCV viruses expressing genotype 3a Core. We infected Huh7.5 cells with either J6/JFH, S52/JFH1 polyclonal 2nd passage viruses, S52/JFH1(T728C;T2718G;T7160C) or S52/JFH1(T2718G;T7160C). Confocal imaging revealed that in all HCV infected cells, in comparison to negative control cells mock infected with J6/JFH(GND) or S52/JFH1(GND) culture supernatants, lipid droplets were often redistributed from the entire cytoplasm to a perinuclear area, typically asymmetrically centered on one side of the nucleus, and co-localized with HCV Core. By confocal microscopy based image analysis we determined the average lipid content per cell in cultures with most cells being HCV positive and with HCV RNA titers of $10^7$ IU/ml in culture supernatant. We could not detect any difference in lipid content between cells infected with the different viruses and mock infected cells. The Core mutation at aa 130, which as a single mutation was found not to influence viability of S52/JFH1, did not appear to influence lipid co-localization with S52 Core or lipid accumulation.

Example 9

S52/JFH1 infection depends on SR-BI

The inventors of the present invention have previously shown that S52/JFH1 infection of Huh7.5 cells was dependent on CD81 (see the previous examples). SR-BI is thought to be part of the HCV receptor complex. Using anti-SR-BI serum the present inventors showed that S52/JFH1(T2718G;A4550C) infection of Huh7.5 cells as well as infection with the reference virus J6/JFH1 depends on SR-BI.
(Figure 9). This further underlines the importance of the developed model system for studies of HCV genotype 3a entry.

Example 10

S52/JFH1 infection is sensitive to treatment with interferon. HCV cell culture systems could be important tools for testing of antiviral therapeutics. Previously, replication of different HCV replicons as well as of the J6/JFH1 virus have been shown to be sensitive to treatment with interferon. Thus Huh7.5 cell cultures infected with J6/JFH1, S52/JFH1(T2718G; A4550C) and S52/JFH1(A4550C) were treated with cell culture medium containing 500IU/ml interferon alfa 2b. After initiation of treatment, a fast decline of HCV antigen positive cells was shown for all cultures. Thus, for J6/JFH1 and S52/JFH1(T2718G; A4550C) the percentage of NS5A positive cells was 90 and 60 the day prior to treatment, whereas only 20% and 10% NS5A positive cells were detected in the respective cultures on day 3 of treatment (Figure 10). Interestingly, prolonged treatment intervals were followed by an increase in NS5A positive cells; thus, on day 6, when the cultures had not been treated for 48 hrs, 40% of cells in the J6/JFH1 and S52/JFH1(T2718G; A4550C) cultures were NS5A positive.
Figure legends

Figure 1

Infection of Huh7.5 cells with intragenotypic 2a/2a and intergenotypic 3a/2a recombinants of HCV. (A). Transfection of Huh7.5 cells with J6/JFH (diamonds) and S52/JFH1 (circles). Huh7.5 cells were transfected with 2.5 µg of RNA transcripts with Lipofectamine 2000. Cells were split every 2 to 3 days and stained for NS5A with anti-NS5A, 9E10 antibody and horseradish peroxidase coupled secondary antibody. The percentage of infected cells was determined by light microscopy. RNA transcripts of pFL-J6/JFH(GND) (squares) served as negative replication control. Arrows indicate supernatants used for 1st viral passages. (B). 1st passage of S52/JFH1 viruses in naïve Huh7.5 cells. Huh7.5 cells were incubated for 48hrs with filter cleared cell culture supernatant from day 11 post-transfection (FIG. 1A), containing 10^{1.8} TCID_{50} of S52/JFH1. Left y-axis: Percentage of NS5A positive cells. Arrow: Supernatant used to inoculate the 2nd viral passage. Right y-axis: HCV RNA titers in supernatants determined by an HCV TaqMan assay. nd; not detected. (C). 2nd passage of S52/JFH1 viruses in naïve Huh7.5 cells. 1st passage supernatant from day 21 post-infection (FIG. 1B) containing 10^{2.4} TCID_{50} was used for infection for 24hrs. On day 11 post-infection cells were plated on chamber slides and stained for NS5A. For comparison, a typical result with Huh7.5 cells inoculated with supernatant from a J6/JFH(GND) transfected culture is shown. Pictures were taken using a Leica confocal microscope at 2Ox magnification.

Figure 2

Infection of Huh7.5 cells with J6/JFH (diamonds) and S52/JFH1 (circles). (A): Transfection of Huh7.5 cells with RNA transcripts. The percentage of infected Huh7.5 cells was determined by NS5A staining. RNA transcripts of pS52/JFH1(GND) (squares) served as negative replication control. Arrow: S52/JFH1 supernatant used for inoculation of the 1st viral passage. (B) 1st passage of S52/JFH1 viruses in naïve Huh7.5 cells. Huh7.5 cells were incubated for 24hrs with filtered day 33 supernatant (FIG. 2A) containing 10^{2.8} TCID_{50}. Supernatant derived from cells transfected with S52/JFH1(GND) served as
negative control. Left y-axis: Percentage of NS5A positive cells. Right y-axis: HCV RNA titers in supernatants. nd; not detected. See also Fig. 1 legend.

Figure 3
5 Comparison of S52/JFH1 (circles and filled bars) and J6/JFH (diamonds and open bars) growth kinetics. Huh7.5 cells were seeded in 6 well plates and incubated for 8hrs with \( \sim 10^4 \) TCID\textsubscript{50} of the respective virus. The inoculum contained S52/JFH1 viruses from day 11 of the 2nd viral passage (polyclonal virus sequence with adaptive mutations compared to pS52/JFH1; TABLE 5) and J6/JFH viruses recovered on day 8 of a 1st viral passage (the consensus sequence of J6/JFH viral genomes was identical to the J6/JFH plasmid sequence). Supernatant derived from J6/JFH1(GND) cultures (squares) served as negative control. The infectivity titers of the inocula were determined as shown in TABLE 4. Left y-axis: Percentage of NS5A positive cells (closed symbols). Right y-axis: HCV RNA titers in supernatants determined in an HCV TaqMan assay (open symbols). nd; not detected. Y-axis on the extreme right: TCID\textsubscript{50} values determined by testing replicates of 10-fold dilutions as described in Material and Methods (bars).

Figure 4
20 Dose dependence of S52/JFH1 growth kinetics. Huh7.5 cells were infected with \( 10^4 \) (same experiment as in FIG. 3), \( 10^3 \), and \( 10^{2.5} \) TCID\textsubscript{50} of S52/JFH1 2nd passage viruses, respectively. Left y-axis: Percentage of NS5A positive cells. Right y-axis: HCV RNA titers in supernatants determined in an HCV TaqMan assay, nd; not detected.

Figure 5
Transfection of Huh7.5 cells with adapted S52/JFH1 recombinants ( SEQ ID NO: 3, 5, 7, 9, 11). (A) After immuno-staining for Core antigen the percentage of infected cells was estimated with confocal fluorescence microscopy (Leica). Arrow: Supernatants used for inoculation of the 1st viral passages (FIG. 6). (B) Comparison of TCID\textsubscript{50} values of viruses recovered at days 3, 7 and 9 post-transfection. *One of 6 replicate wells was showing infection when incubated with undiluted supernatant derived on day 9 from cells transfected with RNA
transcripts of pS52/JFH1 resulting in a non-determinable TCID50. # None of 6 replicate wells was showing infection when incubated with undiluted supernatant derived on day 3, 7, and 9 from Huh7.5 cells transfected with RNA transcripts of pS52/JFH1(GND). P7_2718 indicates the nucleotide change T2718G.

Figure 6
First passage of five adapted S52/JFH1 viruses in naïve Huh7.5 cells. Huh7.5 cells were incubated for 24hrs with ImL (~10^4 TCID50) of filtered supernatant derived from day 9 of the cultures transfected with RNA transcripts of the five S52/JFH1 recombinants (SEQ ID NO: 3, 5, 7, 9, 11) (FIG. 5). (A) After immuno-staining for Core antigen, the percentage of infected cells was determined with confocal fluorescence microscopy (Leica). (B) HCV RNA titers in supernatants were determined by an HCV TaqMan assay. nd; not detected. (C) Twelve days post-infection cells were plated on chamber slides and subsequently stained with anti-Core primary and Alexa594 fluorochrome coupled secondary antibody. Cell nuclei were counterstained with Hoechst reagent. Pictures were taken using a Leica confocal fluorescence microscope. P7_2718 indicates the nucleotide change T2718G.

Figure 7
Infectivity titers after transfection of Huh7.5 cells with S52/JFH1 recombinants with putative adaptive mutations identified in the 1st and 2nd transfection experiment (Table 5). TCID50 values were determined on day 7 supernatant. Data were generated in 5 different experiments (including experiment shown in Figure 5); S52/JFH1(T728C;T2718G;T7160C) (SEQ ID NO: 7) was included in five, S52/JFH1 in four, and S52/JFH1(T7160C) in three experiments. *One/#None of 6 replicate wells infected after incubation with undiluted supernatant.

Figure 8
S52/JFH1 infection depends on CD81. After pre-incubation with anti-CD81 or isotype matched control antibody (anti-p24), 6xIo^3 Huh7.5 cells/well of a 96 well dish were inoculated with 100 TCID50 of S52/JFH1(T728C;T2718G;T7160C). A: Numbers of focus forming units (FFU) per well determined by HCV NS5A staining on day 3. Mean values calculated from four independent infections, error bars
indicate standard errors of the mean. B: HCV RNA titers were determined on pooled supernatants from the four different infection experiments on day 3. *no FFL) observed, nd; not detected.

Figure 9

S52/JFH1 infection depends on SR-BI. After 1 hr pre-incubation with indicated dilutions of anti-SR-BI serum or control serum (Qr), 6 x 10^3 Huh7.5 cells/well of a 96-well dish were inoculated with 150 focus forming units (FFU) of S52/JFH1(T2718G;A4550C) or J6/JFH1. The number of FFUs per well was determined by HCV NS5A staining on day 2. % inhibition was calculated referring to the mean FFU count of non-treated but with the respective virus infected wells. Mean values were calculated from 3 independent infections, error bars indicate standard errors of the mean.

Figure 10

S52/JFH1 viruses are sensitive to treatment with Interferon alfa. 3x10^6 Huh7.5 cells were infected with 10^4 TCID_{50} (MOI 0.003) of the indicated viruses. On day 5 after infection, cultures were evaluated for % of NS5A positive cells (first datapoint) and 4x10^5 cells of the respective culture were plated per well of a 6-well dish. After 24 hrs incubation, treatment with cell culture medium containing 500IU/ml _ interferon alfa 2b was started. Interferon containing medium was replaced at the time points indicated by arrows. Cultures were evaluated for the % of NS5A positive cells at the indicated timepoints.
Tables

TABLE 1

Primers used for amplification of HCV strain S52 (genotype 3a) and generation of amplicons for analysis of S52/JFH1 5’ and 3’ UTR.

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<th>Cloning primers</th>
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<td>HCV3aF1T7NotI-G (SEQ ID NO: 23)</td>
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Underlined: introduced restriction sites. Italics: T7 promoter.
TABLE 2

Primers used for S52/JFH1 long RT-PCR procedure to generate amplicons for direct sequencing of the open reading frame.

<table>
<thead>
<tr>
<th>Amplification step and amplicon</th>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis</td>
<td>9470R(24)_JF H1 (SEQ ID NO: 27)</td>
<td>5’-CTATGGAGTGTACCTAGTGATGC-3’</td>
</tr>
<tr>
<td>1st round PCR</td>
<td>-285S_HCV-MOD (SEQ ID NO: 31) 9470R(24)_JF H1 (SEQ ID NO: 27)</td>
<td>5’-ACTGTTCTCCAGCAGAAAGCGCTAGCCAT-3’ 5’-CTATGGAGTGTACCTAGTGATGC-3’</td>
</tr>
<tr>
<td>2nd round PCR</td>
<td>-84S_HCV-MOD (SEQ ID NO: 39) HCV3aCoSeR1072 (SEQ ID NO: 28)</td>
<td>5’-GTAGCGTTGGGGTTGCAAGGCGCTGGTGATGCTGAT-3’ 5’-GTAGGTGTCACTGCGGGGTCACGC-3’</td>
</tr>
<tr>
<td>Amplicon 1</td>
<td>HCV3aCoSeF918 (SEQ ID NO: 40) HCV3aCoSeR1819 (SEQ ID NO: 41)</td>
<td>5’-GTGGCGGAATACGTCTGGCCTC-3’ 5’-GTCTAGGTGCGTAGTCGTCCAGCAG-3’</td>
</tr>
<tr>
<td>Amplicon 2</td>
<td>HCV3aCoSeF1288 (SEQ ID NO: 42)</td>
<td>5’-CGAATGGCCTGGGATATGATGATGA-3’</td>
</tr>
<tr>
<td><strong>Amplicon 4</strong></td>
<td></td>
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<tr>
<td>----------------</td>
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<tr>
<td>HCV3aCoSeF2</td>
<td>5' - GCAACTGGACCAGGGGGAGC-3'</td>
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<td>288 (SEQ ID NO: 44)</td>
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<tr>
<td>HCV3aCoSeR3</td>
<td>5' - TCCCGATAGTCATCAGCAGGTCC-3'</td>
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<td>416 (SEQ ID NO: 45)</td>
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<table>
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<tr>
<th><strong>Amplicon 5</strong></th>
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<tbody>
<tr>
<td>HCV3aCoSeF3</td>
<td>5' - CGGAGATATTTCCTTCGCGGCTGC-3'</td>
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<tr>
<td>336 (SEQ ID NO: 46)</td>
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<tr>
<td>4118R_JFH1</td>
<td>5' - CGCCCGAGGCCTACCTTTCTATATC-3'</td>
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</tr>
<tr>
<td>(SEQ ID NO: 47)</td>
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<table>
<thead>
<tr>
<th><strong>Amplicon 6</strong></th>
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</thead>
<tbody>
<tr>
<td>3880S_J6</td>
<td>5' - CCCATCACGTACTCCACATATGGC-3'</td>
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<td>(SEQ ID NO: 48)</td>
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<tr>
<td>4796R_JFH1</td>
<td>5' - GCGCACACCGGTAGCTTGATTAGG-3'</td>
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<td>(SEQ ID NO: 49)</td>
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<table>
<thead>
<tr>
<th><strong>Amplicon 7</strong></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4528S_J6</td>
<td>5' - GAGCGAGCCTAGGAATTTTGACA-3'</td>
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<tr>
<td>(SEQ ID NO: 50)</td>
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<td></td>
</tr>
<tr>
<td>5446R_JFH1</td>
<td>5' - TGGATTTGAGAAGGATGGTGATAC-3'</td>
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</tr>
<tr>
<td>(SEQ ID NO: 51)</td>
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<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th><strong>Amplicon 8</strong></th>
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</thead>
<tbody>
<tr>
<td>5272S_JFH1</td>
<td>5' - TGGGCCAAAGTTGGAACATTGG-3'</td>
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<tr>
<td>(SEQ ID NO: 52)</td>
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<td></td>
</tr>
<tr>
<td>6460R_J6</td>
<td>5' - CAACGCAGAAGGAGACCTATTC-3'</td>
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<td>(SEQ ID NO: 53)</td>
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<table>
<thead>
<tr>
<th><strong>Amplicon 9</strong></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>6186S_JFH1</td>
<td>5' - GACCTTCTCATCAATTTGCTACAC-3'</td>
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</tr>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
| Amplicon 10 | 6862S_JFH1 (SEQ ID NO: 55) | 7234R_JFH1 (SEQ ID NO: 35) | 5'-GAAGCTCTACCTGATCAGACTCCA-3'  
| Amplicon 11 | 7741S_J6 (SEQ ID NO: 56)  
| Amplicon 12 | 8137S_JFH1 (SEQ ID NO: 58)  
|            | 7848R_JFH1 (SEQ ID NO: 30) | 5'-TGGGCACGGCCCTGACTACCA-3'  
|            | 8703R_JFH1 (SEQ ID NO: 57) | 5'-GGCCATTTTCTCGCAGACCCGGAC-3'  
|            | 9464R(24)_JFH1 (SEQ ID NO: 59) | 5'-ATGGCCAAAAATGAGGTGTCTGC-3'  
|            |                         | 5'-AAGGTCCAAGGATTCACGGAGTA-3'  
|            |                         | 5'-GGTCAAACCTCGGGTTACAGACGG-3'  
|            |                         | 5'-GTGTACCTAGTGTGTGCCCGCTCTA-3'  

TABLE 3

Primers used for J6/JFH long RT-PCR procedure to generate amplicons for direct sequencing of the open reading frame. *

<table>
<thead>
<tr>
<th>Amplification step and amplicon</th>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd round PCR</td>
<td>-84S_HCV-MOD (SEQ ID NO: 39) 1109R_J6 (SEQ ID NO: 60)</td>
<td>5’-GTAGCGTGGGTTGCGAAGGCCCTTGTTGACTGCTGAT-3’ 5’-TTTGCCCAAGCTCCCTGCAATGAGAA-3’</td>
</tr>
<tr>
<td>Amplicon 1</td>
<td>946S_J6 (SEQ ID NO: 61) 2111R_J6 (SEQ ID NO: 62)</td>
<td>5’-CACCACCATGGCGTGACATGATG-3’ 5’-TGACACGTCCACGATGTTTTGATG-3’</td>
</tr>
<tr>
<td>Amplicon 2</td>
<td>1849S_J6 (SEQ ID NO: 63) 2763R_J6 (SEQ ID NO: 64)</td>
<td>5’-TACAGGCTCTGGCCATTACCCCTGCAC-3’ 5’-AGCGTGGAGCCCTGACGAAGTACGG-3’</td>
</tr>
<tr>
<td>Amplicon 3</td>
<td>2754S_J6 (SEQ ID NO: 65) 3774R_J6 (SEQ ID NO: 66)</td>
<td>5’-TAGCATTGCCCCAACCAGGGCTTGATGTTATGACG-3’ 5’-GGGATGACATCGCGTCCCGGGAACAG-3’</td>
</tr>
<tr>
<td>Amplicon 4</td>
<td>3081S_J6/JFH 1 (SEQ ID NO: 67) 4118R_JFH</td>
<td>5’-GGAGGTCTTTCTCGCTCCCATCACTGC-3’ 5’-CGCCCGAGGCCGTACCTTCTATATC-3’</td>
</tr>
</tbody>
</table>
*For cDNA synthesis, 1st round PCR, and 2nd round PCR amplicons 6-12 see TABLE 2.
TABLE 4

Representative infectivity and HCV RNA titers of J6/JFH and S52/JFH1 cultures.

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>Origin of supernatant</th>
<th>Experimental day</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;/ml</th>
<th>% infectd cells</th>
<th>HCV RNA titer (IU/ml)</th>
<th>Specific infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>J6/JFH</td>
<td>1st viral passage (used for kinetic experiment)</td>
<td>8</td>
<td>10&lt;sup&gt;4.6+&lt;/sup&gt;</td>
<td>95</td>
<td>10&lt;sup&gt;7.2&lt;/sup&gt;</td>
<td>1:398</td>
</tr>
<tr>
<td>J6/JFH</td>
<td>2nd viral passage (inoculated with 10&lt;sup&gt;4&lt;/sup&gt; TCID&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>3</td>
<td>10&lt;sup&gt;4.7+&lt;/sup&gt;</td>
<td>80</td>
<td>10&lt;sup&gt;6.0&lt;/sup&gt;</td>
<td>1:20</td>
</tr>
<tr>
<td>S52/JFH H1</td>
<td>First experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S52/JFH 1</td>
<td>Transfection</td>
<td>11</td>
<td>10&lt;sup&gt;2.3+&lt;/sup&gt;</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S52/JFH 1</td>
<td>2nd viral passage (used for kinetic experiment)</td>
<td>11</td>
<td>10&lt;sup&gt;4.2+&lt;/sup&gt;</td>
<td>95</td>
<td>10&lt;sup&gt;6.9&lt;/sup&gt;</td>
<td>1:501</td>
</tr>
<tr>
<td>S52/JFH 1</td>
<td>3rd viral passage (inoculated with 10&lt;sup&gt;4&lt;/sup&gt; TCID&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>3</td>
<td>10&lt;sup&gt;4.6+&lt;/sup&gt;</td>
<td>90</td>
<td>10&lt;sup&gt;6.0&lt;/sup&gt;</td>
<td>1:25</td>
</tr>
<tr>
<td>S52/JFH 1</td>
<td>3rd viral passage (inoculated with 10&lt;sup&gt;2.5&lt;/sup&gt; TCID&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>12</td>
<td>10&lt;sup&gt;4.7+&lt;/sup&gt;</td>
<td>95</td>
<td>10&lt;sup&gt;6.6&lt;/sup&gt;</td>
<td>1:79</td>
</tr>
<tr>
<td>S52/JFH H1</td>
<td>Second experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S52/JFH 1</td>
<td>Transfection</td>
<td>33</td>
<td>10&lt;sup&gt;3.2+&lt;/sup&gt;</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S52/JFH 1</td>
<td>1st viral passage</td>
<td>8</td>
<td>10&lt;sup&gt;4.3+&lt;/sup&gt;</td>
<td>95</td>
<td>10&lt;sup&gt;7.2&lt;/sup&gt;</td>
<td>1:501</td>
</tr>
</tbody>
</table>

5 For determination of TCID<sub>50</sub> Huh7.5 cells were plated at 6 x 10<sup>3</sup> cells/well on a poly-D-Lysine coated 96 well plate and infected with serial dilutions of virus
containing cell culture supernatant in complete growth medium in replicates of 6
wells per dilution. After incubation for 48 hours cells were stained for NS5A
expression. A well was counted positive, if at least one infected cell was visible.
Calculation of TCID<sub>50</sub> was done as previously described by Reed et al.

Average of 3 independent determinations, * average of 2 independent
determinations, + 1 determination. HCV RNA titers were determined with an
taqMan assay. Supernatants from transfection experiments were not tested in
taqMan due to DNA/RNA input from the transfection procedure. Specific infectivity
was calculated as infectious units (TCID<sub>50</sub>/ml) per genome number (IU/ml).

TABLE 5 (below)

Adaptive nucleotide (A) and amino acid mutations (B) acquired by S52/JFH1
viruses during viral passage.

Direct sequence analysis was performed on viruses recovered from the first
transfection experiment and the consecutive 1st, 2nd and 3rd viral passages
(Table 4; Figure 1; Figure 4); on 1st viral passage viruses from the second
transfection experiment (Table 4, Figure 2); and on mutated viruses after 1st
passage or transfection as indicated. Clonal analysis was performed on 2nd
passage virus from the 1st transfection; all clones had in addition to the
mutations shown changes at other positions, which could partly have resulted
from errors in PCR. At the positions with evidence of mutations the S52 consensus
sequence had the original pS52/JFH1 sequence with no heterogeneity among 5
clones. Capital letters indicate the presence of one determinate sequence peak.

Two capital letters separated by a slash indicate the presence of a 50/50
quasispecies, whereas a capital letter separated by a slash from a lowercase letter
indicates a quasispecies with a predominant versus a minor sequence. Criterion
for listing of nt positions was the occurrence of a quasispecies in direct sequencing
with the new sequence showing at least a 50/50 distribution in at least one
experiment. Names of adapted S52/JFH1 recombinants engineered to contain
mutations singly or in combination refer to the respective nt changes. # additional
mutation C64T in the 5' LTR. Grey shading: engineered nt and deduced aa
changes. • nt/aa identical with pS52/JFH1 sequence. Numbers of nt/aa positions
refer to pS52/JFH1. * nt/aa position in analogy to the H77 reference genome
(accession number AF009606) determined as described. For aa positions the absolute (abs. ref. num.; referring to H77 polyprotein) and relative (rel. ref. num.; referring to the individual H77 protein) reference numbers are given.
<table>
<thead>
<tr>
<th>Core</th>
<th>E2</th>
<th>p7</th>
<th>NS2</th>
<th>NS3</th>
<th>NS4A</th>
<th>NS5A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide position S52/JFH1</td>
<td>728</td>
<td>1527</td>
<td>1553</td>
<td>1907</td>
<td>2297</td>
<td>2718</td>
</tr>
<tr>
<td>Nucleotide position H77 (A700960G)</td>
<td>729</td>
<td>1528</td>
<td>1554</td>
<td>1905</td>
<td>2280</td>
<td>2701</td>
</tr>
</tbody>
</table>

**Sequence of p52/JFH1**

| T | C | A | A | T | T | A | A | A | G | A | A | A | C | A | T |

Sequence of viruses recovered from infected Huh7.5 cells

---

**First transfection experiment with S52/JFH1**

**Direct sequencing**

- Transfection, day 11
- 1st passage, day 21
- 2nd passage, day 11
- 3rd passage: inoculum 1E2.5 TCD50, day 12
- 3rd passage: inoculum 1E4 TCD50, day 3

**Clonal sequence analysis of 2nd passage virus**

- clone 5, 12 and 17
- clone 6, 13 and 18
- clone 11
- clone 2
- clone 1

**Transfection experiment with mutated S52/JFH1**

- SEQ ID NO 3: T728C;A1553G;A1907C;T2718G;T7160C
- SEQ ID NO 5: T728C;A1553G;A1907C;A2721G;T7160C
- SEQ ID NO 7: T728C;T2718G;T7160C
- SEQ ID NO 9: A1553G;A2721G;A4845T: 1st passage
- SEQ ID NO 19: T7218G;T7160C: 1st passage
- SEQ ID NO 11: A2721G;A4845T: 1st passage

**Transfection, day 32**

- T728C: Transfection, day 32
- A1553G: Transfection, day 24
- A1907C: Transfection, day 24
- SEQ ID NO 15: T7218G: 1st passage
- A2721G: 1st passage
- A4845T: 1st passage
- T7160C: 1st passage

**Second transfection experiment with S52/JFH1**

**Direct sequencing**

- 1st passage, day 8

**Transfection experiment with mutated S52/JFH1**

- SEQ ID NO 21: T7218C;A4550C: 1st passage
- T7218C: 1st passage
- SEQ ID NO 17: A4550C: 1st passage
<table>
<thead>
<tr>
<th>Core</th>
<th>E3</th>
<th>p7</th>
<th>NS2</th>
<th>NS3</th>
<th>NS4A/NS5A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid position S52/JFH1</td>
<td>130 396 405 523 653 793 794</td>
<td>985 1012 1036 1074 1104 1129 1136</td>
<td>1375</td>
<td>1404</td>
<td>1502 1559 1698 1774 2274</td>
</tr>
<tr>
<td>Amino acid position H77 (abs. ref. num.) *</td>
<td>130 396 405 522 647 787 788</td>
<td>889 1130 1369 1398</td>
<td>1496</td>
<td>1683 2270 2272</td>
<td></td>
</tr>
<tr>
<td>Amino acid position H77 (rel. ref. num.) *</td>
<td>130 13 22 139 264 41</td>
<td>42 80 104 343 372 470 26 298 300</td>
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<td></td>
</tr>
</tbody>
</table>

**Sequence of S52/JFH1 polyprotein**

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<tr>
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<th>A</th>
<th>M</th>
<th>K</th>
<th>T</th>
<th>I</th>
<th>Y</th>
<th>T</th>
<th>L</th>
<th>R</th>
<th>K</th>
<th>Q</th>
<th>I</th>
<th>I</th>
<th>S</th>
</tr>
</thead>
</table>
| **Sequence of viruses recovered from infected Vii7.5 cells**

**First transfection experiment with S52/JFH1**

Direct sequencing

<table>
<thead>
<tr>
<th>Transfection, day 11</th>
<th>L/F</th>
<th>L/V</th>
<th>L/I</th>
<th>Q/Y</th>
<th>L/Q</th>
<th>P/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st passage, day 21</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>2nd passage, day 11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd passage, inoculum 1E2.5 TCID50, day 12</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3rd passage, inoculum 1E4 TCID50, day 3</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Clonal sequence analysis of 2nd passage virus

| clone 5, 12 and 17 | L | S |     |     |     |     |     |     |     |     |     |     |     |     |
| clone 6, 13 and 18 | L | V | Q |     |     |     |     |     |     |     |     |     |     |     |
| clone 11          | L | V |     | S |     |     |     |     |     |     |     |     |     |     |
| clone 2           | L | V | Q |     | C |     |     |     |     |     |     |     |     |     |
| clone 1           | L | V |     | C |     |     |     |     |     |     |     |     |     |     |

**Second transfection experiment with S52/JFH1**

Direct sequencing

<table>
<thead>
<tr>
<th>Transfection, day 8</th>
<th></th>
<th>T/I</th>
<th></th>
<th></th>
<th>Q</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1st passage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Transfection experiment with mutated S52/JFH1**

| SEQ ID NO 1 T272G,A1553G,A1907C |     | V |     | N/Y | L |     |
| SEQ ID NO 1 T272G,A1553G,A1907C,A272G |     | V | Q |     | P/T | Q/R | K/N |
| SEQ ID NO 1 T2718G,A4845T |     | S |     | C |     |     |     |
| SEQ ID NO 1 T2718G,A4845T |     | V/a | S/I |     | L |     |
| SEQ ID NO 20 T2718G,A4845T |     |     | S/I | T/I |     |     |     |     |     |     |     |     |     |     |
| SEQ ID NO 20 T2718G,A4845T |     |     | S/I | T/I |     |     |     |     |     |     |     |     |     |     |
| SEQ ID NO 20 T2718G,A4845T |     |     | S/I | T/I |     |     |     |     |     |     |     |     |     |     |
| SEQ ID NO 20 T2718G,A4845T |     |     | S/I | T/I |     |     |     |     |     |     |     |     |     |     |

**Second transfection experiment with S52/JFH1**

Direct sequencing

<table>
<thead>
<tr>
<th>1st passage, day 8</th>
<th></th>
<th>T/I</th>
<th></th>
<th></th>
<th>Q</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1st passage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Transfection experiment with mutated S52/JFH1**

| SEQ ID NO 22 T2718G,A4550C |     | T |     | Q |     |
| SEQ ID NO 22 T2718G,A4550C |     |     | T |     | K/N | L/I |     |     |     |     |     |     |     |     |
| SEQ ID NO 18 A4550C |     |     |     |     | Q |     |     |     |     |     |     |     |     |     |     |
References


**Claims**

1. A replicating RNA comprising the structural genes (Core, E1, E2), p7 and non-structural genes NS2 of genotype 3a and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B from the JFH1 strain.

2. A replicating RNA according to claim 1, wherein the structural genes originates from genotype 3a strain S52.

3. An isolated nucleic acid molecule which encodes human hepatitis C virus of genotype 3a/JFH1 (e.g. S52/JFH1), wherein said molecule is capable of expressing said virus when transfected into cells and further capable of infectivity *in vivo* and wherein said molecule encodes the amino acid sequence with at least 90% sequence identity to that of SEQ ID NO: 2.

4. A nucleic acid molecule according to claim 3, wherein said molecule comprises at least 90% sequence identity to that of SEQ ID NO: 1.

5. A nucleic acid molecule according to any of claims 1-4, where in said molecule comprises adaptive mutations in Core, E2, p7, NS3 and NS5A singly or in combination.


7. A nucleic acid molecule according to claim 5, wherein said adaptive mutation is at least one of the replacements of the first said amino acid at the said position of SEQ ID NO: 2 by the following said amino acid selected from the group consisting of F130L, I162V, V284A, A396V, M405V, K523Q, N533D, T653S, I793S, I793T,
Y794C, Y794N, C859Y, T895P, R1375L, R1375Q, K1404N, K1404Q, Q1502L, I2272L, S2274P and S2281N.

8. A nucleic acid molecule according to any of claims 3-7, 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.

9. A nucleic acid molecule according to any of claims 2-11, wherein said molecule, following transfection and/or subsequent viral passage, is capable of generating a HCV infectivity titer of $10^2$ TCID$_{50}$/ml or above.

10. A composition comprising a nucleic acid molecule according to any of claims 3-9 suspended in a suitable amount of a pharmaceutical acceptable diluent or excipient.

11. A cassette vector for cloning viral genomes, comprising, inserted therein, the nucleic acid sequence according to any of claims 3-9 and having an active promoter upstream thereof.

12. A method for producing a cell which replicates HCV 3a/JFH1 RNA and produces a virus particle comprising introducing the said RNA according to claim 1-2 into a cell.

13. A method according to claim 12, wherein the cell is Huh7.5.


15. A method for producing a hepatitis C virus particle, comprising culturing the cell according to claim 12 to allow the cell to produce the virus.

16. A hepatitis C virus particle obtainable by the method according to claim 15.

17. A method for *in vitro* producing a hepatitis C virus-infected cell comprising culturing the cell according to claim 14 and infecting other cells with the produced virus particle in the culture.
18. A hepatitis C virus infected cell obtainable by the method according to claim 17.

19. 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 claim 14, a hepatitis C virus infected cell according to claim 18 and a hepatitis C virus particle obtainable by claim 15 together with a hepatitis C virus permissive cell, and

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

20. A hepatitis C vaccine comprising a hepatitis C virus particle according to claim 16 or a part thereof.

21. A method for producing a hepatitis C virus vaccine comprising using a hepatitis C virus particle obtained from claim 16 as an antigen.

22. An antibody against the hepatitis C virus particle according to claim 16.
Fig. 1
Fig. 2
Fig. 4
Fig. 5
Fig. 6
Fig. 8
Fig. 10
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) and the national classification and IPC

INV. C12N7/00 C12N7/02 C12N15/51 C12N5/10 C07K14/18
A61K39/29

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C07K A61K

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 practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>GOTTWEIN J M ET AL.: &quot;Robust Hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses&quot; GASTROENTEROLOGY, vol. 133, November 2007 (2007-11), pages 1614-1626, XP002461123 published online 3.8.2007. the whole document</td>
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Date of the actual completion of the international search

7 August 2008

Date of mailing of the international search report

19/08/2008

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl
Fax: (+31-70) 340-3016

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Galli, Ivo

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