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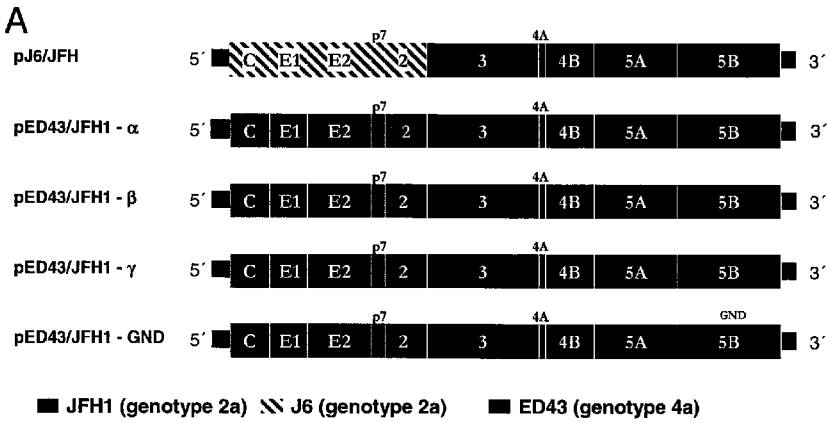
(54) Title: ADAPTIVE MUTATIONS ALLOW ESTABLISHMENT OF JFH1-BASED CELL CULTURE SYSTEMS FOR HEPATITIS C VIRUS GENOTYPE 4A



(57) Abstract: The present inventors developed three 4a/2a intergenotypic recombinants in which the JFHl structural genes (Core, El and E2), p7 and all of or part of NS2 were replaced by the corresponding genes of the genotype 4a reference strain ED43. The 4a/2a junction in NS2 was placed after the first transmembrane domain (α), in the cytoplasmic part (β) or at the NS2/NS3 cleavage site (y). Following transfection of Huh7.5 cells with RNA transcripts, infectious viruses were produced in the ED43/JFHl-β and -y cultures only. Compared to the 2a control virus, production of infectious viruses was significantly delayed. However, in subsequent passages efficient spread of infection and high HCV RNA titers were obtained. Infectivity titers were approximately 10-fold lower than for the 2a control virus. Sequence analysis of recovered 4a/2a recombinants from 3 serial passages and subsequent reverse genetic studies revealed a vital dependence on a mutation in the NS2 4a part. ED43/JFHl-γ further depended on a second NS2 mutation. Infectivity of the 4a/2a viruses was CD81 dependent. Conclusion : The developed 4a/2a viruses provide a robust in vitro tool for research in HCV genotype 4, including vaccine studies and functional analyses of an increasingly important genotype in the Middle East and Europe.

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ADAPTIVE MUTATIONS ALLOW ESTABLISHMENT OF JFH1-BASED CELL CULTURE SYSTEMS FOR HEPATITIS C VIRUS GENOTYPE 4A

Field of the invention

This invention provides infectious recombinant hepatitis C genotype 4 viruses (HCV), and vectors, cells and animals comprising the same. The present invention provides methods of producing the infectious recombinant HCV genotype 4, 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, is one of the most widespread infectious diseases in the world. About 170 million people are infected with hepatitis C virus (HCV) worldwide with a yearly incidence of 3-4 million. While the acute phase of infection is mostly asymptomatic, the majority of acutely infected individuals develops chronic hepatitis and is at increased risk of developing liver cirrhosis and hepatocellular carcinoma. Thus, HCV infection is a major contributor to end-stage liver disease and in developed countries to liver transplantation.

HCV is a small, enveloped virus classified as a member of the Flaviviridae family. Its genome consists of a 9.6 kb single stranded RNA of positive polarity composed of 5´ and 3´ untranslated regions (UTR) and one long open reading frame (ORF) encoding a polyprotein, which is co- and 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 in nucleotide and amino acid sequence composition by 31-35% (Bukh et al. 1993). In addition, there are numerous subtypes (a, b, c, etc.). In the Middle East, particularly in Egypt, up to 15% of the population are infected with HCV. From this geographic region HCV genotype 4 constitute about 90% of the cases diagnosed. The high prevalence of HCV genotype 4 and in particular HCV genotype 4a in Egypt is believed to be caused by unintended transmission to the

population through parenteral intervention against schistosomiasis. The prevalence HCV genotype 4 in Western countries has traditionally been low, but in certain European regions this genotype has been shown to be significant mainly among intravenous dug users. At present the incidences continues to increase.

The only approved therapy for HCV comprises a combination therapy with interferon and ribavirin. Such therapy 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 and 4. 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 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 *in vivo*.

In 2001, a genotype 2a isolate (JFH1) 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 JFH1 full-length consensus cDNA clone into Huh7 cells (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 JFH1 were replaced by the respective genes of pJ6CF, 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*.

Despite the importance of the described cell culture systems they represent only a single subtype (genotype 2a) of HCV. It is important to develop cell culture systems for representative strains of other HCV genotypes, 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.

Pietschmann et al. 2006 disclose construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus recombinants. The authors created a series of recombinant genomes allowing production of infectious genotype 1a, 1b, 2a and 3a particles by constructing hybrid genomes between the JFH1 isolate and the HCV isolates: H77 (genotype 1a), Con1 (genotype 1b), J6 (genotype 2a) and 452 (genotype 3a) respectively. Thus, disclosing both genotypes completely different from the genotype disclosed in the present application and relating to completely different strains of origin.

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 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 TCID50/mL contain insufficient amounts of infectious virus.

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 develop 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

In the present study, the inventors used the ED43 reference isolate (genotype 4a) to construct a viable, JFH1-based genome. The present inventors serially passaged ED43/JFH1 virus in cell culture, obtained relatively high HCV RNA titers and infectivity titers, and identified adaptive mutations required for efficient growth.

The present inventors have developed a robust cell culture system for HCV genotype 4a. This is an important advance for the study of HCV, since genotype 4a is highly prevalent especially in the Middle East, and since it permits detailed molecular studies of HCV and enhances the potential for developing broadly reactive reagents against HCV, including but not limited to small molecule drugs, antibodies and vaccines. Accordingly, the present invention may be used for individualised treatment of patients infected with one of the six major genotypes.

In one aspect the present invention relates to a replicating RNA comprising the structural genes (Core, E1, E2), p7 and the non-structural gene NS2 of genotype 4a and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B and part of NS2 from the JFH1 strain.

In another aspect the present invention pertains to an isolated nucleic acid molecule which encodes human hepatitis C virus of genotype 4a/JFH1, wherein said molecule is capable of expressing said virus when transfected into cells.

In yet another aspect the present invention pertains to a composition comprising a nucleic acid molecule according to the present invention, a cassette vector for cloning viral genomes, methods for producing a cell which replicates HCV 4a/JFH1 RNA and cells obtainable there from.

In another aspect the present invention pertains to methods for producing a hepatitis C virus particle, methods for *in vitro* producing a hepatitis C virus-infected cell.

In a further aspect the present invention pertains to methods for screening an anti-hepatitis C virus substance, hepatitis C vaccines comprising a hepatitis C

virus particle, methods for producing a hepatitis C virus vaccine and antibodies against hepatitis C virus.

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.

The present inventors have established the first cell culture system for studying HCV genotype 4. Except for the HCV retroviral pseudo particle system (HCVpp) incorporating E1 and E2 of genotype 4a isolates, this is the first functional in vitro model system for studying genotype 4, which is an increasingly important genotype dominating the Middle East, regions of Africa and currently spreading to Europe. This is a significant step in extending the current HCV cell culture systems to all six major genotypes.

Nucleic 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 (ED43/JFH1-α or -β) comprising the structural genes (core, E1, E2), p7 and part of the non-structural gene NS2 of genotype 4a (e.g. strain ED43, genbank accession number Y11604) and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B and part of NS2 from the JFH1 strain (genbank accession number AB047639) as well as a replicating RNA (ED43/JFH1-γ) comprising the structural genes (core, E1, E2), p7 and non-structural gene NS2 of genotype 4a (e.g. strain ED43) and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B from the JFH1 strain.

Thus in one embodiment, the present invention relates to a replicating RNA comprising the structural genes (Core, E1, E2), p7 and the non-structural gene NS2 of genotype 4a and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B from the JFH1 strain.

In another embodiment part of the non-structural gene NS2 is of genotype 4a and part is of the JFH1 strain.

In another embodiment the genotype 4a is of the strain ED43.

In yet another embodiment the strains are ED43/JFH1-α , ED43/JFH1-β or ED43/JFH1-γ.

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 genes (NS2) or parts hereof from a first HCV strain, and sequences encoding the 5' untranlated region (UTR), nonstructural genes (NS2) 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 ED43, and in another embodiment, the second HCV strain is strain JFH1.

The present inventors constructed three ED43/JFH1 viruses (α, β and γ) with different C-terminal genotype junctions. As expected, since replication depend on JFH1 non-structural sequences, all JFH1-based recombinants replicated in Huh7.5 cell culture after transfection. For ED43/JFH1-α, the junction was placed after the first TM domain of NS2.

Interestingly, in difference to a study on JFH1-based inter- and intra-genotypic recombinants by Pietschmann et al. suggesting using the α-junction in obtaining the most efficient phenotype, no infectious ED43/JFH1-α viruses were produced. Thus, our results suggest that the conclusion, that the α-junction in general is favourable for construction of JFH1-based recombinants, is strongly dependent on the considered isolate.

ED43/JFH1-β and -γ virus production was significantly delayed compared to the control 2a virus following initial transfection but not in subsequent passages. Four cell-free passages with immediate spread of ED43/JFH1-β and -γ, confirmed the robust genotype 4a in vitro infection.

We mimicked a natural 2k/1b isolate in the construction of ED43/JFH1-β, placing the junction in the cytoplasmic protease domain and hereby generated an in vitro infectious virus. Some controversy has been whether the NS2 C-terminal part is located cytoplasmic preceded by three TM domains or luminal, preceded by four TM domains. Anyhow, our selection of genotype junction shows the advantage in considering natural evolution for in vitro research. The fact that ED43/JFH-β but not -α generated infectious viruses indicates that ED43 sequences between the two junction sites are needed for interaction with upstream sequence in the Core-p7 region or the N-terminal part of NS2 during assembly and/or release. NS2 TM regions possibly require genotype specific interactions with each other and/or TM regions of E1, E2 or p7, while the protease domain can be of a different genotype. This is in accordance with findings in a study on H77/JFH1 recombinants, that homogenous genotype in the Core-p7 region and the NS2 sequence N-terminal to the β-junction is necessary for infectivity, but in contradiction to Pietschmann et al.'s findings on similar JFH1-based constructs of genotype 1a and 1b.

The viability of ED43/JFH1-γ underlines the permissiveness of intergenotypic recombinants harbouring an NS2/NS3 junction similar to the J6/JFH virus and some natural occurring isolates. Importantly, this construct also contains the longest ED43 region tested. The production of infectious viruses from ED43/JFH1-γ supports others and our findings, that the unique ability of JFH1 to efficiently replicate HCV RNA is compatible with replacement of Core-NS2 of other genotypes

In one embodiment, the HCV nucleic acid molecule of the present invention comprises the nucleic acid sequence (cDNA) of ED43/JFH1-β, SEQ ID NO 1 and of ED43/JFH1-γ, SEQ ID NO 2 and of ED43/JFH-α, SEQ ID NO 5. In another embodiment the nucleic acid molecule has at least a functional portion of a sequence as shown in SEQ ID NO: 1 and SEQ ID NO 2 and SEQ ID NO 5, which represents a specific embodiment of the present invention exemplified herein.

In yet an embodiment the nucleic acid molecule comprises the nucleic acid sequence with a sequence identity of at least 90% to that of SEQ ID NO 1 and/or SEQ ID NO 2 and/or SEQ ID NO 5.

In another embodiment, the nucleic acid comprises a sequence sharing at least 90 % identity with that set forth in SEQ ID NO 1 or SEQ ID NO 2 or SEQ ID NO 5, such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

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, SEQ ID NO 2 and SEQ ID NO 5 are DNA sequences, 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 or SEQ ID NO 2 or SEQ ID NO 5.

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, SEQ ID NO 2 and SEQ ID NO 5. Various modifications for example of the 5' and 3' UTR are also contemplated by the invention. In another embodiment, the nucleic acid further comprises a reporter gene, which, in one embodiment, is a gene encoding neomycin phosphotransferase, Renilla luciferase, secreted alkaline phosphatase (SEAP), Gaussia luciferase or the green fluorescent protein.

Naturally, as noted above, the HCV nucleic acid sequence of the invention is selected from the group consisting of double stranded DNA, positive-sense cDNA, or negative-sense cDNA, or positive-sense RNA or negative-sense RNA or double stranded RNA. Thus, where particular sequences of nucleic acids of the invention are set forth, both DNA and corresponding RNA are intended, including positive and negative strands thereof.

In a further embodiment, the nucleic acid sequence of SEQ ID NO 1 or SEQ ID NO 2 or SEQ ID NO 5 or the said nucleic acid sequence with any mutation described in this document is obtained by any other means than what is described above.

In another embodiment, the complementary DNA (cDNA) provided by the present invention encodes human hepatitis C virus of genotype 4a/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 ED43/JFH1-β, SEQ ID NO 3 or ED43/JFH1-γ, SEQ ID NO 4 or ED43/JFH-α, SEQ ID NO 6.

According to various aspects of the invention, HCV nucleic acid, including the polyprotein coding region, can be mutated or engineered to produce variants or derivatives with, e.g., silent mutations, conservative mutations, etc. In a further preferred aspect, 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.

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 3, 4, and 6.

In yet an embodiment the isolated nucleic acid molecule encodes the amino acid sequence with a sequence identity of at least 90% to that of SEQ ID NO 3 and/or SEQ ID NO 4 and/or SEQ ID NO 6.

In another embodiment, the amino acid sequences comprises a sequence sharing at least 90 % identity with that set forth in SEQ ID NO 3 or SEQ ID NO 4 or SEQ ID NO 6, such as 90 % identity, 91 % identity, 92 % identity, 93 % identity, 94 % identity, 95 % identity, 96 % identity, 97 % identity, 98 % identity, or 99 % identity.

It should 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.

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

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

Adaptive Mutations

Adapted mutants of a HCV-cDNA construct or HCV-RNA full-length genome with improved abilities to generate infectious viral particles in cell culture compared to the original HCV-cDNA construct or the original HCV-RNA full-length genome are characterized in that they are obtainable by a method in which the type and number of mutations in a cell culture adapted HCV-RNA genome are determined through sequence analysis and sequence comparison and these mutations are

introduced into a HCV-cDNA construct, particularly a HCV-cDNA construct according to the present invention, or into an (isolated) HCV-RNA full-length genome, either by site-directed mutagenesis, or by exchange of DNA fragments containing the relevant mutations.

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

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

ED43/JFH1-β and -γ virus production was significantly delayed compared to the control 2a virus following initial transfection but not in subsequent passages, indicating a need for adaptive mutations.

Sequencing 1st, 2nd and 3rd passage viruses, the present inventors observed a complete changed mutation in the first TM domain of NS2 (A2819G, amino acid T827A) in both infectious ED43/JFH1 recombinants.

ED43/JFH1-γ A3269T (amino acid T977S) further changed completely in the γ-specific ED43 cytoplasmic part of NS2.

The requirement for these adaptations was confirmed by reverse genetics. Thus, introducing these mutation only, leading to the constructs pED43/JFH1-βA2819G and pED43/JFH1-γA2819G,A3269T (SEQ ID NO 7 and 8 with resulting amino acid sequences SEQ ID NO 18 and 19), led to immediate production of infectious viral particles after transfection in cell culture. Introduction of one the two mutations found for ED43/JFH1-γ only, leading to the constructs pED43/JFH1-γA2819G and pED43/JFH1-γA3269T (SEQ ID NO 9 and 10 with resulting amino acid sequences

SEQ ID NO 20 and 21), did not lead to production of infectious particles after transfection.

Adaptation was not caused by suboptimal sequences inserted during construction, since the patient ED43 sequence (Chamberlain et al 1997) and ED43 viruses passaged in a chimpanzee (cloning source in the present invention) are identical at these positions.

The E1 mutation A1325T (amino acid T329S) was dominating in 1st viral passage, but continued only as a mixture in 2nd and 3rd passage, and was not at all detected in the 3rd passage clonal analysis. Furthermore reverse genetics studies showed A1325T to cause a slightly attenuated phenotype and a need for further genomic adaptation. Hence, A1325T was probably co-selected with A2819G and A3269T and later reversed to the more fit wild-type sequence.

In the 3rd and 4th passage of ED43/JFH1-γ most cells stayed infected for up to 30 days accompanied by continuance of high RNA titers and reduced cell death compared to earlier passages and J6/JFH, which showed massive infection for approximately 10 days only, followed by proliferation of uninfected cells (Figure 2A and B).

Zhong et al. earlier reported on virus-host interaction patterns for JFH1 infecting Huh7 derived cells, showing that the expansion of non-infected cells could be ascribed cellular resistance mechanisms.

The prolonged infection was not observed with adapted recombinants harbouring A1325T, A2819G or A3269T. This could be indicative of a virus-host adapted phenotype introduced in 3rd passage.

The amino acid C2270R (DNA T7148C) mutation in NS5A, not seen earlier than 3rd passage and present in 7/10 3rd passage clones, could be the determinant of this virus-host optimized phenotype. For instance, NS5A is speculated to be involved in regulation of host cell defence mechanisms such as the IFN-α induced dsRNA dependent protein kinase, PKR as well as 2,5 oligoadenylate synthetase, that has been found to interact with NS5A. The PKR interaction domain has been mapped

to include AA 2209-2274 of the H77 reference sequence AF009606 including C2270R. 3rd passage ED43/JFH1-β also showed NS5A mutations in the N-terminal membrane anchor sequence (C6306T, amino acid T1989I) and in the very C-terminal region (G7646T, amino acid V2436L).

The ED43/JFH1-γ E1 mutation, G986A (amino acid A216T), was also only found in 3rd passage. A number of known genotype 1 and 4 sequences but not ED43 have threonine at position 216 too. We did not analyse non-coding mutations in reverse genetic studies, but still succeeded in generating adapted infectious cDNA clones. Silent mutations could possibly represent co-selection, and have earlier been shown to be functional in infectious cDNA clones. However, non-coding mutations could be important for functional RNA elements e.g. in Core.

For the first time we demonstrated that HCV of genotype 4 utilizes CD81 for entry into host cells.

The infectivity titer of the ED43/JFH1 viruses was diminished by 10-100 fold compared to J6/JFH and earlier studies on J6/JFH, but significantly greater than similar constructs of H77/JFH1 (1a/2a), Con1/JFH1 (1b/2a) and 452/JFH1 (3a/2a) constructed by Pietschmann et al.

In a study using HCV pseudo particles (HCVpp) with HCV E1 and E2 glycoproteins of genotype 4a, McKeating et al. find a significantly lower incorporation of glycoproteins and infectivity in Hep3B cells compared to J6 HCVpp. This could reflect a generally lower infectivity of genotype 4a viruses. On the other hand, the chosen 4a E1 and E2 proteins deviate from ED43 at four AA positions, and large infectivity deviations are seen among different HCVpp isolates of same subtype. In another study on ED43 HCVpp with proteins identical to the ones in our constructs, infectivity was apparently not reduced compared to J6 HCVpp. It has been showed that HCV virions are produced in a spectrum of different boyant densities showing large variation in infectivity. Hence steps of assembly, where ED43 proteins such as Core, E1, E2 and NS2 have to interact with JFH1 proteins could lower production ratio of infectious to non-infectious viral particles. The new possibilities of studying HCV infection in cell culture calls for further studies on variations in infectivity among different isolates, and elucidation of the biological relevance of such will be important for future studies on genotype 4.

Thus in one embodiment, the present invention relates to nucleic acid molecules according to the present invention, wherein said molecule comprises adaptive mutations in E1 and NS2 and NS5A singly or in combination.

In the context of the present invention the term "adaptive mutation" is meant to cover mutations identified in passaged ED43/JFH1 viruses that provides the original ED43/JFH1 genome and any other HCV sequence the ability to grow efficiently in culture. Furthermore all introductions of mutations into the ED43/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. This also include other HCV genomes with adaptive mutations, all of them, combinations of them or individual mutations, that grows 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 of 3rd passage viruses showed the conversion of the input plasmid sequence into a quasispecies population of viruses, in accordance to what is seen in HCV in vivo infections. In the limited prepared clonal analysis we detected 6 subpopulations of ED43/JFH1-β and 3 of ED43/JFH1-γ, when positions mutated in at least 5/10 clones were taken into account.

The 3rd passage dominating quasispecies containing amino acid mutations, E989K (NS2), T1989I and V2436L (both NS5A) (β) or A216T (E1) and C2270R (NS5A) (γ) in addition to the NS2 mutations T827A (β) or T827A and T977S (γ) found already

in 1st passage, did not enhance infectivity titers compared to earlier passages. Selected single 3rd passage mutations can be of importance for vaccine development. Hence, future studies on ED43 cell culture systems should include analyses of biological function of the identified NS2 mutations as well as relevance of 3rd passage mutations and the prolonged cell culture infection.

Furthermore a number of additional mutations were found by direct sequencing of 1st, 2nd and 3rd passage as well as in 10 clones of ED43/JFH1-β and ED43/JFH-γ from 3rd passage each. Mutations found in at least two clones are likely to occur because the virus has adapted to cell culture. Mutations found in less than two clones could be due to PCR errors and are not listed.

To test various combinations of adaptive mutations, the following constructs were made, and in addition to SEQ ID NO 7 and 8 found to efficiently produce infectious viral particles in culture after transfection (see Example 4); pED43/JFH1-γA1325T,A2819G,A3269T, pED43/JFH1-βT827A,E989K, pED43/JFH1-βT827A,V2436L, pED43/JFH1-βT827A,E989K,T1989I,V2436L, pED43/JFH1-γA216T,T827A,T977S, pED43/JFH1-γT827A,T977S,C2270R and pED43/JFH1-γA216T,T827A,T977S,C2270R (SEQ ID NO 11, 12, 13, 14, 15, 16 and 17 with resulting amino acid sequences SEQ ID NO 22, 23, 24, 25, 26, 27 and 28).

This study demonstrates the possibility for development of recombinant ED43/JFH1 viruses with different genotype junctions. It further underlines the importance in choosing the junction-site for compatibility between the genotypes. As such it presents important information for further studies implementing larger regions of ED43 in the process of obtaining genotype 4 full-length cell culture systems. The developed systems are the first for studying HCV replication genotype 4 in vitro, and could be important for development of inactivated vaccine candidates, since the high-endemic Egypt, a potential region for clinical vaccine trials, mainly have genotype 4a infections. Furthermore the developed systems for the first time allow for genotype 4 specific functional studies and studies of neutralising antibodies and anti-viral drugs in a true cell culture system.

When sequencing HCV genomes from the supernatant of ED43/JFH1-β infected cell cultures, the following changes at the nucleotide level were observed at least

once; G787A, C1944G, T2021C, C2206T, A2772G, A2819G, G3305A, T3392C, C4222T, T5836C, C6306T, G7147C, G7198A, A7640G, G7646T and G8150A. These mutations caused the amino acid changes T535S, F561L, D811G, T827A, E989K, Y1018H, T1989I, E2269D, T2434A, V2436L and G2604S.

Thus, 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 G787A, C1944G, T2021C, C2206T, A2772G, A2819G, G3305A, T3392C, C4222T, T5836C, C6306T, G7147C, G7198A, A7640G, G7646T and G8150A.

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 G787A, C2206T, A2819G, G3305A, C4222T, C6306T and G7646T.

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 A2819G.

When sequencing HCV genomes from the supernatant of ED43/JFH1-γ infected cell cultures, the following changes at the nucleotide level were observed at least once; C373T, A387C, C436A, G723A, C781A, G986A, G1026A, T1150C, T1211G, A1325T, A1336G, T1369C, T2093A, A2114G, G2251A, C2480T, T2727C, T2731C, A2785G, A2819G, T2916C, T2937C, A2995G, C3001G, G3154A, G3208A, A3269T, A4152G, C4459T, T4540C, G4918A, C4944T, G5079A, A5592G, A5668G, T6184G, A6248G, G7022A, A7103G, T7125C, A7128G, T7148C, G7291A, G7534A, G7584A, T7809C, T7879C, T7985C and A8212G.
These mutations caused the amino acid changes N16T, C128Y, A216T, C229Y, F291V, T329S, C585S, T592A, L714F, L796P, T827A, V859A, V866A, D887E, M956I, T977S, T1531I, G1580E, Q1751R, I1970V, D2228N, M2255V, L2262P, E2263G, C2270R, G2415E and V2490A.

Thus, 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 2 by the following said nucleotide selected from the group consisting of C373T, A387C, C436A, G723A, C781A, G986A, G1026A, T1150C, T1211G, A1325T, A1336G, T1369C, T2093A, A2114G, G2251A, C2480T, T2727C, T2731C, A2785G, A2819G, T2916C, T2937C, A2995G, C3001G, G3154A, G3208A, A3269T, A4152G, C4459T, T4540C, G4918A, C4944T, G5079A, A5592G, A5668G, T6184G, A6248G, G7022A, A7103G, T7125C, A7128G, T7148C, G7291A, G7534A, G7584A, T7809C, T7879C, T7985C and A8212G.

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 2 by the following said nucleotide selected from the group consisting of G986A, A1325T, A1336G, A2785G, A2819G, A3269T, C4459T, G4918A, G7022A, A7128G, T7148C.

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 2 by the following said nucleotide selected from the group consisting of A2819G and A3269T.

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 3 by the following said amino acid selected from the group consisting of T535S, F561L, D811G, T827A, E989K, Y1018H, T1989I, E2269D, T2434A, V2436L and G2604S.

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 3 by the following said amino acid selected from the group consisting of T827A, E989K, T1989I and V2436L.

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 3 by the following said amino acid selected from the group consisting of T827A.

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 4 by the following said amino acid selected from the group consisting of N16T, C128Y, A216T, C229Y, F291V, T329S, C585S, T592A, L714F, L796P, T827A, V859A, V866A, D887E, M956I, T977S, T1531I, G1580E, Q1751R, I1970V, D2228N, M2255V, L2262P, E2263G, C2270R, G2415E and V2490A

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 4 by the following said amino acid selected from the group consisting of A216T, T329S, T827A, T977S, D2228N, E2263G and C2270R.

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 4 by the following said amino acid selected from the group consisting of T827A and T977S.

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 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 1a/2a recombinants cannot be directly compared to that of the recombinants used in this study, because it has not been clarified, how different measures of infectivity (FFU versus TCID50) compare

The present study also points to a low infectivity of the original ED43/JFH1 viruses. First, the present inventors found low infectivity titers shortly after transfection with pED43/JFH1 *in vitro* transcripts, which eventually became undeterminable. Second, the original pED43/JFH1 sequence could not be detected in clonal analysis of virus genomes derived from a 3rd viral passage.

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 4a recombinants, which would allow *in vitro* studies of any patient 4a isolate of interest.

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 assay become infected and is given in $TCID_{50}$/ml.

One embodiment of the present invention releates 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 TCID50/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.

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

Compositions

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

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

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

parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

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

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

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

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

"Pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans.

The term " excipient " refers to a diluent, adjuvant, carrier, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or

synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response. Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvmm. Preferably, the adjuvant is pharmaceutically acceptable.

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 4a/JFH1 RNA and produces a virus particle comprising introducing the said RNA according to the invention into a cell.

In one embodiment the 4a strain is ED43.

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

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

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

In another embodiment the cell is Huh7.5.

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

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

Virus particle

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

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

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

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

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

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 the viability of the developed viruses may be determined *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 variant genomes obtained in the present study may prove useful for different research topics. Genomes with the original ED43 Core could be applied to examine genotype 4a specific features of Core.

The systems developed in this study are ideal candidates for the genotype 4a specific testing of therapeutics targeting viral entry, assembly and release. Genomes with the original ED43 E1 and E2 are valuable for testing of neutralizing antibodies and other drugs acting on entry level, such as fusion inhibitors.

The present inventors conducted cross-genotype neutralization studies in HCV cell culture systems recapitulating the entire viral life cycle using JFH1-based viruses

with envelope sequences of all 6 major genotypes, which has previously not been possible. HCV E1/E2 assembled on HCV pseudo particles (HCVpp), used in previous neutralization studies could show an unphysiological confirmation, glycosylation pattern and/or lipoprotein association due to the nature of the HCVpp as well as the non-hepatic producer cell-lines used in such experiments. In such studies the viral particles are incubated with the neutralizing substance, e.g. patient derived antibodies present in serum, prior to incubation with cells permissive and susceptible to viral infection. The neutralizing effect, i.e. the inhibitory effect on viral entry, is measured e.g. by relating the number of focus forming units (FFUs, defined as foci of adjacent infected cells) to the equivalent count in a control experiment done under same circumstances without the active inhibitor molecule.

The inventors of the present invention showed that JFH1-based viruses of genotype 1a, 4a, 5a and 6a were efficiently neutralized by chronic phase H06 genotype 1a serum derived from reference Patient H (Table 6). Neutralization of the ancestral H77C/JFH1 virus, whose sequence originates from acute phase Patient H serum, is in agreement with an extensive longitudinal study on neutralizing antibodies in Patient H carried out in the HCVpp system showing neutralization by serum samples taken later but not concurrently or earlier than the envelope sequence used for HCVpp. The results in the cell culture systems compare well to neutralization experiments using Patient H serum from year 26 (H03) carried out in HCVpp systems with envelope proteins of the same prototype isolates of all 6 HCV genotypes as used in the present application, and heterogeneity between the genotypes is thus as previously reported by Meunier et al. 2005.

In addition the present inventors found that cross-genotype neutralization extended to a chronic phase genotype 4a serum (AA), which efficiently neutralized genotype 4a, 5a and 6a and to a lesser extent 1a (Table 6). Accordingly, the JFH1-based cell culture systems which has been developed for HCV genotype 1a/1b, 2a, 3a, 4a, 5a and 6a provides a valuable tool for efficiently screening for and identifying new candidate HCV genotype 1a/1b, 2a, 3a, 4a, 5a and 6a inhibitors e.g. of entry e.g. in serum derived from infected patients. Accordingly this invention, allows identification and raise of cross-neutralizing antibodies,

which is important for the development of active and passive immunization strategies. Furthermore the availability of cell culture grown HCV particles bearing envelope proteins of the six major genotypes enables the development of inactivated whole virus vaccines and comprehensive virus neutralization studies.

Furthermore the present inventors showed neutralization of 4a/JFH virus (ED43/JFH1-$\gamma_{T827A,T977S}$) with sera from patients infected with genotype 1a and 4a and 5a as can be seen in Figure 5 and Table 6 and 7.

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

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

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

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

In one embodiment the present invention related to a method for screening new HCV genotype 1a/1b, 2a, 3a, 4a, 5a and/or 6a 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 1a/1b, 2a, 3a, 4a, 5a and/or 6a infected patient, and

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

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

As proof of principle of using the present invention in testing of anti-hepatitis C virus substances, the effects of interferon-α, currently used in combination therapy for HCV, were tested on the infected cell culture. As shown in Figure 9, addition of interferon-α to a 4a/JFH1 infected culture immediately and significantly reduced the percentage of infected cells. In this in vitro system, prolonged treatment of the culture with interferon-α resulted in complete absence of HCV protein expression as determined by immuno staining.

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

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

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

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 the present invention, a hepatitis C virus infected cell

according to the present invention and a hepatitis C virus particle obtainable by the present invention together with a hepatitis C virus permissive cell,

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

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

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

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

In one embodiment, the method may be conducted be in vitro or in vivo. In one embodiment, the cells as described may be in an animal model, or a human subject, entered in a clinical trial to evaluate the efficacy of a candidate molecule. In one embodiment, the molecule is labelled for easier detection, including radio-labelled, antibody labelled for fluorescently labelled molecules, which may be detected by any means well known to one skilled in the art.

In one embodiment, the candidate molecule is an antibody.

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, 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 genotype 4a cell culture system developed of the present invention will be a valuable tool to address different research topics. It will allow the genotype specific study of functions of the structural proteins (Core, E1, E2) as well as p7 and NS2 using reverse genetics. While the replicon systems facilitated testing of drugs interfering with replication such as NS3/4A protease and polymerase inhibitors, the system developed in this study is ideal for the genotype 4 specific testing of new drugs, such as drugs interfering with viral entry, such as fusion inhibitors, as well as assembly and release.

Accordingly the genotype 1a/1b, 2a, 3a, 4a, 5a and 6a developed cell culture systems allows individual patient targeting. This means that when a new potential therapeutic candidate is discovered it is possible to test this particular candidate or combination of candidates on each of the individual genotypes. Knowing which specific genotype(s) the candidate is functioning towards, it allows an individual treatment of each patient dependent on which specific genotype the patient is infected with. Furthermore these cell culture systems allow the development of antibodies and vaccines targeting individual patients.

In addition new therapeutics targeting the putative p7 ion-channel and protease inhibitors targeting NS2 can be tested specifically for genotype 4 thus allowing individual patient targeting.

As ED43/JFH1 viability does not seem to depend on mutations in the envelope glycoproteins, these recombinant viruseses will be well suited for screenings for broadly reactive neutralizing antibodies, thus aiding vaccine development.

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 in vitro culture systems. According to this aspect of the invention, and in some embodiments, cells contacted with the variants are characterized by reduced infection, as compared to cells contacted with the chimeric HCV.

Kits

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

Furthermore the invention also provide test kits, for screening for new HCV genotype 1a/1b, 2a, 3a, 4a, 5a and 6a inhibitors, neutralizing and cross neutralizing antibodies, comprising HCV virus components.

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 be 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 do 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 be 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

Sequence ID	DNA/amino acid(AA)	Name
SEQ ID NO 1	DNA	ED43/JFH1-β
SEQ ID NO 2	DNA	ED43/JFH1-γ
SEQ ID NO 3	AA	ED43/JFH1-β
SEQ ID NO 4	AA	ED43/JFH1-γ
SEQ ID NO 5	DNA	ED43/JFH1-α
SEQ ID NO 6	AA	ED43/JFH1-α
SEQ ID NO 7	DNA	pED43/JFH1-β_{A2819G}
SEQ ID NO 8	DNA	pED43/JFH1-$\gamma_{A2819G,A3269T}$
SEQ ID NO 9	DNA	pED43/JFH1-γ_{A2819G}
SEQ ID NO 10	DNA	pED43/JFH1-γ_{A3269T}
SEQ ID NO 11	DNA	pED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$
SEQ ID NO 12	DNA	pED43/JFH1-βT827A,E989K
SEQ ID NO 13	DNA	pED43/JFH1-βT827A,V2436L
SEQ ID NO 14	DNA	pED43/JFH1-βT827A,E989K,T1989I,V2436L
SEQ ID NO 15	DNA	pED43/JFH1-γA216T,T827A,T977S
SEQ ID NO 16	DNA	pED43/JFH1-γT827A,T977S,C2270R
SEQ ID NO 17	DNA	pED43/JFH1-γA216T,T827A,T977S,C2270R
SEQ ID NO 18	AA	pED43/JFH1-β_{A2819G}

SEQ ID NO 19	AA	pED43/JFH1-$\gamma_{A2819G,A3269T}$
SEQ ID NO 20	AA	pED43/JFH1-γ_{A2819G}
SEQ ID NO 21	AA	pED43/JFH1-γ_{A3269T}
SEQ ID NO 22	AA	pED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$
SEQ ID NO 23	AA	pED43/JFH1-βT827A,E989K
SEQ ID NO 24	AA	pED43/JFH1-βT827A,V2436L
SEQ ID NO 25	AA	pED43/JFH1-βT827A,E989K,T1989I,V2436L
SEQ ID NO 26	AA	pED43/JFH1-γA216T,T827A,T977S
SEQ ID NO 27	AA	pED43/JFH1-γT827A,T977S,C2270R
SEQ ID NO 28	AA	pED43/JFH1-γA216T,T827A,T977S,C2270R
SEQ ID NO 29	DNA	4aF193
SEQ ID NO 30	DNA	4aF1G-NotI-T7
SEQ ID NO 31	DNA	4aF2676
SEQ ID NO 32	DNA	4aF2719
SEQ ID NO 33	DNA	4aF309
SEQ ID NO 34	DNA	4aF5446
SEQ ID NO 35	DNA	4aF741
SEQ ID NO 36	DNA	4aF9251
SEQ ID NO 37	DNA	4aF9271-HindIII
SEQ ID NO 38	DNA	4aR262
SEQ ID NO 39	DNA	4aR489
SEQ ID NO 40	DNA	4aR5664
SEQ ID NO 41	DNA	4aR862
SEQ ID NO 42	DNA	4aR9406
SEQ ID NO 43	DNA	4aR9491-Xba
SEQ ID NO 44	DNA	4aR9504
SEQ ID NO 45	DNA	JF2879
SEQ ID NO 46	DNA	JF2962
SEQ ID NO 47	DNA	JF3198
SEQ ID NO 48	DNA	JR345
SEQ ID NO 49	DNA	JR3593
SEQ ID NO 50	DNA	JR8368
SEQ ID NO 51	DNA	JR8688

SEQ ID NO 52	DNA	RU-O-5720
SEQ ID NO 53	DNA	RU-O-5721
SEQ ID NO 54	DNA	JVF12328
SEQ ID NO 55	DNA	2aR2905/4aR2866
SEQ ID NO 56	DNA	2aR3220/4aR3185
SEQ ID NO 57	DNA	2aR3451/4aR3419
SEQ ID NO 58	DNA	-285s-HCV-MOD
SEQ ID NO 59	DNA	9470R_JFH1
SEQ ID NO 60	DNA	consR268
SEQ ID NO 61	DNA	consR312
SEQ ID NO 62	DNA	consR337
SEQ ID NO 63	DNA	-84S_HCV-MOD
SEQ ID NO 64	DNA	4aF965
SEQ ID NO 65	DNA	4aF1910
SEQ ID NO 66	DNA	4aF2719
SEQ ID NO 67	DNA	4aR705
SEQ ID NO 68	DNA	4aR1080
SEQ ID NO 69	DNA	4aR2010
SEQ ID NO 70	DNA	4aR2871
SEQ ID NO 71	DNA	3329R_JFH1-MOD
SEQ ID NO 72	DNA	946S_J6
SEQ ID NO 73	DNA	1849S_J6
SEQ ID NO 74	DNA	2754S_J6
SEQ ID NO 75	DNA	JR513
SEQ ID NO 76	DNA	1109R_J6
SEQ ID NO 77	DNA	2111R_J6
SEQ ID NO 78	DNA	2763R_J6
SEQ ID NO 79	DNA	3774R_J6
SEQ ID NO 80	DNA	3081S_J6/JFH1
SEQ ID NO 81	DNA	3880S_J6
SEQ ID NO 82	DNA	4528S_J6
SEQ ID NO 83	DNA	5272S_JFH1
SEQ ID NO 84	DNA	6186S_JFH1

SEQ ID NO 85	DNA	6862S_JFH1
SEQ ID NO 86	DNA	7741S_J6
SEQ ID NO 87	DNA	8137S_JFH1
SEQ ID NO 88	DNA	4118R_JFH1
SEQ ID NO 89	DNA	4796R_JFH1
SEQ ID NO 90	DNA	5446R_JFH1
SEQ ID NO 91	DNA	6460R_J6
SEQ ID NO 92	DNA	7234R_JFH1
SEQ ID NO 93	DNA	7848R_JFH1
SEQ ID NO 94	DNA	8703R_JFH1
SEQ ID NO 95	DNA	9464R(24)_JFH1
SEQ ID NO 96	DNA	4aF2261

Examples

MATERIALS AND METHODS

Source of HCV genotype 4a and HCV plasmids

A plasma pool of strain ED43 was prepared from acute-phase plasmapheresis units collected from an experimentally infected chimpanzee. This plasma pool has an HCV RNA titer of approximately 105.5 IU/ml and an infectivity titer of approximately 105 chimpanzee infectious doses/ml. (Bukh et al, unpublished data). The ED43 strain originated from an Egyptian genotype 4a patient and virus recovered from this patient was originally sequenced by Chamberlain et al 1997.

For construction of the ED43 constructs, a fragment of an ED43 E1 and E2 expression vector (pCMV-ED43(4a-1))was used to make HCV pseudo particles. pJFH1 and pJFH1/GND used for recombinant 4a/2a constructs was a generous gift from Dr. Wakita

RNA extraction

RNA was extracted from plasma and cell culture supernatant using either the High Pure Viral Nucleic Acid Kit (Roche) or the TRIzol (Invitrogen) procedure according to manufacturers protocol. Extracted RNA was diluted in 10mM DTT with 5%

(vol/vol) RNasin (20-40units/μL) (Promega) and used directly for cDNA synthesis or stored at -80°C.

Reverse transcription, PCR and cloning

All reverse transcription-polymerase chain reactions (RT-PCR) were carried out using RNA extracted from 100μL plasma or cell culture supernatant. Primers (TAG Copenhagen) were 1,25μM and dNTPs (Invitrogen) were 0,5mM in RT reactions. For denaturation, RNA was incubated for 2' at 65°C together with primer and dNTPs and placed on ice. cDNA syntheses was done in a 20μL volume with enzyme and incubation times as described. The final RT reaction was treated with 1-4U RNase H (Invitrogen) and 1000U RNase T1 (Ambion) for 20' at 37°C to degrade RNA.

All PCR reactions were done using 2,5μL cDNA reaction as template in a 50μL reaction volume. Final concentrations of primer and dNTPs in PCR were 0,2μM and 0,25mM respectively. Enzyme and cycle parameters are described. Primer sequences are given in Table 1.

All DNA purification including gel extraction was done using Wizard SV Gel and PCR Clean-Up System (Promega). Restriction endonucleases were from New England Biolabs and ligation reactions were done using Rapid DNA ligation kit (Roche) according to protocol. TOP10 chemically competent bacteria (Invitrogen) were used for all bacterial cloning. After ligation or TA-TOPO reaction (Invitrogen, TA-TOPO cloning kits) 2μL of the reaction was incubated with 50μL competent bacteria for 30' on ice before heat-shocking 30' at 42°C. 250μL SOC-media (Invitrogen) were added to the bacteria and the suspension was incubated for 1h shaking at 37°C. Bacteria were plated on agar plates with selection as described. DNA preparations were carried out using QIAGEN QIAprep spin miniprep kit or QIAfilter plasmid maxi kit. DNA stocks of plasmids containing the final virus constructs were prepared using QIAGEN Endofree plasmid maxi kit.

Construction of ED43/JFH1 intergenotypic recombinants

The ED43 sequence was amplified from the chimpanzee plasma using RT primer 4aR5664 (SEQ ID NO 40) and 200U SuperScriptII (Invitrogen) for 1h at 42°C.

Enzyme was inactivated for 15′ at 70°C. A PCR reaction was set up using the Advantage 2 PCR Enzyme System (Clontech) and primers 4aF1g-NotI-T7 (SEQ ID NO 30) and 4aR5664 (SEQ ID NO 40). Cycling parameters were 1′ at 95°C, 35 cycles of 30″ at 95° and 9′ at 68°C followed by a final elongation of 9′ at 68°C. The PCR product was analyzed by standard agarose gel electrophoresis techniques and TOPO-cloned using TOPO-XL PCR Cloning kit (Invitrogen). From 4 clones we assembled an ED43 sequence deviating from consensus at the two non-coding positions A2458G and A2593G only. pED43/JFH1-γ (SEQ ID NO 2) was constructed by inserting a fusion PCR product containing the ED43 region and the correct junctions directly into pJFH1 8. Templates included a pJFH1 5′UTR PCR fragment, the ED43 consensus sequence and a fusion PCR product containing the ED43/JFH1-γ junction. The JFH1 5′UTR fragment was amplified from pJFH1 using primers JVF12328 (SEQ ID NO 54) (vector sequence) and JR345 (SEQ ID NO 48). Cycling parameters were 45″ at 95°C, 20 cycles of 45″ at 95°C, 45″ at 60° and 1′ at 72° followed by a final 10′ at 72°C. The γ-junction product was obtained by fusion PCR on two 1st round PCRs. One was using pJFH1 template and forward primer RU-O-5720 (SEQ ID NO 52) at the NS3 5′end and reverse primer RU-O-5721 (SEQ ID NO 53) in NS3. Another was using the ED43 consensus sequence and forward primer 4aF2719 (SEQ ID NO 32) and reverse primer 2aR3451/4aR3419 (SEQ ID NO 57) at the NS2 3′end tailed with 21nts of the JFH1 NS3 5′end. Cycling parameters in 1st round PCRs were 45″ at 95°C, 30 cycles of 45″ at 95°C, 45″ at 60° and 1′30″ at 72° followed by a final 10′ at 72°C. Overlapping 1st round PCR products were fused using primers 4aF2719 (SEQ ID NO 32) and RU-O-5721 (SEQ ID NO 53). Cycling parameters were 45″ at 95°C, 35 cycles of 45″ at 95°C, 45″ at 60° and 5′ at 72° followed by a final 10′ at 72°C. The final fusion PCR was set up using primers JVF12328 (SEQ ID NO 54) and RU-O-5721 (SEQ ID NO 53). Cycle parameters were 45″ at 95°C, 35 cycles of 45″ at 95°C, 45″ at 60° and 5′ at 72°, followed by a final 10′ at 72°C. The obtained product was directly digested by AgeI and SpeI in the JFH1 5′UTR and NS3 respectively and ligated into pJFH1. All PCR reactions were carried out using proofreading polymerase Pfu (Stratagene).

pED43/JFH1-α and –β (SEQ ID NO 5 and 1) were constructed by inserting a fusion PCR product containing the specific ED43/JFH1 junction into pED43/JFH1-γ. To introduce the correct junction 1st round PCR was done on pJFH1 using forward

primer JF2879 (SEQ ID NO 45) (α) or JF3198 (SEQ ID NO 47) (β) and reverse primer RU-O-5721 53. On the ED43 consensus sequence another PCR was done using forward primer F2261 (SEQ ID NO 96) and reverse primer with JFH1 overhang 2aR2905/2aR2866 (SEQ ID NO 55) (α) or 2aR3220/2aR3185 (SEQ ID NO 56) (β). Cycling parameters were 45'' at 95°C, 26 cycles of 45'' at 95°C, 45'' at 62° and 1'30'' at 70°, followed by a final 10' at 70°C. Fusion PCR was done on the 1st PCR reactions using primers F2261(SEQ ID NO 96)/RU-O-5721(SEQ ID NO 53) with cycling parameters as above extending cycle-number to 35.

As a negative control a viral polymerase (NS5B) mutant with the active site GDD changed to GND, pED43/JFH1-GND, was created in analogy to pED43/JFH-γ ligating the ED43 containing region into pJFH1/GND digested with AgeI and SpeI. The complete HCV sequence of all final plasmid preparations was sequenced.

ED43/JFH1 (4a/2a) intergenotypic recombinants were constructed to retain the unique cell culture replication abilities of the genotype 2a isolate JFH1, while the structural and part of the non-structural genes were replaced by the genotype 4a reference strain ED43. Three ED43/JFH1 recombinants all containing the amino acid (AA) consensus sequence of Core, E1, E2, p7 and part of or all of NS2 from ED43 were constructed. The 5' untranslated region (UTR, differing from the sequence provided for JFH1 (accession number AB047639) at C301T) as well as the sequence downstream from the NS2-junction, including the nonstructural proteins NS3, NS4A, NS4B, NS5A, NS5B and the 3'UTR was from JFH1 (Figure 1A). pED43/JFH1-α was constructed with the C-terminal genotype junction between the first and second putative transmembrane (TM) domain of NS2 in analogy to JFH1-based intra- and inter-genotypic recombinants of genotype 1a, 1b, 2a and 3a. pED43/JFH1-β was constructed comprising the internal NS2-junction at a parental hairpin structure in the NS2 protease domain in analogy to the first described natural occurring HCV recombinant. pED43/JFH1-γ contained the entire NS2 gene from ED43. As a negative control, a replication deficient construct mutated in the polymerase active site, pED43/JFH1-GND was constructed. The final ED43 sequences deviated from the consensus sequence at 2 noncoding positions only (A2458G and A2593G).

In vitro transcription

For in vitro transcription 5µg plasmid was XbaI-linearized and treated with mung bean nuclease (New England Biolabs) according to protocol in order to remove XbaI-overhang. This procedure leaves the linearized clone with the exact HCV 3'end. Transcription was carried out for 2hrs with T7 RNA polymerase (Promega) according to protocol. RNA production was evaluated by gel electrophoresis.

Culturing of Huh7.5 cells

The human hepatoma cell line Huh7.5 is an INF-α cured clone of the Huh7 hepatoma cell line, with increased HCV replication abilities. Cells were cultured in D-MEM + 4500 mg/L Glucose + GlutaMAX-I + Pyruvate (Invitrogen) containing 10% heat inactivated fetal bovine serum (FBS) (Sigma), penicillin at 100 units/ml and streptomycin at 100 mg/ml (Invitrogen) at 5% CO2 and 37°C. Every 2-3 days cells were split after washing with PBS, trypsinizing (Trypsin/EDTA, Invitrogen) and centrifuging for 5' at 1000rpm. Supernatants were sterile filtered to exclude cells and debris and stored at -80°C.

Transfection of RNA transcripts into Huh7.5 cells

Naïve Huh7.5 cells were plated at 3*105/well in 6-well plates the day before transfection. Prior to transfection 1-2,5µg of unpurified RNA transcripts were incubated with Lipofectamine2000 (Invitrogen) in 500µL Opti-MEM (Invitrogen) for 20' at room temperature. RNA-Lipofectamine2000 transfection complexes were left on cells for 16-24hrs before washing.

Infection of Huh7.5 cells with supernatants

To prove the production of infectious viruses, sterile filtered supernatant from infected cultures was used to infect naïve Huh7.5 cells. Unless other is described, 1mL supernatant was used for infection of Huh7.5 cells plated in 6-well plates at 3*105/well the day before. Supernatants were left on cells for 6-24 hrs as described in figure legends.

Immunohistochemistry

For staining, cells grown over night on 4- or 8-well chamberslides (Nunc) were washed 2x with PBS and fixed for 5 minutes with acetone. After washing 2x with

PBS and 1x with PBS/Tween-20 (0.1%), slides were incubated with 1° antibody (MAB Murine Anti-Human HCV, Core Protein, Clone B2 (Anogen) used at 1:200 in PBS containing 5% bovine serum albumine (BSA)) for 20' at room temperature. After washing as above, 2° antibody (Alexa Fluor 594 goat anti-mouse IgG (H+L)) and Hoechst33342 (both Invitrogen) for cell nuclei counterstaining, used at 1:500 and 1:10000 dilutions, respectively in PBS/Tween, was added for 5min. Finally slides were washed with PBS, mounted with Fluoromount-G (Southern Biotech) and cover slipped. Staining was visualized using a Leica TCS SP5 confocal microscope. Percentage of infected cells was evaluated by assigning values of 0% (no cells infected), 1% (or below), 5%, 10-90% in steps of 10, 95% and 100% (all cells infected).

Infectivity titration

Viral titers were determined by the tissue culture infectious dose 50 (TCID50). 6*103/well naïve Huh7.5 cells were plated out in a poly-D-lysine coated 96-well plate (Nunc) the day before infection. Cells were then incubated with 10-fold dilutions of cell culture supernatants in replicates of 6 for 2-3 days. Hereafter cells were washed 2x with PBS and fixed and permeabilized for 5' with cold methanol. After washing 2x with PBS and 1x with PBS/Tween-20, blocking was carried out for 20' with sterile filtered 1%BSA/0,2% skim milk in PBS followed by a 5' blocking of endogenous peroxidase activity using 3% H2O2. Cells were washed as above and a 1:200 dilution of 1°Ab α-NS5A (9E10) in PBS/0,1%tween-20 and incubated on the plate over night at 4°C. After washing, a 1:300 dilution of 2°Ab HRP-goat anti-mouse IgG (H+L) (Amersham Biosciences) in PBS/0,1%tween-20 was added and incubated for 30' at room temperature. Staining was developed using DAB substrate kit (DAKO) for 30' after washing. Wells were scored positive if one or more cells were infected, and the TCID50 was calculated according to the Reed and Muench method.

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

RNA was purified from 200 µL of heat inactivated (56°C for 30') 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 106 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′ UTR 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, unpublished data). 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′, 60°C for 30′ and 95°C for 5′ followed by 45 cycles of 94°C for 20″ and 62°C for 1′. 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. 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.

Diagnostic PCR and direct sequencing of complete ORF

To confirm the identity of the replicating recombinant viruses a diagnostic PCR was developed. RNA extraction was done as described above and reverse transcription was carried out using 200U SuperScriptIII (Invitrogen) and JFH1 specific RT-primer JR3593 (SEQ ID NO 49) for 1h at 50°C in a 20µL volume. Enzyme was inactivated for 15′ at 70°C. The cDNA reaction was treated with 1-4U RNase H (Invitrogen) and 1000U RNase T1 (Ambion) for 20′ at 37°C to degrade RNA. PCR was carried out in 50µL on 2,5µL cDNA reaction mixture using AmpliTaq gold (Applied Biosystems) the same reverse primer (JR3593 (SEQ ID NO 49)) and a genotype specific forward primer (JF2962 (SEQ ID NO 46) or 4aF2676 (SEQ ID NO 31)) yielding genotype specific band lengths. Sequencing determined NS2 junction for ED43/JFH1 recombinants.

Direct sequencing of complete ORF was done to identify adaptive mutations. RNA extraction and reverse transcription was done as described above using 400U SuperScriptIII (Invitrogen) and RT-primer 9470R_JFH1 (SEQ ID NO 59). 1st round PCR was performed in a 50µL volume on 2,5µL of the cDNA reaction using the Advantage 2 PCR Enzyme System (Clontech), the same reverse primer (9470R_JFH1 (SEQ ID NO 59)) and forward primer -285s-HCV-MOD (SEQ ID NO 58). Cycle parameters were 5 cycles of 35'' at 99°C, 30'' at 67°C and 10' at 68°C, 10 cycles of 35'' at 99°C, 30'' at 67°C and 11' at 68°C, 10 cycles of 35'' at 99°C, 30'' at 67°C and 12' at 68°C and 10 cycles of 35'' at 99°C, 30'' at 67°C and 13' at 68°C. 12 ~1kb products were synthesized in a nested PCR covering the entire ORF (nt 297-9427) using primer pairs 1-12 (Table 2). PCR was set up as above using 2,5µL of the 1st round PCR for each reaction. Initial denaturation was 35sec at 99ºC followed by 35 cycles with 35sec at 99ºC, 30sec at 67ºC and 6min at 68ºC. For determination of 5'UTR nt86-296 of selected virus pools another nested reaction was setup using the same 1st round product and primer pair 0 (Table 2). PCR products were agarose gel purified and directly sequenced in both directions.

5'RACE (rapid amplification of cDNA ends)

The extreme 5' end of selected viral genomes was determined following the protocol of the kit '5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0' (Invitrogen) after RNA extraction as above. First strand synthesis was carried out using RT-primer consR337 (SEQ ID NO 62) and by replacing SuperScriptII by SuperscriptIII (Invitrogen), with reverse transcription of 40' at 50°C and 30' at 55°C followed by 15' enzyme inactivation at 70°C. To optimize binding on S.N.A.P. cDNA purification columns, samples were reloaded twice, and 16,5µL of the eluate were used for TdT-tailing according to protocol. 1st round PCR was done according to protocol using reverse primer consR312 (SEQ ID NO 61) and AmpliTaq Gold (Applied Biosystems). Cycle parameters were as following: 10' at 94°C, 40 cycles of 45'' at 94°C, 45'' at 55°C and 1'30'' at 72°C followed by a final 10' at 72°C. A nested PCR was done using reverse primer consR268 (SEQ ID NO 60) and cycle parameters as above.

PCR products were agarose gel purified and directly sequenced.

Clonal sequence analysis of 3rd passage ED43/JFH1-ß and γ viruses.
RNA extraction was done as described above and reverse transcription was carried out using 400U SuperScriptIII (Invitrogen) and RT-primer JR8688 (SEQ ID NO 51) for 1h at 50°C in a 20µL volume. Enzyme was inactivated for 15' at 70°C. The cDNA reaction was treated with 1-4U RNase H (Invitrogen) and 1000U RNase T1 (Ambion) for 20' at 37°C to degrade RNA. A long PCR product was generated with primers -285S_HCV-MOD (SEQ ID NO 58) and JR8368 (SEQ ID NO 50) using the Advantage 2 PCR Enzyme System (Clontech) with the following cycling parameters: 1min at 95ºC, 40 cycles with 35sec at 95ºC, 35sec at 67ºC, 9min at 68ºC, and final extension of 9min at 68ºC. The resulting PCR product was gel purified, subcloned into pCR-XL-TOPO (Invitrogen), and 10 clones per construct were sequenced.

Sequencing, sequence analysis and databases
All sequence reactions was performed at Macrogen Inc., Seoul, South Korea. Sequence analysis was performed with 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 (euHCVdb; http://euhcvdb.ibcp.fr/euHCVdb/)(48) and the American HCV database (LANL; http://hcv.lanl.gov/content/hcv-db/index).

Example 1

In vitro infectivity of ED43/JFH1 recombinants depends on location of the recombination point.
In vitro transcribed RNA from pED43/JFH1-α, -β, -γ (SEQ ID NO 5, 1 and 2) and -GND as well as pJ6/JFH (positive control), was transfected into Huh7.5 hepatoma cells. ED43/JFH1-α, -β and -γ replicated as evidenced by anti-Core immunostaining at day 1 (Figure 1B and C). While J6/JFH infected most Huh7.5 cells (80-100%) within 3 days, the percentage of ED43/JFH1 antigen positive cells decreased. Subsequent viral spread to most cells in the culture was observed for ED43/JHF1-β at day 16 and for ED43/JFH1-γ at day 43 (Figure 1B and C). Cytopathic effects followed by proliferation of infection resistant Huh7.5 cells, occurred after infection spread to most cells. Full culture infection was generally seen for approximately 5-10 days. The delayed production of infectious

ED43/JFH1-β and -γ viruses was confirmed by infectivity titration using the tissue culture infectious dose-50 method ($TCID_{50}$, Table 3). ED43/JFH1-α replication declined until day 19. Hereafter replication was not observed following the culture until the end of the experiment at day 66. Furthermore, no virus production was observed throughout the experiment, as measured by $TCID_{50}$. 1mL of filtered supernatant from J6/JFH (day 6, $10^{4,4}$ $TCID_{50}$), ED43/JFH1-β (day 19, $10^{2,9}$ $TCID_{50}$), ED43/JFH1-γ (day 45, $10^{2,9}$ $TCID_{50}$) and ED43/JFH1-GND (day 19) transfection cultures, was used for 1st passage to naive Huh7.5 cells. Both viable ED43/JFH1 constructs readily infected naïve cells, and required only 8 and 10 days respectively to infect the whole culture (Figure 1C). J6/JFH spread to most cells within 3 days only, reflecting the higher infectious dose of the inoculum.

Example 2

ED43/JFH1 viral spread kinetics resembles J6/JFH while the specific infectivity titers appear to be lower.

J6/JFH and ED43/JFH1-β and -γ in a total of four cell free passages were serially passaged . Peak genome titers around 10^7 IU/mL (IU, International Units) were obtained in all experiments, with J6/JFH peaking at $10^{7,7}$ IU/mL in 4th passage, ED43/JFH1-β at $10^{7,3}$ IU/mL in 1st and 4th passage and ED43/JFH1-γ at $10^{7,2}$ IU/mL in 4th passage. J6/JFH showed infectivity titers of up to $10^{5,1}$ $TCID_{50}$/mL, while the genotype 4 recombinants peaked at $10^{3,5}$ and $10^{3,6}$ $TCID_{50}$/mL, respectively (Table 4). Inconsistency between genome titers and infectivity titers in J6/JFH and the two genotype 4 recombinants was reflected by a difference in specific infectivity (infectious doses per genomes measured in IU). For J6/JFH specific infectivities of 1:100 were observed. Specific infectivities of 1:1600 only was observed for ED43/JFH1-β and -γ.

In order to directly compare the spread and infectivity of the viruses, approximately 10^3 $TCID_{50}$ of 1st passage J6/JFH and 3rd passage J6/JFH, ED43/JFH1-β and -γ were inoculated for 6hrs on naïve Huh7.5 cells. Similar infection spread kinetics was observed for the four virus cultures, and the spread was reflected in genome titers rising above 10^7 IU/mL for all cultures (Figure 2A). Apart from for J6/JFH, 3rd passage inoculum, which produced infectious progeny slightly faster, the initial rise in infectivity titer was similar for J6/JFH and the two

ED43/JFH1 viruses. The ED43/JFH1 production stagnated and titers peaked at $10^{3,4}$ $TCID_{50}$/mL (β) and $10^{3,2}$ $TCID_{50}$/mL (γ) while J6/JFH titers continued to rise to $10^{4,9}$ $TCID_{50}$/mL (Figure 2B), thus confirming observations from earlier passages (Table 4). As the consensus ORF sequence of both J6/JFH pools was confirmed to be identical to the plasmid sequence, the kinetics differences may be ascribed to variation in the assay.

Example 3

Identification of putative adaptive mutations in recovered ED43/JFH1 viruses. The significantly extended time needed for the 4a/2a recombinants to spread in transfection culture relative to subsequent passages may be indicative of requirements of adaptation. Thus, viral RNA was isolated from supernatant at high-titer time points (same used for passage) in 1st, 2nd and 3rd passage, and the complete ORF sequences were analyzed by direct sequence analyses of overlapping nested RT-PCR amplicons. Putative adaptive mutations were found as indicated in Table 5 (A-B). Noteworthy regarding recovered ED43/JFH1-β viruses is the occurrence of one coding mutation in the ED43 part of NS2 (A2819G), which changed completely already in 1st passage. No other dominating coding mutations where observed, except for an NS5A mutation (G7646T) present only in virus recovered from 3rd passage. The NS2 mutation A2819G appeared to be essential for the viability of ED43/JFH1 recombinants, since the same mutation occurred also in ED43/JFH1-γ 1st passage virus. Two other dominating coding mutations were observed in 1st passage ED43/JFH1-γ; A1325T in the E1 gene and A3269T in the γ-specific ED43 part of NS2. Again, additional dominating coding mutations were only observed in 3rd passage (G986A in E1 and T7148C in NS5A). Dominating mutations occurring in 1st passage were all consistently seen in 2nd and 3rd passage viruses, except for the γ-specific A1325T continuing only as a mixture with the original sequence. Additional positions showed co-existence of original and mutant sequence in direct sequencing of 1st, 2nd and 3rd passage. The complete 5'UTR of 1st passage viruses was sequenced, and no mutations were found.

Clonal analysis of 3rd passage viral quasispecies

Co-existence of different viral quasispecies in the 3rd passage was investigated by amplifying nt86-8335 of ED43/JFH1-β and -γ in a single long RT-PCR. 10 clones of each were sequenced (Table 5, A-B).

Analysis confirmed the complete change of A2819G, which was found in all clones from both constructs. 9/10 ED43/JFH1-β clones acquired the coding G7646T NS5A mutation. 5 and 6 of these 9 clones further carried coding mutations G3305A in the JFH1 part of NS2 and C6306T in the NS5A membrane anchor, respectively. Non-coding mutations were observed in Core, E2 and NS3 as indicated in table 5A. All 10 ED43/JFH1-γ clones, in addition to A2819G, confirmed the complete change of A3269T. Furthermore, 7/10 clones carried a distinct pattern of mutations including two coding mutations (G986A in E1 and T7148C in NS5A). By direct sequencing these were observed in 3rd passage only. Interestingly, A1325T was not observed in the 10 sequenced 3rd passage γ-clones. Non-coding mutations were observed in E1 and NS3.

Example 4

Identification of ED43/JFH1 vital adaptive mutations by reverse genetics

Adaptive mutations were investigated by introducing selected mutations back into the ED43/JFH1-β and -γ cDNA clones. Coding mutations being dominant already in 1st passage virus pools was included as such mutations were likely to prove vital for infectivity. Accordingly pED43/JFH1-β_{A2819G} (SEQ ID NO 7), pED43/JFH1-γ_{A2819G} (SEQ ID NO 9), pED43/JFH1-γ_{A3269T} (SEQ ID NO 10), pED43/JFH1-$\gamma_{A2819G,A3269T}$ (SEQ ID NO 8) and pED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$ (SEQ ID NO 11) were generated. RNA transcripts from mutated plasmids were transfected into Huh7.5 cells along with RNA transcripts from the original ED43/JFH1 plasmids, J6/JFH and ED43/JFH1-GND. While the percentage of cells infected with the original constructs decreased as previously observed, J6/JFH, ED43/JFH1-β_{A2819G}, ED43/JFH1-$\gamma_{A2819G,A3269T}$ and ED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$ infection immediately spread in the culture as apparent from Figure 3A. This was supported by infectivity titers measured on day 3, 5, 7 and 10 (Figure 3B). J6/JFH titers peaked at approximately 10^4 $TCID_{50}$/mL, while ED43/JFH1-β_{A2819G} and ED43/JFH1-$\gamma_{A2819G,A3269T}$ peak titers were approximately 10-fold below the J6/JFH titer.

ED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$ peaked at $10^{2,4}$ $TCID_{50}$/mL. Though sufficient for ED43/JFH1-β, single introduction of A2819G into the γ-construct (pED43/JFH1-γ_{A2819G}) was not sufficient to confer viability in cell culture (Figure 3A and B). In analogy to transfection of the original construct, efficient spread in culture was delayed, here by 42 days. Sequencing of the complete ORF at day 42 revealed the occurrence of the A3269T mutation, thus emphasized the need for the two described NS2 mutations. In addition the virus acquired four completely changed non-coding mutations (G3154A, A4152G, T6184G and T7879C) and two positions with presence of both the original and the mutated sequence (A387C coding and C436A non-coding). The ED43/JFH1-γ_{T977S} transfection culture produced low or undeterminable infectivity titers (Fig. 6), until the virus finally infected most cells on day 30 (Figure 7) where it had acquired the T827A amino acid change as well as C4944T and T7125C coding for T1535I and L2262P. Introduction of the E1 mutation A1325T indicated a reduction of viral fitness as reflected by percentage of infected cells, infectivity titer (Figures 3A and B) and cytopathic effects observed in cell culture (data not shown). At day 5 transfection supernatants of ED43/JFH1-β_{A2819G}, ED43/JFH1-$\gamma_{A2819G,A3269T}$ and ED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$ were passed to naïve Huh7.5 cells along with J6/JFH and ED43/JFH1-GND. Infection was confirmed by anti-Core staining. The complete ORF of the infectious progeny from day 17 was sequenced to investigate the genetic stability of the mutated constructs. ED43/JFH1-β_{A2819G} and ED43/JFH1-$\gamma_{A2819G,A3269T}$ both showed the expected sequence after 17 days of 1st passage infection with no additional mutations. Introduction of A1325T conferred need for genomic adaptation; ED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$ acquired two dominating changes (T7809C coding and C781A noncoding) as well as a position with presence of both original and changed sequence (A5592G).

Thus introduction of A2819G into pED43/JFH1-β and A2819G combined with A3269T into pED43/JFH1-γ immediately rescued the production of infectious viruses in Huh7.5 cells. These adapted constructs showed genetic stability during transfection and 1st viral passage. Introduction of A1325T resulted in an attenuated phenotype and requirements for further genomic adaptation.

To test whether the additional coding mutations observed in 3rd passage clonal analysis of ED43/JFH1-β and ED43/JFH1-γ (Tables 5A and 5B) could further

improve viral infectivity, pED43/JFH1-$\beta_{T827A,E989K}$(SEQ ID NO 12), pED43/JFH1-$\beta_{T827A,V2436L}$(SEQ ID NO 13) and pED43/JFH1-$\beta_{T827A,E989K,T1989I,V2436L}$(SEQ ID NO 14) as well as pED43/JFH1-$\gamma_{A216T,T827A,T977S}$(SEQ ID NO 15), pED43/JFH1-$\gamma_{T827A,T977S,C2270R}$(SEQ ID NO 16) and pED43/JFH1-$\gamma_{A216T,T827A,T977S,C2270R}$(SEQ ID NO 17) were constructed. However, no significant increase of peak titers was observed after transfection of Huh7.5 cells (Fig. 6).

Example 5

ED43/JFH1 infection depend on CD81

The tetraspanin cell surface molecule CD81 is expressed on various cells including hepatocytes and has been shown to interact with the E2 protein. Studies have shown that CD81 antibodies block infection of Huh7 cells with HCV pseudo particles as well as JFH1 viruses. In order to investigate whether ED43/JFH1 infection depend on CD81, Huh7.5 cells were incubated with CD81 antibodies in different concentrations before infection with approximately 100 $TCID_{50}$ 3rd passage and -γ viruses as well as ED43/JFH1-β_{A2819G} and ED43/JFH1-$\gamma_{A2819G,A3269T}$ viruses from transfection cultures. CD81 antibodies specifically blocked infection of all viruses, and ≥95% inhibition was achieved by incubation with 2,5µg/mL CD81 antibody. Except for one of three wells with ED43/JFH1-β infection, 12,5µg/mL CD81 antibody prevented infection entirely (Figure 4).

EXAMPLE 6

Testing of cross-genotype neutralization of genotype 1-6 recombinant viruses with 1a and 4a anti-sera.

To further investigate the biological relevance of ED43/JFH1 viruses, ~100 $TCID_{50}$ ED43/JFH1-γ were incubated with serial 2-fold dilutions of chronic phase serum from a genotype 4a infected patient (AA) before infection of Huh7.5 cells. In contrast to incubation with HCV negative control serum, incubation with AA serum reduced the number of FFUs in a concentration dependent manner, yielding a 50% neutralization titer of 1:6400 (Fig. 5, Table 6). Thus, the 4a/JFH1 viruses could be efficiently neutralized with homologous patient serum.

Homologous neutralization of recombinant H77C/JFH1 virus was demonstrated with serum from Patient H, taken 29 years after acute infection (H06). Serial 2-fold dilutions of H06 serum were used to neutralize ~100 $TCID_{50}$ of H77C/JFH1, yielding a 50% neutralization titer of 1:1600 (Table 6). The H06 1a serum efficiently neutralized ED43/JFH1-γ (4a/JFH1) with a 50% titer of 1:12800, while the AA 4a serum showed low-level neutralization of H77C/JFH1 with a 50% titer of 1:50 (Table 6). To further broaden the investigation of cross-genotype neutralization, serial 2-fold dilutions of 1a and 4a sera were tested against ~100 $TCID_{50}$ of JFH1-based recombinant viruses expressing the envelope proteins of genotype 2a, 3a, 5a, and 6a. Genotype 2a and 3a viruses could not be neutralized at a 1:50 dilution of either serum. However, genotype 5a and 6a viruses were efficiently neutralized by both sera with 50% neutralization titers of at least 1:3200 (Table 6).

Example 7

4a/JFH1 infection depends on SR-BI.

Blockage of the cell surface molecule SR-BI was previously shown to inhibit entry of HCV pseudo particles or cell culture grown virus of genotype 2a into Huh7 and derived cell lines. To investigate the SR-BI dependence of genotype 4a entry, Huh7.5 cells were incubated with anti-SR-BI serum before infection with ~300 TCID50 (150 FFUs) ED43/JFH1-γ. Anti-SR-BI serum but not a control serum reduced the number of focus forming units (FFUs) in a concentration dependent manner (Fig. 8).

Example 8

Use of the 4a/JFH1 system in drug testing: infected cells can be cured using the currently used antiviral IFN-α.

As an example of the use of the 4a/JFH1 system in test of antiviral drugs, the inventors of the present invention applied the antiviral drug IFN-α 2b, which is a constituent of the currently used combination therapy against HCV also involving ribavirin, to 4a/JFH1 infected cell cultures. Huh7.5 cells were infected with 4a/JFH1 (MOI 0,003). On day 5, when infection had spread to approximately 40%

of the cells, 500 IU/mL IFN-α were applied every 1-3 days. Already from the third day of the treatment period, the percentage of infected cells was strongly diminished. A complete curing of the culture, as determined by the absence of NS5A antigen positive staining cells, was achieved after 38 days of treatment with 500 IU/mL IFN-α (Fig. 9).

Example 9

Cross-genotype neutralization potential of genotype 5a sera.

The inventors of the present invention found that chronic phase sera from genotype 1a and 4a infected patients could cross-neutralize intergenotypic recombinant viruses of genotype 1a, 4a, 5a and 6a, but failed to neutralize recombinants of genotype 2a and 3a. Thus, the ability of 5a sera (SA1, SA3, SA13, two-fold dilutions starting at 1:100) to cross-neutralize intergenotypic recombinants of the different genotypes were examined. The SA1 serum had the highest reciprocal 50% cross-neutralization titers, being 1,600, 400 and >51,200 against genotype 1a, 4a, and 6a viruses, respectively (Table 7). The SA3 and SA13 sera had limited or no cross-neutralization activity against genotype 1a and 4a viruses, but both sera had relative high titers of neutralizing antibodies against the 6a virus (Table 7). The 5a sera had no detectable cross-neutralizing activity against the genotypes 2a and 3a viruses at the 1:100 dilution. However, when subsequently testing a 1:50 dilution of the SA1 serum, which had the highest homologous neutralization titer, the inventors of the present invention observed >50% neutralization of the genotype 2a and 3a viruses (data not shown).

Figure legends

Figure 1

Intergenotypic recombinant 2a/2a and 4a/2a constructs and their replication in Huh7.5 cells. (A) Genome map of J6/JFH (genotype 2a/2a) and ED43/JFH1 (genotype 4a/2a) cDNA clones. The 4a/2a constructs contain the Core, E1, E2, p7 and part of or all of NS2 genes from isolate ED43 (genotype 4a). pED43/JFH1-α (SEQ ID NO 5) has its 3' junction between the first and second TM domain (nt2866/2867, H77 reference (AF009606) nt2867/2868) of NS2, while pED43/JFH1-ß (SEQ ID NO 1) has the junction placed in the cytoplasmic part of NS2 (nt 3185/3186 H77 reference (AF009606) nt3186/3187) in analogy to a naturally occurring 2k/1b isolate. The complete NS2 gene from ED43 is included in pED43/JFH1-γ (SEQ ID NO 2) and the negative replication control ED43/JFH1-GND. All constructs contain the 5'UTR, NS3 through NS5B and the 3'UTR of the JFH1 genome. (B) After immuno-staining for HCV Core, the percentage of infected cells was scored. Supernatant from day 6 (J6/JFH), day 19 (ED43/JFH1-ß) and 45 (ED43/JFH1-γ) was used for 1st passage (arrows). A decreasing number of ED43/JFH1-α Core positive cells was observed until day 19. During continuation of the experiment hereafter and until day 66, no positive cells could be detected. The ED43/JFH1-GND transfected culture remained negative throughout the experiment. (C) Huh7.5 cells were transfected in parallel with 1,5µg RNA transcripts from pJ6/JFH (positive control), pED43/JFH-GND (negative control) and pED43/JFH1-α , -ß and –γ (SEQ ID NO 5, 1 and 2), respectively. Immunostainings for HCV Core in transfection and 1st passage experiments are shown. Core antigen is visualized in red by confocal fluorescence microscopy. Cell nuclei were counterstained using Hoechst reagent (blue).

Figure 2

Comparison of J6/JFH and ED43/JFH1-ß and -γ infection kinetics. Naïve Huh7.5 cells were inoculated for 6hrs with approximately 10^3 TCID50 of 1st passage J6/JFH (J6/JFH#, titer measured in 4 replicates) and 3rd passage J6/JFH (J6/JFH§) and ED43/JFH-ß and -γ (titers measured in duplicates). (A) Viral RNA titers of culture supernatant were monitored by a quantitative HCV TaqMan RT-PCR assay. nd: not determinable; below assay detection limit of 500 IU/mL. (B)

After immuno-staining for Core antigen, the percentage of infected cells was visualized and scored using confocal fluorescence microscopy. (C) Infectivity titers were determined for supernatant collected on day 3, 6, 8 and 10 by the TCID50 assay (dark coloured coloumns). To visualize the difference between HCV RNA- and infectivity- titers (reciprocal specific infectivity), RNA titers are shown in IU/mL (light coloured coloumns).

Figure 3

Transfection of Huh7.5 cells with 2,5µg RNA transcripts of ED43/JFH1 intergenotypic recombinants with putative adaptive mutations (SEQ ID NO 7, 8, 9, 11). RNA transcripts from J6/JFH (positive control), ED43/JFH1-GND (negative control) and the original pED43/JFH1-ß and -γ constructs (SEQ ID NO 1 and 2) were tested in parallel. (A) After immuno-staining for Core antigen the percentage of infected cells was visualized and scored using confocal fluorescence microscopy. As indicated, ED43/JFH1-γ_{A2819G} did not spread within the depicted timeframe. However, the infection spread from below 1% at day 31 to more than 50% at day 42 (data not shown). Day 5 supernatants from J6/JFH, ED43/JFH1-GND, ED43/JFH1-$ß_{A2819G}$, ED43/JFH1-$\gamma_{A2819G,A3269T}$ and ED43/JFH1-$\gamma_{A1325T,A2819G,A3269T}$ cultures were used for inoculation of the 1st passage (arrow). (B) Comparison of TCID50 values of viruses recovered from supernatant at days 3, 5, 7 and 10 post-transfection. nd: not determined. §: One of six replicates infected by undiluted supernatant only; TCID50 value undeterminable. #: None of 6 replicate wells infected by undiluted supernatant.

Figure 4

Blocking of CD81 inhibits ED43/JFH1-ß and -γ infection. Huh7.5 cells growing in a 96 well plate were incubated for one hour at 37°C with anti-CD81 antibodies or anti-HIV-p24 isotype-matched control antibodies in concentrations as indicated. Approximately 100 TCID50 (measured in duplicates) of ED43/JFH1-ß 3rd passage virus (A), ED43/JFH1-γ 3rd passage virus (B), ED43/JFH1-$ß_{A2819G}$ from transfection culture (C) or ED43/JFH1-$\gamma_{A2819G,A3269T}$ from transfection culture (D) was added and incubated with the cells for 4 hours at 37°C. The count of focus forming units (FFU) per well after 2 days of growth is indicated. Each data point was determined in triplicates. Error bars indicate standard error of the mean.

Figure 5

Neutralization of ED43/JFH1 virus. ~100 $TCID_{50}$ of ED43/JFH1-γ 1st passage virus were incubated with serial 2-fold dilutions of genotype 4a (AA, gray) or genotype 1a (H06, white) chronic phase patient samples or a mixture of sera from four HCV negative controls (black) in final dilutions as indicated, prior to incubation with Huh7.5 cells. The count of FFUs per well after an incubation period of 2 days is indicated. Each data point represents triplicate experiments. Error bars indicate standard errors of the mean.

Figure 6

Transfection of RNA transcripts from pJ6/JFH, pED43/JFH1-GND and pED43/JFH1-γ_{T977S} (SEQ ID NO 10), as well as pED43/JFH1-β_{T827A} and pED43/JFH1-$\gamma_{T827A,T977S}$ constructs with or without mutations observed in 3rd passage (SEQ ID NO 7, 8, 12, 13, 14, 15, 16 and 17). $TCID_{50}$ determinations on transfection supernatants are shown. ‡, two ($TCID_{50}$ <0.7) of 6 replicates infected by undiluted supernatant. ED43/JFH1-GND was confirmed negative.

Figure 7

Transfection of Huh7.5 cells with ED43/JFH1 recombinants with putative adaptive mutations. ED43/JFH1-β_{T827A} (SEQ ID NO 7), ED43/JFH1-$\beta_{T827A,E989K}$ (SEQ ID NO 12), ED43/JFH1-$\beta_{T827A,V2436L}$ (SEQ ID NO 13) and ED43/JFH1-$\beta_{T827A,E989K,T1989I,V2436L}$ (SEQ ID NO 14) as well as ED43/JFH1-$\gamma_{T827A,T977S}$ (SEQ ID NO 8), ED43/JFH1-γ_{T977S} (SEQ ID NO 10), ED43/JFH1-$\gamma_{A216T,T827A,T977S}$ (SEQ ID NO 15), ED43/JFH1-$\gamma_{T827A,T977S,C2270R}$ (SEQ ID NO 16) and ED43/JFH1-$\gamma_{A216T,T827A,T977S,C2270R}$ (SEQ ID NO 17) RNA transcripts were transfected into naïve Huh7.5 cells. After immunostaining, the percentage of HCV Core positive cells was scored using confocal fluorescence microscopy.

Figure 8

Blocking of SR-BI inhibits ED43/JFH1 infection.
Huh7.5 cells growing in a 96 well plate were incubated for one hour at 37°C with SR-BI anti-serum or a control serum in dilutions as indicated. Approximately 300 TCID50 ED43/JFH1-γ were added and incubated with the cells for 3 hours at

37°C. The count of focus forming units (FFU) per well after 2 days of growth is indicated. Each data point was determined in triplicates. Error bars indicate standard error of the mean.

Figure 9

INF-α treatment significantly reduces 4a/JFH1 infection of Huh7.5 cells. Huh7.5 cells were infected with ED43/JFH1 (MOI 0,003). On day 5, when infection had spread to approximately 40% of the cells, 500 IU/mL IFN-α were applied every 1-3 days. A complete curing of the ED43/JFH1 culture, as determined by the absence of NS5A antigen positive staining cells, was achieved after 38 days of treatment with 500 IU/mL IFN-α.

Tables

TABLE 1

Primers and primer sequences for PCR

Primer	SEQ ID NO	5'-3' Sequence
4aF193	29	TTTCTTGGATTAACCCGCTCAATG
4aF1G-NotI-T7	30	**TTTTTTTTGCGGCCGC*TAATACGACTCACTATA***GACCTGCTCTCTATGAGAGCAACACTCC
4aF2676	31	AGGGCCGGTTCCCAGCTGCT
4aF2719	32	GTGGCCCTGTTTTCTCCTGCTTC
4aF309	33	AGTGCCCCGGGAGGTCTCGTAG
4aF5446	34	CCAACAGTTCGACGAAATGGAGGAGTGTTC
4aF741	35	TGGGATACATCCCGCTCGTAGG
4aF9251	36	GGCGCCGGCGGGGGAGACATTTATCACAGC
4aF9271-HindIII	37	**GTCCAAGCTT**ATCACAGCATGTCTCATGCCCGACCCCG
4aR262	38	ACACTACTCGGCTAGCAGTCTTGC
4aR489	39	CGAGTCGCGCGCACACCCAATC
4aR5664	40	AGATATTGAATGCCGCTGATGAAATTCCACATG
4aR862	41	AAAGGAGCAACCGGGGAGATTC

4aR9406	42	AAAAAACAAGGGGACCCTAAGGTCGGAGTG
4aR9491-Xba	43	**CGTCTCTAGA**GGACCTTTCACAGCTAGCCGTGACTAGGG
4aR9504	44	TCATGCGGCTCACGGACCTTTCACAGCTAG
JF2879	45	CTGTGGTGGTTGTGCTATCTCC
JF2962	46	TGATGGCATCATATGGGCCGTC
JF3198	47	TCTATGACCACCTCACACCTATG
JR345	48	CTCATGGTGCACGGTCTACGAGA
JR3593	49	TTGCCAGCTCCGTGGTAAAC
JR8368	50	CCTGATGTCTCTCTCAGTGAC
JR8688	51	TCCGTGAAGGCTCTCAGGTTC
RU-O-5720	52	GCTCCCATCACTGCTTATGCC
RU-O-5721	53	GCTACCGAGGGGTTAAGCACT
JVF12328	54	CGTTGTAAAACGACGGCCAGTGA
2aR2905/4aR2866	55	*GGAGATAGCACAACCACCACAG*TCCCCTAGCCAGCCATAACTTG
2aR3220/4aR3185	56	*CATAGGTGTGAGGTGGTCATAGA*TGTAAGTACCAGTCAGGGCCCC
2aR3451/4aR3419	57	*GGCATAAGCAGTGATGGGAGC*AAGGAGTCTCCACCCCTTTG
-285s-HCV-MOD	58	ACTGTCTTCACGCAGAAAGCGCCTAGCCAT

9470R_JFH1	59	CTATGGAGTGTACCTAGTGTGTGC
consR268	60	ACCCAACGCTACTCGGCTA
consR312	61	CGCAAGCRCCCTATCAGGCAGTACC
consR337	62	GGTCTACGAGRCCTCCCGGGGCAC

Bold: non-HCV sequence. Bold italics: T7 promotor sequence. Italics: intergenotype primers, genotype 2a sequence.
Underlined: Restriction sites.

TABLE 2

Primers and primer sequences used for generation of amplicons for sequencing as described in Materials and Methods.

Primer pair	Forward	5'-3' Sequence	SEQ ID NO	Reverse	5'-3' Sequence	SEQ ID NO
ED43/JFH1						
0	-285S_HCV-MOD	ACTGTCTTCACGCAGAAAGCGCCTAGCCAT	58	4aR705	ACCTTACCCAAATTGCGGGACCTC	67
1	-84S_HCV-MOD	GTAGCGTTGGGTTGCGAAAGGCCTTGTGGTACTGCCTGAT	63	4aR1080	GCGACGGTAGGAGTAAGGGCCACCC	68
2	4aF965	AATTCAAGCATAGTGTATGAGGCCGAC	64	4aR2010	TTCATCCACACGCATCCAAACC	69
3	4aF1910	GGGGTCCCTACTTACACCTGGGG	65	4aR2871	CACAATCCCCTAGCCAGCCATAAC	70
4	4aF2719	GTGGCCCTGTTTTCTCCTGCTTC	66	3329R_JFH1-MOD	CCCTCAGCACTCGAGTACATCTG	71
J6/JFH						
0	-285S_HCV-	ACTGTCTTCACGCAGAAAGCGCCTAGCCAT	58	JR513	gctgggaccgctccgaag	75

	MOD					TTTGCCCACGCTCCCTG	
	-84S_HCV-	AGGCCTTGTGGTACTGCC		1109R_J6	CATAGAGAA		76
1	MOD	TGAT	63			TGCACGTCCACGATGTT	
		CACCGCATGGCGTGGGA		2111R_J6	TTGGTG		77
2	946S_J6	CATGATG	72			AGCGTGAGCCCTGACG	
		TACAGGCTCTGGCATTAC		2763R_J6	AAGTACGG		78
3	1849S_J6	CCCTGCAC	73			GGGATGACATCAGCGTT	
		TAGCATTGCCCCAACAGG		3774R_J6	CCGCGTGACCAG		79
4	2754S_J6	CTTATGCTTATGACG	74				
JFH1				4118R_JFH	CGCCCGAGGCCTACCTC		
	3081S_J6/J	GGAGTCTTCTCGCTCCCA		1	TTCTATATC		88
5	FH1	TCACTGC	80	4796R_JFH	GCGCACACCGTAGCTTG		
		CCCATCACGTACTCCACA		1	GTAGG		89
6	3880S_J6	TATGGC	81	5446R_JFH	TGATGTTGAGAAGGATG		
		GAGCGAGCCTCAGGAAT		1	GTGGTAC		90
7	4528S_J6	GTTTGACA	82		CAACGCAGAACGAGAC		
	5272S_JFH	TGGCCCAAAGTGGAACAA		6460R_J6	CTCATCCC		91
8	1	TTTTGG	83	7234R_JFH	GAAGCTCTACCTGATCA		
	6186S_JFH	GACCTTTCCTATCAATTG		1	GACTCCA		92
9	1	CTACAC	84				

	6862S_JFH	TGGGCACGGCCTGACTA		7848R_JFH	GGCCATTTTCTCGCAGA	
10	1	CAA	85	1	CCCGGAC	93
		ATGGCCAAAAATGAGGTG		8703R_JFH	AAGGTCCAAAGGATTCA	
11	7741S_J6	TTCTGC	86	1	CGGAGTA	94
	8137S_JFH	GGTCAAACCTGCGGTTAC		9464R(24)	GTGTACCTAGTGTGTGC	
12	1	AGACGTTG	87	_JFH1	CGCTCTA	95

TABLE 3

Tranfection culture of J6/JFH and ED43/JFH1-α, -ß and -γ was monitored for viral infectivity by the TCID50 assay. This titration method gives an endpoint, based on the virus dilution infecting half of the six replicates tested (1). Results are given in infectious doses per mL. The infectivity titer for each culture was measured on day 6, 19 and 45 (time points where J6/JFH, ED43/JFH1-ß and ED43/JFH1-γ infection, respectively, spread to most cell in the culture) or until percentage of infected cells peaked. nd: not determined. §: One of six replicates infected by undiluted supernatant only; TCID50 value undeterminable. #: None of 6 replicate wells infected by undiluted supernatant.

Infectivity titer / Log(TCID-50/mL)	**Day**		
	6	19	45
J6/JFH	4,4	nd	nd
ED43/JFH1-α	#	#	#
ED43/JFH1-ß	§	2,9	nd
ED43/JFH1-γ	#	#	2,9

TABLE 4

Infectivity titer, RNA titer and specific infectivity for J6/JFH and ED43/JFH1-ß and -γ. Viruses were serially passaged in four cell free passages. In each passage, infectivity titer in supernatant was determined at selected timepoints during the period with most cells infected. For each passage selected samples and correlating RNA titers are shown. The reciprocal specific infectivity (viral genome titer / dose TCID50) is given.

Virus construct	**Passage**	**Days post infection**	**Infectivity titer / log($TCID_{50}$/mL)**	**RNA titer / log(IU/mL)**	**Reciprocal specific infectivity, log-transformed**
J6/JFH	1st	7	5,1	7,1	2,0
	2nd	12	4,3	6,7	2,4
	3rd	6	3,5	6,9	3,4
	4th	8	4,9	7,6	2,7
ED43/JFH-ß	1st	10	3,5	6,7	3,2
	2nd	7	3,3	6,8	3,5
	3rd	10	3,1	7,0	3,9
	4th	10	3,4	7,2	3,8
ED43/JFH-γ	1st	12	3,6	6,8	3,2
	2nd	15	2,7	6,8	4,1
	3rd	19	3,4	6,8	3,4
	4th	10	3,2	6,6	3,4

TABLE 5A

Mutations of ED43/JFH1-β during serial passages in Huh7.5 cells.

HCV gene*	**Core**	**E2**	**NS2**	**NS2**	**NS3**	**NS5A**	
Nucleotide number†							
ED43/JFH1-β	**787**	**2206**	**2819**	**3305**	**4222**	**6306**	**7646**
H77 abs. ref.	**788**	**2207**	**2820**	**3306**	**4223**	**6307**	**7593**
pED43/JFH1-β	**G**	**C**	**A**	**G**	**C**	**C**	**G**
Direct sequencing‡							
1st passage	**.**	**.**	**G**	**.**	**.**	**.**	**.**
2nd passage	**G/a**	**.**	**G**	**G/a**	**C/t**	**.**	**G/t**
3rd passage	**A/g**	**C/T**	**G**	**G/A**	**C/T**	**C/T**	**T/g**
3rd passage clonal distribution§							
3 / 10	**A**	**T**	**G**	**A**	**T**	**T**	**T**
2 / 10	**A**	**T**	**G**	**A**	**T**	**.**	**T**
2 / 10	**.**	**.**	**G**	**.**	**.**	**T**	**T**
1 / 10	**A**	**.**	**G**	**.**	**.**	**T**	**T**
1 / 10	**.**	**.**	**G**	**.**	**.**	**.**	**T**
1 / 10	**.**	**.**	**G**	**.**	**.**	**.**	**.**
Amino acid number†							
ED43/JFH1-β			**827**	**989**		**1989**	**2436**
H77 abs. ref.			**827**	**989**		**1989**	**2418**
Change			**T → A**	**E → K**		**T → I**	**V → L**

*Mutations within the region of nucleotide 341-3185, both incl. are in the ED43 region, others are in the JFH1 region

†Positions are numbered according to the HCV sequence of pED43/JFH1-β. Corresponding H77 reference positions (AF009606) are given.

‡Mutations representing ≥ 50% of the sequence read in at least one passage. Positions with mixtures are written with the dominant sequence in capital and the minor sequence in lower case letters, or with both capitalized wherever a dominant nucleotide was not determinable. Dots indicate identity with the original plasmid sequence.

§ In addition to indicated mutations, G7147C (E2269D) and A7640G (T2434A) were present in 3 clones and C1944G (T535S), T2021C (F561L), A2772G (D811G), T3392C (Y1018H), and T5836C (nc) were present in 2 clones. In each clone a number of single mutations were found, yielding an average of 22 mutations in total per clone. Thus, all clones had unique sequences.

TABLE 5B

Mutations of ED43/JFH1-γ during serial passages in Huh7.5 cells*.

HCV gene	E1			NS2			NS3		NS5A		
Nucleotide number†											
ED43/JFH1-γ	**986**	**1325**	**1336**	**2785**	**2819**	**3269**	**4459**	**4918**	**7022**	**7128**	**7148**
H77 abs. ref.	**987**	**1326**	**1337**	**2786**	**2820**	**3270**	**4460**	**4919**	**7023**	**7141**	**7161**
pED43/JFH-γ	**G**	**A**	**A**	**A**	**A**	**A**	**C**	**G**	**G**	**A**	**T**
Direct sequencing											
1st passage	.	**T/a**	**G**	**A/G**	**G**	**T**	.	.	**G/A**	**A/g**	.
2nd passage	.	**A/T**	**G/a**	**A/G**	**G**	**T**	**C/T**	**G/A**	**G/a**	**A/G**	.
3rd passage	**A/g**	**A/t**	**G/a**	**A/g**	**G**	**T**	**T**	**A**	.	.	**C**
3rd passage clonal distribution‡											
7 / 10	**A**	.	**G**	.	**G**	**T**	**T**	**A**	.	.	**C**
2 / 10	.	.	**G**	.	**G**	**T**	.	.	.	.	.
1 / 10	.	.	.	.	**G**	**T**	.	.	.	.	.
Amino Acid number											
ED43/JFH1-γ	**216**	**329**			**827**	**977**			**2228**	**2263**	**2270**
H77 abs. ref.	**216**	**329**			**827**	**977**			**2228**	**2267**	**2274**
Change	**A → T**	**T → S**			**T → A**	**T → S**			**D → N**	**E → G**	**C → R**

* Mutations within the region of nucleotide 341-3418, both incl. are in the ED43 region, others are in the JFH1 region. See Table 6 legend for further details.

†Positions are numbered according to the HCV sequence of pED43/JFH1-γ.

‡In addition to indicated mutations, G1026A (C229Y), T1150C (nc), C2480T (L714F), A2995G (nc), C3001G (D887E), G7291A (nc) and T7985C (nc) were present in 3 clones; G723A (C128Y), T1211G (F291V), T1369C (nc), A2114G (T592A), G2251A (nc), T2916C (V859A), T2937C (V866A), G3208A (M956I), T4540C (nc), A5668G (nc), A6248G (I1970V), A7103G (M2255V), G7534A (nc) and G7584A (G2415E) were present in 2 clones.

TABLE 6

Reciprocal titers of neutralizing antibodies in chronic phase serum from patients infected with HCV genotype 1a (H06) and 4a (AA) against JFH1-based viruses representing the 6 HCV genotypes.

	90% reciprocal serum neutralizing antibody titer		**50% reciprocal serum neutralizing antibody titr**	
Envelope genotype	**1a (H06)**	**4a (AA)**	**1a (H06)**	**4a (AA)**
1a	50	<50	1600	50
2a	<50	<50	<50	<50
3a	<50	<50	<50	<50
4a	800	400	12800	6400
5a	3200	800	25600	3200
6a	25600	3200	204800	25600

~100 $TCID_{50}$ of JFH1-based recombinant virus containing Core-NS2 of each of the 6 major genotypes were incubated in triplicates with a 2-fold dilution series of genotype 1a or 4a chronic phase patient serum or a mixture of sera from four HCV negative controls and tested in Huh7.5 cells. Reciprocal neutralization titers are indicated as the highest dilution showing a reduction in FFUs of at least 90% or 50% compared to HCV negative controls.

Table 7

Neutralization of JFH1-based HCV recombinants of genotypes 1-6 with genotype 5a sera.

	Envelope genotype of JFH1-based recombinants					
	1a	2a	3a	4a	5a	6a
	Reciprocal 50% neutralization titer					
SA1 serum	1,600	<100	<100	400	25,600	>51,200 [#]
SA3 serum	<100	<100	<100	200	6,400	12,800
SA13 serum	<100	<100	<100	<100	1,600	3,200

Neutralization of HCV was performed against 100-200 $TCID_{50}$ of JFH1-based recombinants containing Core-NS2 of genotypes 1-6. The recombinant viruses were incubated in triplicates with 2-fold serial dilutions of chronic phase sera of patients infected with HCV genotype 5a (SA1, SA3 and SA13) or 2-fold serial dilutions of a control serum mixture before testing on Huh7.5 cells. Reciprocal neutralization titers were determined as the highest serum dilution showing a reduction of 50% FFUs compared to the average FFUs counts for the control serum. The average FFUs in the controls in the different genotype experiments ranged from 10 to 70 FFUs/well. In control serum experiments for a particular genotype there was no significant difference in the FFU counts between the different dilutions (data not shown). [a] Neutralization ~75% at a 1:51,200 dilution.

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Claims

1. A replicating RNA comprising the structural genes (Core, E1, E2), p7 and the non-structural gene NS2 of genotype 4a and the non-structural genes NS3, NS4A, NS4B, NS5A and NS5B and part of NS2 from the JFH1 strain.

2. A replicating RNA according to claim 1, wherein part of the non-structural gene NS2 is of genotype 4a and part is of the JFH1 strain.

3. A replicating RNA according to any of claims 1-2, wherein the genotype 4a is of the strain ED43.

4. A replicating RNA according to any of claims 1-3, wherein the strains are ED43/JFH1-α , ED43/JFH1-β or ED43/JFH1-γ.

5. An isolated nucleic acid molecule which encodes human hepatitis C virus of genotype 4a/JFH1, wherein said molecule is capable of expressing said virus when transfected into cells and wherein said molecule encodes the amino acid sequence with a sequence identity of at least 90% to that of SEQ ID NO 3 and/or SEQ ID NO 4 and/or SEQ ID NO 6.

6. An isolated nucleic acid molecule according to claim 5, wherein said isolated nucleic acid molecule is further capable of infectivity *in vivo*.

7. A nucleic acid molecule according to claim 5, wherein said molecule comprises the nucleic acid sequence with a sequence identity of at least 90% to that of SEQ ID NO 1 and/or SEQ ID NO 2 and/or SEQ ID NO 5.

8. A nucleic acid molecule according to any of claims 5-7, wherein said molecule comprises adaptive mutations in E1 and NS2 and NS5A

9. A nucleic acid molecule according to claim 8, 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 G787A, C1944G, T2021C, C2206T, A2772G, A2819G, G3305A, T3392C, C4222T, T5836C, C6306T, G7147C, G7198A, A7640G, G7646T and G8150A.

10. A nucleic acid molecule according to claim 8, 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 G787A, C2206T, A2819G, G3305A, C4222T, C6306T and G7646T.

11. A nucleic acid molecule according to claim 8, wherein said adaptive mutation is at least one of the replacements of the first said nucleotide at the said position of SEQ ID NO 2 by the following said nucleotide selected from the group consisting of C373T, A387C, C436A, G723A, C781A, G986A, G1026A, T1150C, T1211G, A1325T, A1336G, T1369C, T2093A, A2114G, G2251A, C2480T, T2727C, T2731C, A2785G, A2819G, T2916C, T2937C, A2995G, C3001G, G3154A, G3208A, A3269T, A4152G, C4459T, T4540C, G4918A, C4944T, G5079A, A5592G, A5668G, T6184G, A6248G, G7022A, A7103G, T7125C, A7128G, T7148C, G7291A, G7534A, G7584A, T7809C, T7879C, T7985C and A8212G.

12. A nucleic acid molecule according to claim 8, 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 3 by the following said amino acid selected from the group consisting of T535S, F561L, D811G, T827A, E989K, Y1018H, T1989I, E2269D, T2434A, V2436L and G2604S

13. A nucleic acid molecule according to claim 8, 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 4 by the following said amino acid selected from the group consisting of N16T, C128Y, A216T, C229Y, F291V, T329S, C585S, T592A, L714F, L796P, T827A, V859A, V866A, D887E, M956I, T977S, T1531I, G1580E, Q1751R, I1970V, D2228N, M2255V, L2262P, E2263G, C2270R, G2415E and V2490A

14. A nucleic acid molecule according to any of claims 5-13, wherein said molecule is capable of generating a HCV RNA titer not represented by input of said molecule itself of 10^4 IU/ml or above following transfection and/or subsequent viral passage.

15. A nucleic acid molecule according to any of claims 5-13, wherein said molecule is capable of generating a HCV infectivity titer of 10^2 $TCID_{50}$/ml (50% tissue culture infectious doses)/ml or above following transfection and/or subsequent viral passage.

16. A composition comprising a nucleic acid molecule according to any of claims 5-15 suspended in a suitable amount of a pharmaceutical acceptable diluent or excipient.

17. A cassette vector for cloning viral genomes, comprising, inserted therein, the nucleic acid sequence according to any of claims 5-15 and having an active promoter upstream thereof.

18. A method for producing a cell which replicates HCV 4a/JFH1 RNA and produces a virus particle comprising introducing the said RNA according to any of claims 1-4 into a cell.

20. A method according to claim 18, wherein the cell is Huh7.5.

21. A cell obtainable by the method of claim 18.

22. A method for producing a hepatitis C virus particle, comprising culturing the cell according to claim 21 to allow the cell to produce the virus.

23. A hepatitis C virus particle obtainable by the method according to claim 22.

24. A method for *in vitro* producing a hepatitis C virus-infected cell comprising culturing the cell according to claim 21 and infecting other cells with the produced virus particle in the culture.

25. A hepatitis C virus infected cell obtainable by the method according to claim 24.

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

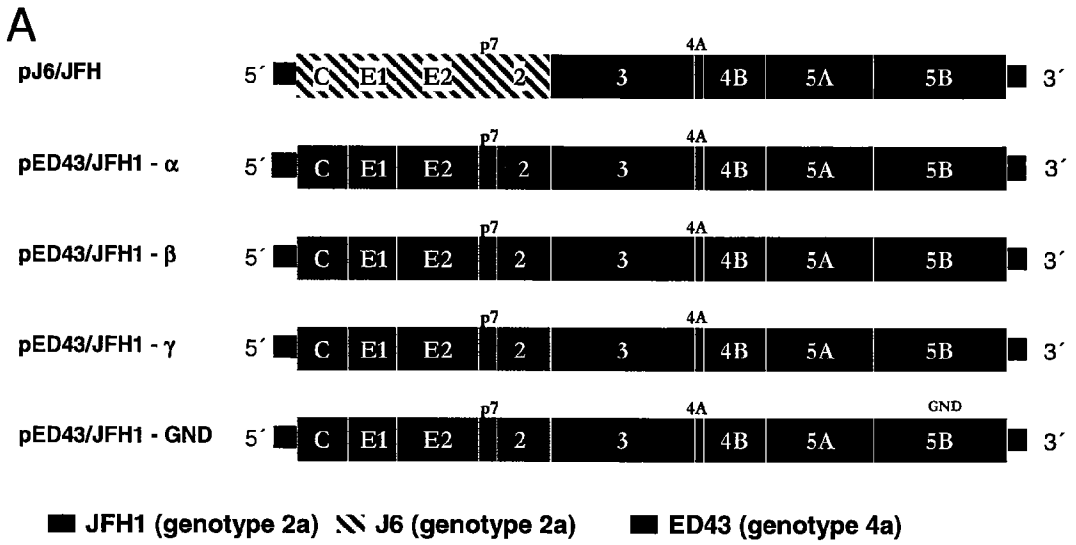
a) culturing at least one selected from the group consisting of a cell according to claim 21, a hepatitis C virus infected cell according to claim 25 and a hepatitis C virus particle obtainable by claim 22 together with a hepatitis C virus permissive cell, and

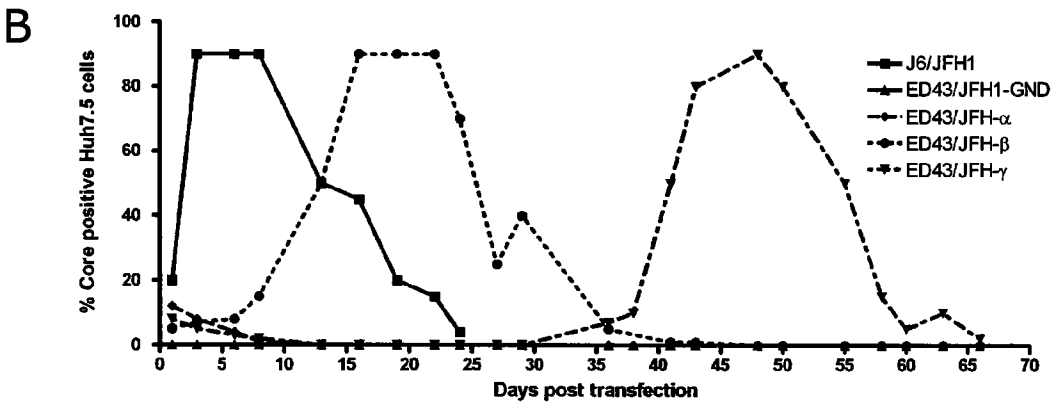
b) detecting the replicating RNA or the virus particles in the resulting culture.

27. A hepatitis C vaccine comprising a hepatitis C virus particle according to claim 23 or a part thereof.

28. A method for producing a hepatitis C virus vaccine comprising using a hepatitis C virus particle obtained from claim 23 as an antigen.

29. An antibody against the hepatitis C virus particle according to claim 23.


A
pJ6/JFH
pED43/JFH1 - α
pED43/JFH1 - β
pED43/JFH1 - γ
pED43/JFH1 - GND
5´
3´
C
E1
E2
p7
2
3
4A
4B
5A
5B
GND
JFH1 (genotype 2a)
J6 (genotype 2a)
ED43 (genotype 4a)


B
% Core positive Huh7.5 cells
Days post transfection
J6/JFH1
ED43/JFH1-GND
ED43/JFH-α
ED43/JFH-β
ED43/JFH-γ

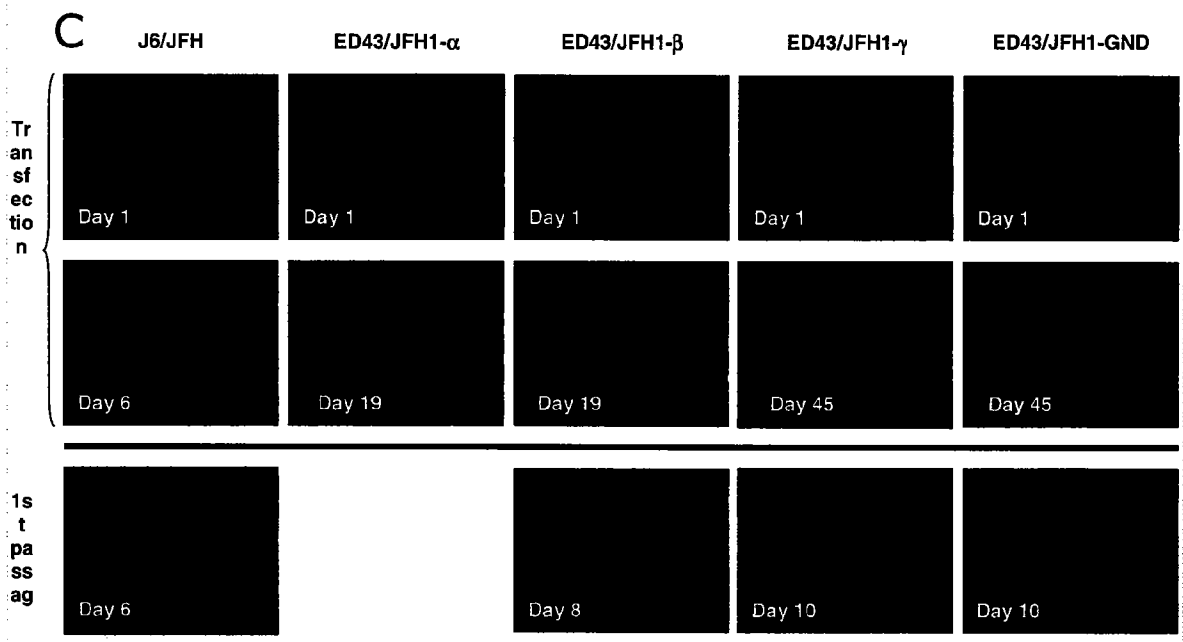

C
J6/JFH
ED43/JFH1-α
ED43/JFH1-β
ED43/JFH1-γ
ED43/JFH1-GND
Transfection
Day 1
Day 6
Day 19
Day 45
1st passag
Day 6
Day 8
Day 10

Fig. 1

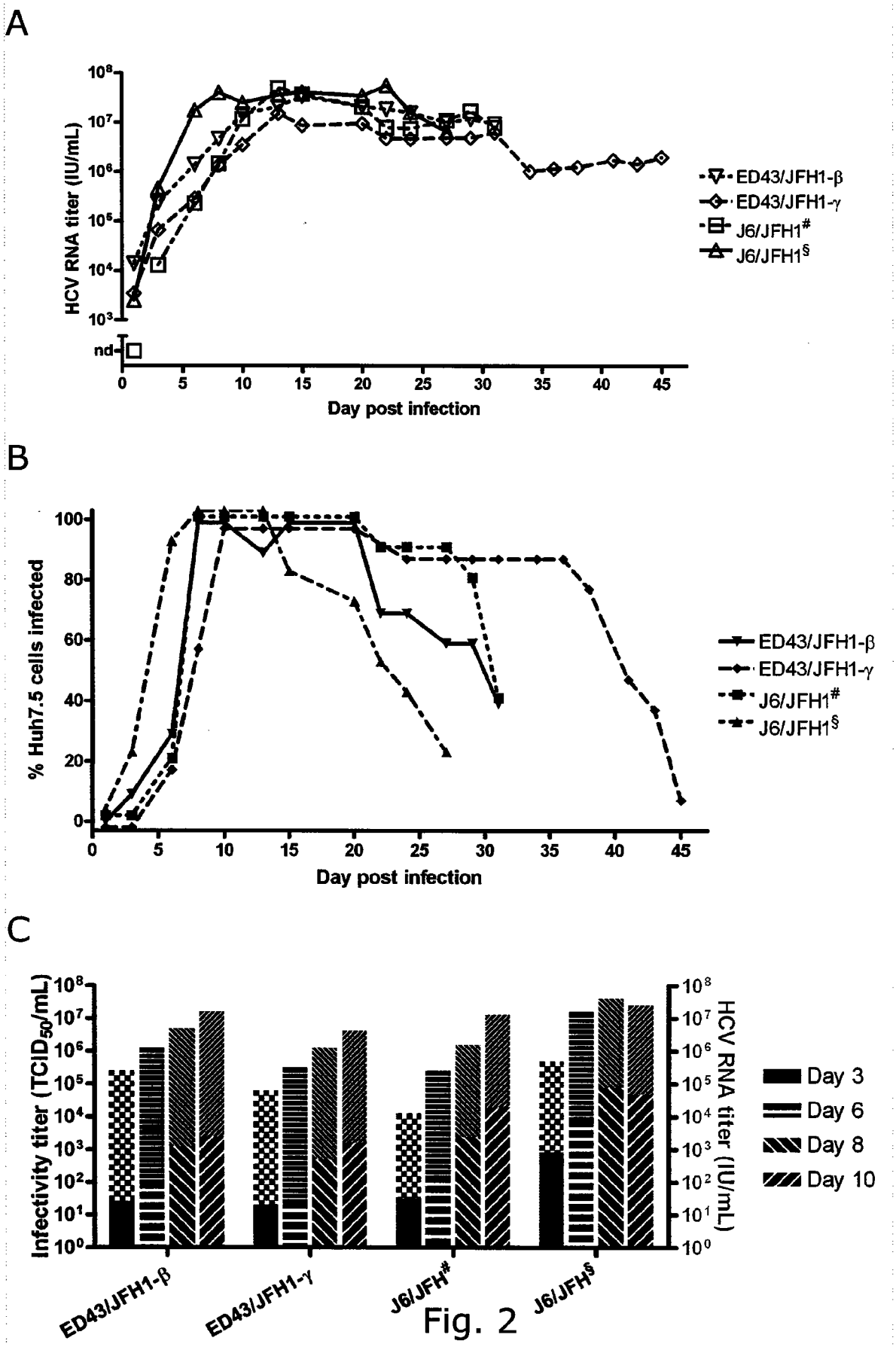

A
HCV RNA titer (IU/mL)
nd
Day post infection
ED43/JFH1-β
ED43/JFH1-γ
J6/JFH1#
J6/JFH1§
B
% Huh7.5 cells infected
Day post infection
ED43/JFH1-β
ED43/JFH1-γ
J6/JFH1#
J6/JFH1§
C
Infectivity titer (TCID50/mL)
HCV RNA titer (IU/mL)
Day 3
Day 6
Day 8
Day 10
ED43/JFH1-β
ED43/JFH1-γ
J6/JFH#
J6/JFH§

Fig. 2

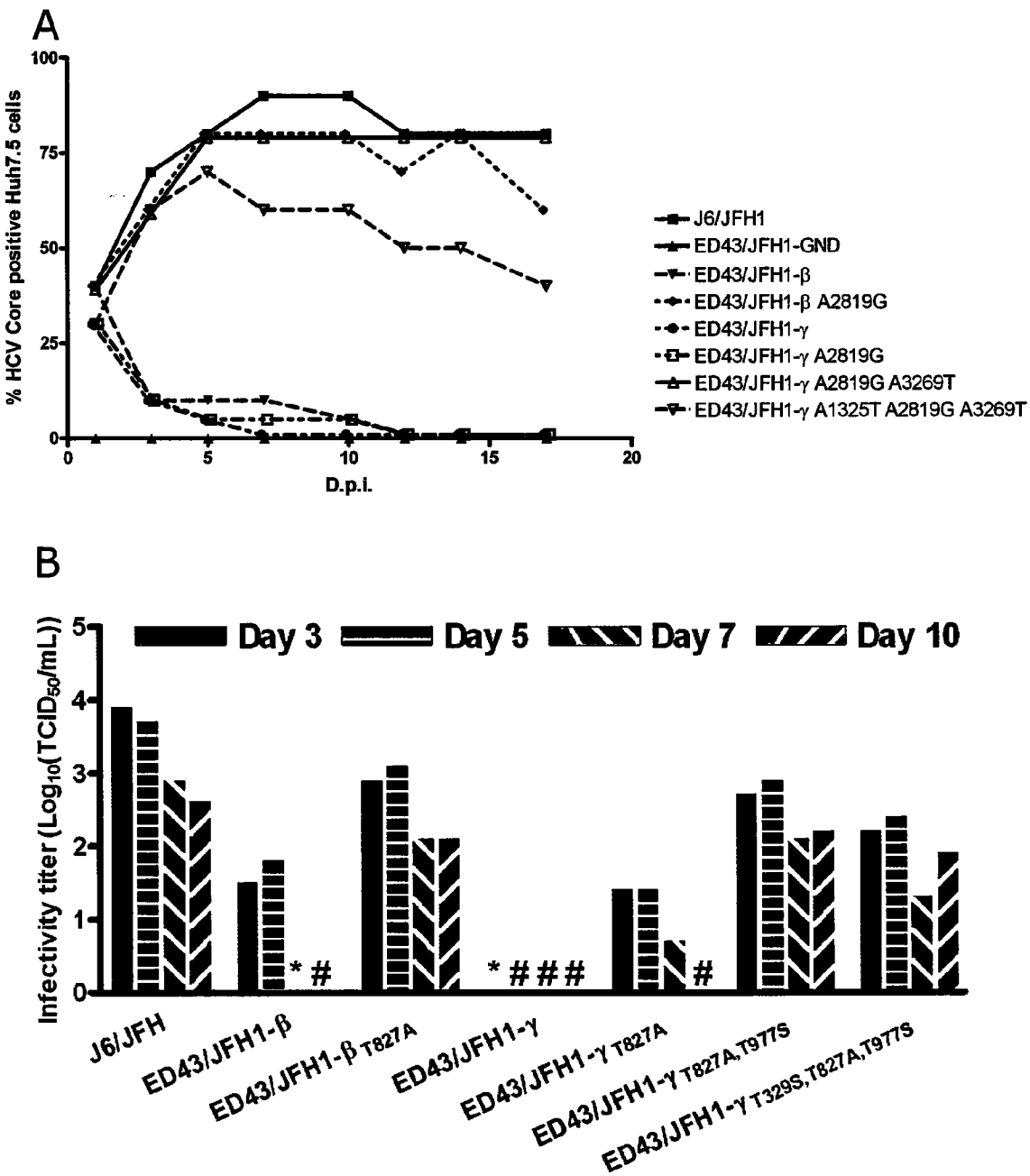

A
% HCV Core positive Huh7.5 cells
100
75
50
25
0
0
5
10
15
20
D.p.i.
J6/JFH1
ED43/JFH1-GND
ED43/JFH1-β
ED43/JFH1-β A2819G
ED43/JFH1-γ
ED43/JFH1-γ A2819G
ED43/JFH1-γ A2819G A3269T
ED43/JFH1-γ A1325T A2819G A3269T
B
Infectivity titer (Log10(TCID50/mL))
Day 3
Day 5
Day 7
Day 10
5
4
3
2
1
0
* #
* # # #
#
J6/JFH
ED43/JFH1-β
ED43/JFH1-β T827A
ED43/JFH1-γ
ED43/JFH1-γ T827A
ED43/JFH1-γ T827A,T977S
ED43/JFH1-γ T329S,T827A,T977S

Fig. 3

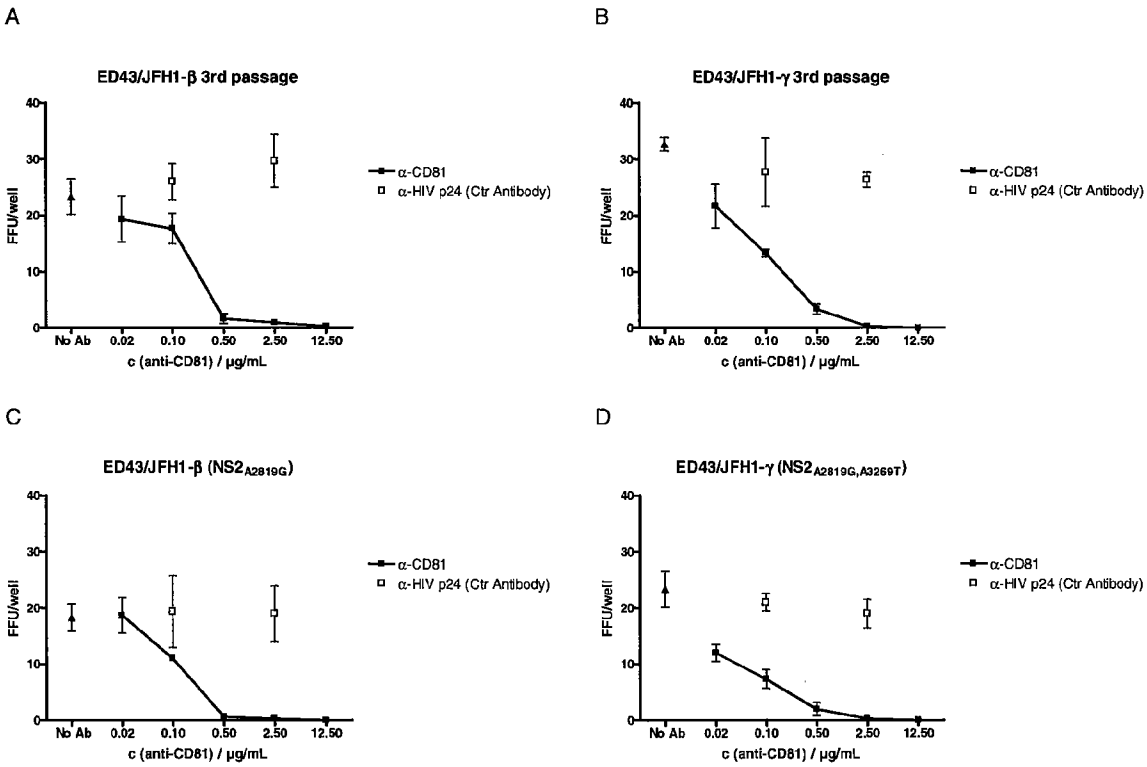

A
ED43/JFH1-β 3rd passage
FFU/well
c (anti-CD81) / µg/mL
No Ab
0.02
0.10
0.50
2.50
12.50
α-CD81
α-HIV p24 (Ctr Antibody)
B
ED43/JFH1-γ 3rd passage
C
ED43/JFH1-β (NS2A2819G)
D
ED43/JFH1-γ (NS2A2819G,A3269T)

Fig. 4

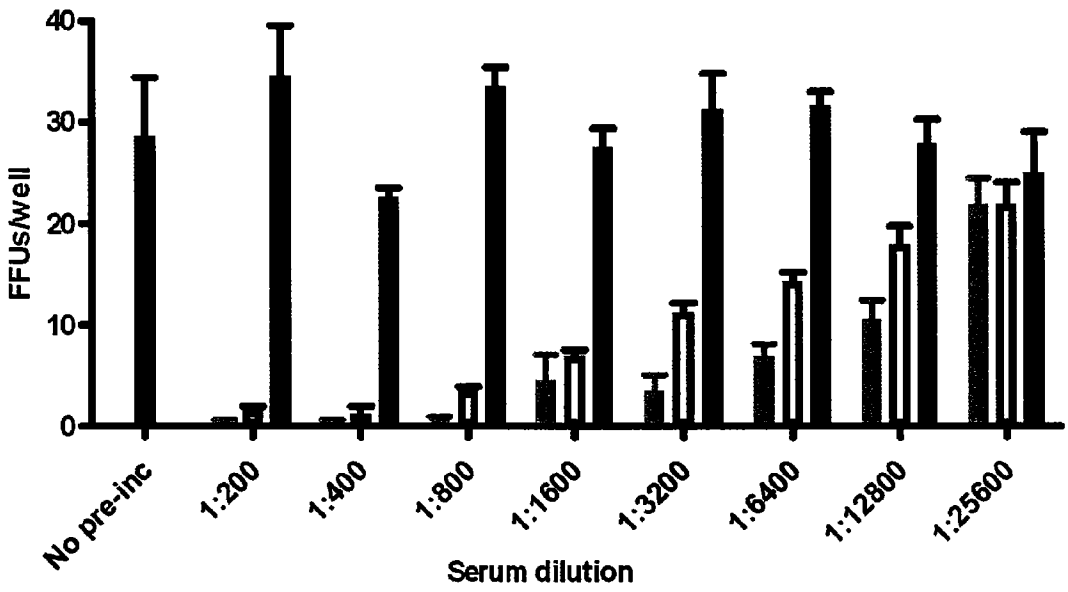

40
30
20
10
0
FFUs/well
No pre-inc
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
Serum dilution

Fig. 5

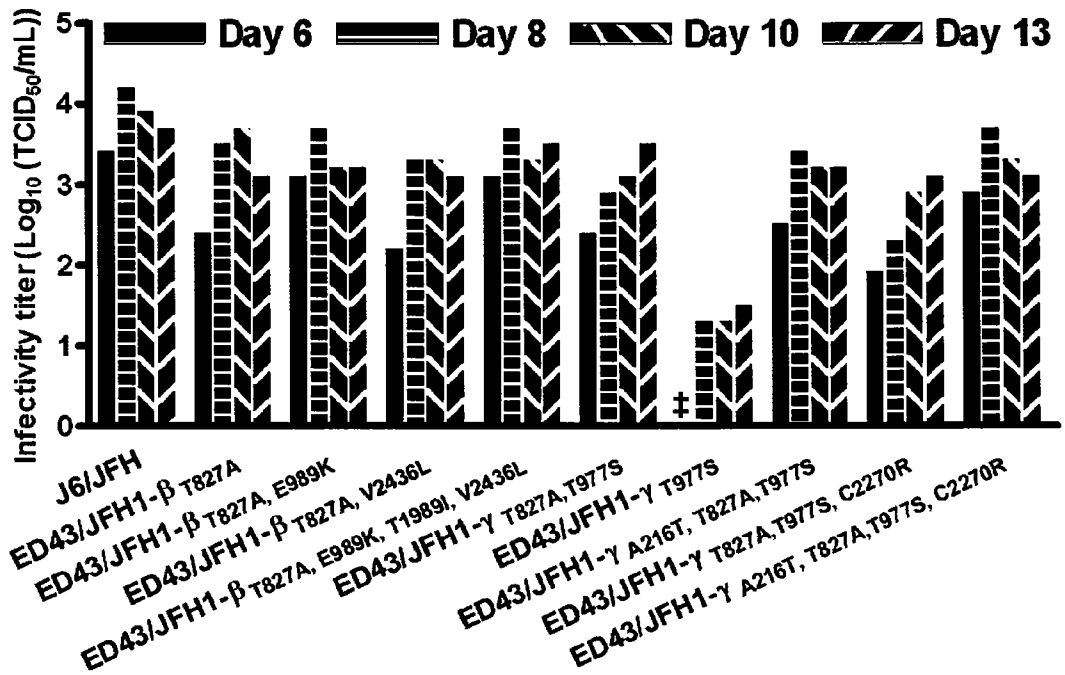

Day 6
Day 8
Day 10
Day 13
Infectivity titer (Log10 (TCID50/mL))
5
4
3
2
1
0
‡
J6/JFH
ED43/JFH1-β T827A
ED43/JFH1-β T827A, E989K
ED43/JFH1-β T827A, V2436L
ED43/JFH1-β T827A, E989K, T1989I, V2436L
ED43/JFH1-γ T827A,T977S
ED43/JFH1-γ T977S
ED43/JFH1-γ A216T, T827A,T977S
ED43/JFH1-γ T827A,T977S, C2270R
ED43/JFH1-γ A216T, T827A,T977S, C2270R

Fig. 6

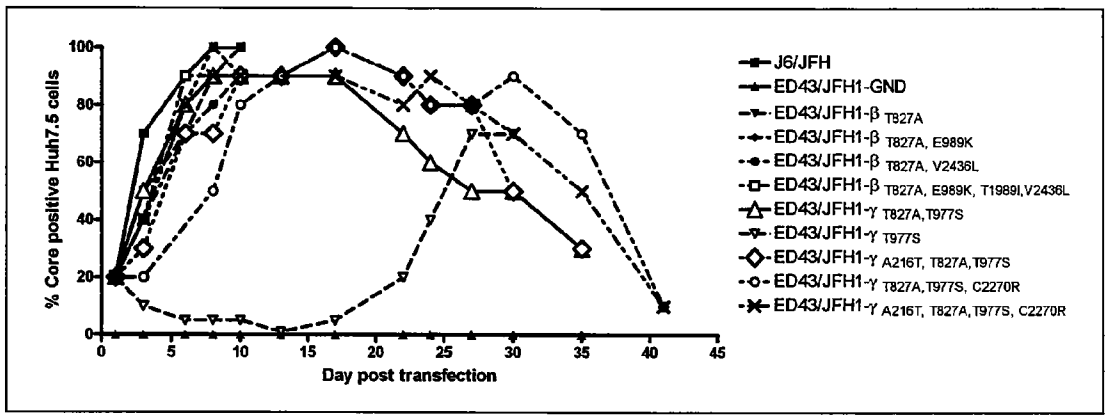

% Core positive Huh7.5 cells
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
Day post transfection
J6/JFH
ED43/JFH1-GND
ED43/JFH1-β T827A
ED43/JFH1-β T827A, E989K
ED43/JFH1-β T827A, V2436L
ED43/JFH1-β T827A, E989K, T1989I,V2436L
ED43/JFH1-γ T827A,T977S
ED43/JFH1-γ T977S
ED43/JFH1-γ A216T, T827A,T977S
ED43/JFH1-γ T827A,T977S, C2270R
ED43/JFH1-γ A216T, T827A,T977S, C2270R

Fig. 7

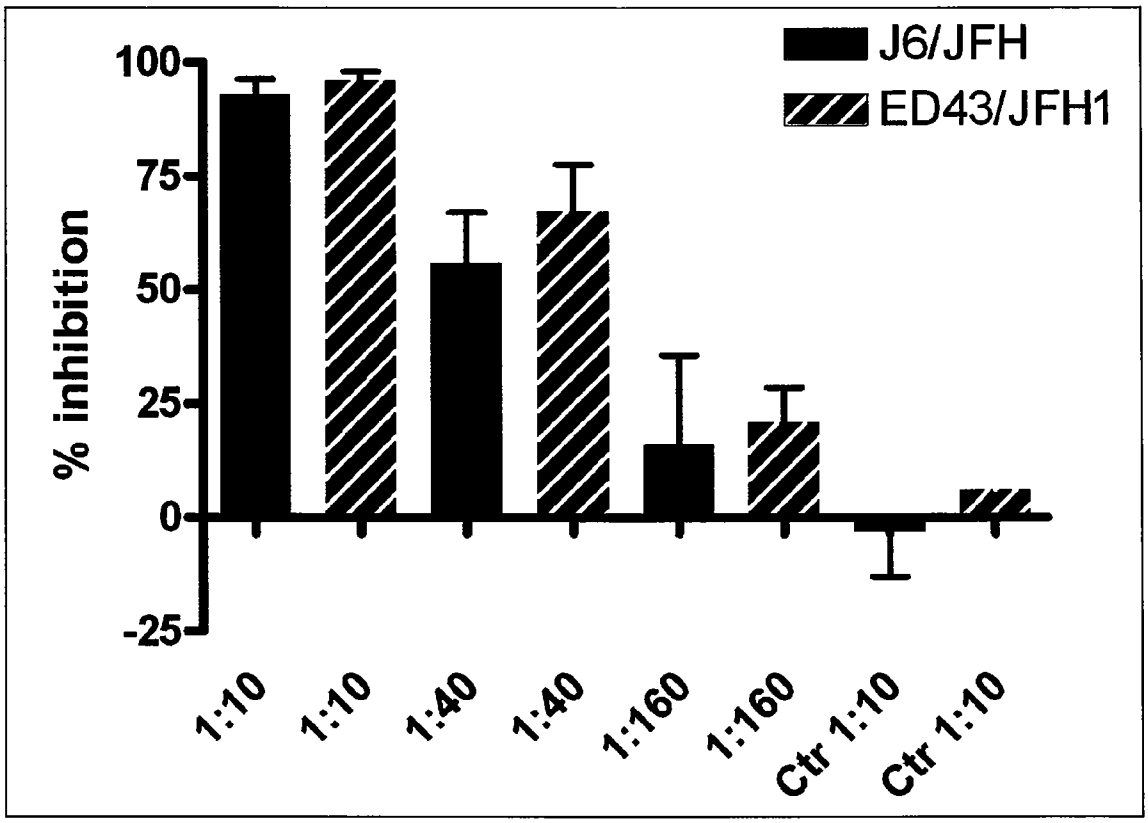

J6/JFH
ED43/JFH1
% inhibition
100
75
50
25
0
-25
1:10
1:10
1:40
1:40
1:160
1:160
Ctr 1:10
Ctr 1:10

Fig. 8

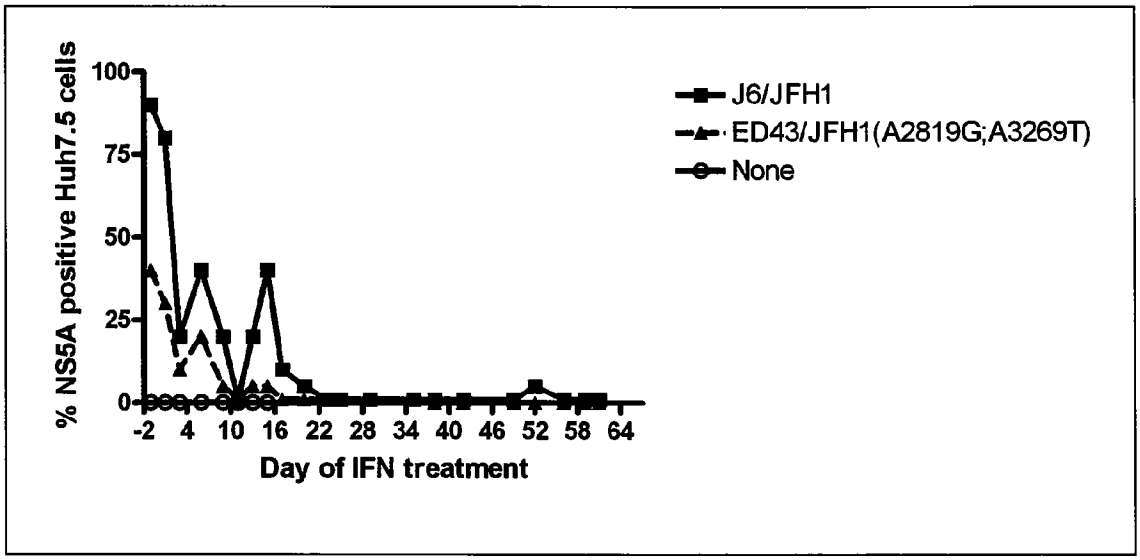

% NS5A positive Huh7.5 cells
100
75
50
25
0
-2 4 10 16 22 28 34 40 46 52 58 64
Day of IFN treatment
J6/JFH1
ED43/JFH1(A2819G;A3269T)
None

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2008/050085

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/00 C12N7/02 C12N15/51 C12N5/10 C07K14/18
A61K39/29

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

B. FIELDS SEARCHED

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

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X, O	GOTTWEIN J.M. ET AL.: "Novel chimeric cell culture system for hepatitis C genotypes 1A, 1B, 3A and 4A." J. HEPATOLOGY, vol. 46, no. Suppl, 28 April 2007 (2007-04-28), page S30, XP002461115 [42nd annual meeting of the European Association for the Study of the Liver; Barcelona, Spain, April 11-15, 2007. Oral presentation on Friday, 13.4.2007] abstract ----- -/--	1-29

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 2008	21/08/2008

Name and mailing address of the ISA/	Authorized officer
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	Galli, Ivo

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2008/050085

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PIETSCHMANN T. ET AL: "Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras" PROC. NATL. ACAD. SCI. USA, vol. 103, no. 19, 9 May 2006 (2006-05-09), pages 7408-7413, XP002461116 the whole document	1-29
A	CHAMBERLAIN R.W. ET AL.: "Complete nucleotide sequence of a type 4 hepatitis C virus variant, the predominant genotype in the Middle East" J. GENERAL VIROL., vol. 78, 1997, pages 1341-1347, XP002461127 the whole document	3,5,7
A	KRIEGER N ET AL: "Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 75, no. 10, May 2001 (2001-05), pages 4614-4624, XP002426793 ISSN: 0022-538X the whole document	9-13
A	YI MK ET AL: "Compensatory mutations in E1, p7, NS2 and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus." J. VIROL., vol. 81, no. 2, January 2007 (2007-01), pages 629-638, XP002461117 2 the whole document	9-13
A	BUKH J. ET AL: "Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees." PROC. NATL. ACAD. SCI. USA, vol. 99, no. 22, 29 October 2002 (2002-10-29), pages 14416-14421, XP002461118 the whole document	9-13
A	WO 02/059321 A (ANGELETTI P IST RICHERCHE BIO [IT]; DE FRANCESCO RAFFAELE [IT]; MIGLIA) 1 August 2002 (2002-08-01) * claims *	9-13

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2008/050085

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2005/047463 A (MERCK & CO INC [US]; ANGELETTI P IST RICHERCHE BIO [IT]; LUDMERER STEV) 26 May 2005 (2005-05-26) * claims *	9-13
A	WO 2005/053516 A (BOARDS OF REGENTS THE UNIVERSI [US]; LEMON STANLEY M [US]; YI MINKYUNG) 16 June 2005 (2005-06-16) * claims *	9-13
A	WO 02/052015 A (BOEHRINGER INGELHEIM CA LTD [CA]; KUKOLJ GEORGE [CA]; PAUSE ARNIM [CA]) 4 July 2002 (2002-07-04) * claims *	9-13
T	GOTTWEIN J.M. ET AL.: "Robust Hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses" GASTROENTEROLOGY, vol. 133, November 2007 (2007-11), pages 1614-1626, XP002461123 published online 3.8.2007. the whole document	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2008/050085

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 2006304803 A	09-11-2006
		MX PA03005638 A	05-04-2004
		NZ 527035 A	24-02-2006