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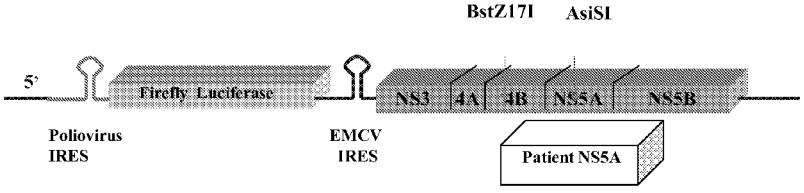

FIG. 1

(57) Abstract: The present invention provides for novel HCV NS5A replicon shuttle vectors useful for cloning in HCV polynucleotide sequences from samples of HCV-infected patients and testing the resulting replicons for drug susceptibility.

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HCV NS5A REPLICON SHUTTLE VECTORS

This invention pertains to novel NS5A HCV replicon shuttle vectors which are useful for screening, testing and evaluating HCV inhibitors.

Hepatitis C virus is a major health problem and the leading cause of chronic liver disease throughout the world. (Boyer, N. *et al. J. Hepatol.* **2000** 32:98-112). Patients infected with HCV are at risk of developing cirrhosis of the liver and subsequent hepatocellular carcinoma and hence HCV is the major indication for liver transplantation.

According to the World Health Organization, there are more than 200 million infected individuals worldwide, with at least 3 to 4 million people being infected each year. Once infected, about 20% of people clear the virus, but the rest can harbor HCV the rest of their lives. Ten to twenty percent of chronically infected individuals eventually develop liver-destroying cirrhosis or cancer. The viral disease is transmitted parenterally by contaminated blood and blood products, contaminated needles, or sexually and vertically from infected mothers or carrier mothers to their offspring. Current treatments for HCV infection, which are restricted to immunotherapy with recombinant interferon-alpha alone or in combination with the nucleoside analog ribavirin, are of limited clinical benefit particularly for genotype 1. There is an urgent need for improved therapeutic agents that effectively combat chronic HCV infection

HCV has been classified as a member of the virus family Flaviviridae that includes the genera *flaviviruses*, *pestiviruses*, and *hepaciviruses* which includes hepatitis C viruses (Rice, C. M., *Flaviviridae: The viruses and their replication*, in: *Fields Virology*, Editors: Fields, B. N., Knipe, D. M., and Howley, P. M., Lippincott-Raven Publishers, Philadelphia, Pa., Chapter 30, 931-959, 1996). HCV is an enveloped virus containing a positive-sense single-stranded RNA genome of approximately 9.4 kb. The viral genome consists of a 5'-untranslated region (UTR), a long open reading frame encoding a polyprotein precursor of-approximately 3011 amino acids, and a short 3' UTR. The 5' UTR is the most highly conserved part of the HCV genome and is important for the initiation and control of polyprotein translation.

Genetic analysis of HCV has identified six main genotypes showing a >30% divergence in the

DNA sequence. Each genotype contains a series of more closely related subtypes which show a 20-25 % divergence in nucleotide sequences (Simmonds, P. **2004** *J. Gen. Virol.* 85:3173-88) . More than 30 subtypes have been distinguished. In the US approximately 70% of infected individuals have type 1a and 1b infection. Type 1b is the most prevalent subtype in Asia. (X. Forns and J. Bukh, *Clinics in Liver Disease* **1999** 3:693-716; J. Bukh *et al.*, *Semin. Liv. Dis.* **1995** 15:41-63). Unfortunately Type 1 infections are less responsive to the current therapy than either type 2 or 3 genotypes (N. N. Zein, *Clin. Microbiol. Rev.*, **2000** 13:223-235).

The genetic organization and polyprotein processing of the nonstructural protein portion of the ORF of pestiviruses and hepaciviruses is very similar. These positive stranded RNA viruses possess a single large open reading frame (ORF) encoding all the viral proteins necessary for virus replication. These proteins are expressed as a polyprotein that is co- and post-translationally processed by both cellular and virus-encoded proteinases to yield the mature viral proteins. The viral proteins responsible for the replication of the viral genome RNA are located towards the carboxy-terminal. Two-thirds of the ORF are termed nonstructural (NS) proteins. For both the pestiviruses and hepaciviruses, the mature nonstructural (NS) proteins, in sequential order from the amino-terminus of the nonstructural protein coding region to the carboxy-terminus of the ORF, consist of p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

The NS proteins of pestiviruses and hepaciviruses share sequence domains that are characteristic of specific protein functions. For example, the NS3 proteins of viruses in both groups possess amino acid sequence motifs characteristic of serine proteinases and of helicases (Gorbalenya *et al. Nature* **1988** 333:22; Bazan and Fletterick *Virology* **1989** 171:637-639; Gorbalenya *et al. Nucleic Acid Res.* **1989** 17.3889-3897). Similarly, the NS5B proteins of pestiviruses and hepaciviruses have the motifs characteristic of RNA-directed RNA polymerases (Koonin, E. V. and Dolja, V. V. *Crit. Rev. Biochem. Molec. Biol.* **1993** 28:375-430).

The actual roles and functions of the NS proteins of pestiviruses and hepaciviruses in the lifecycle of the viruses are directly analogous. In both cases, the NS3 serine proteinase is responsible for all proteolytic processing of polyprotein precursors downstream of its position in the ORF (Wiskerchen and Collett *Virology* **1991** 184:341-350; Bartenschlager *et al.* J. Virol. **1993** 67:3835-3844; Eckart *et al. Biochem. Biophys. Res. Comm.* **1993** 192:399-406; Grakoui *et al. J. Virol.* **1993** 67:2832-2843; Grakoui *et al. Proc. Natl. Acad. Sci. USA* **1993** 90:10583-10587; Ilijikata *et al. J. Virol.* **1993** 67:4665-4675; Tome *et al. J. Virol.* **1993** 67:4017-4026). The NS4A

protein, in both cases, acts as a cofactor with the NS3 serine protease (Bartenschlager *et al. J. Virol.* **1994** 68:5045-5055; Failla *et al. J. Virol.* **1994** 68: 3753-3760; Xu *et al. J Virol.* **1997** 71:53 12-5322). The NS3 protein of both viruses also functions as a helicase (Kim *et al. Biochem. Biophys. Res. Comm.* **1995** 215: 160-166; Jin and Peterson *Arch. Biochem. Biophys.* **1995**, 323:47-53; Warrener and Collett *J. Virol.* **1995** 69:1720-1726). Finally, the NS5B proteins of pestiviruses and hepaciviruses have the predicted RNA-dependent RNA polymerase activity (Behrens et al. EMBO **1996** 15:12-22; Lechmann *et al. J. Virol.* **1997** 71:8416-8428; Yuan *et al. Biochem. Biophys. Res. Comm.* **1997** 232:231-235; Hagedorn, PCT WO 97/12033; Zhong *et al. J. Virol.* **1998** 72:9365-9369).

Currently there are a limited number of approved therapies are currently available for the treatment of HCV infection. New and existing therapeutic approaches to treating HCV and inhibition of HCV NS5B polymerase have been reviewed: R. G. Gish, *Sem. Liver. Dis.*, **1999** 19:5; Di Besceglie, A. M. and Bacon, B. R., *Scientific American*, October: **1999** 80-85; G. Lake-Bakaar, Current and Future Therapy for Chronic Hepatitis C Virus Liver Disease, *Curr. Drug Targ. Infect Dis.* **2003** 3(3):247-253; P. Hoffmann *et al.*, Recent patents on experimental therapy for hepatitis C virus infection (1999-2002), *Exp. Opin. Ther. Patents* **2003** 13(11):1707-1723; F. F. Poordad *et al.* Developments in Hepatitis C therapy during 2000-2002, *Exp. Opin. Emerging Drugs* **2003** 8(1):9-25; M. P. Walker *et al.*, Promising Candidates for the treatment of chronic hepatitis C, *Exp. Opin. Investig. Drugs* **2003** 12(8):1269-1280; S.-L. Tan *et al.*, Hepatitis C Therapeutics: Current Status and Emerging Strategies, *Nature Rev. Drug Discov.* **2002** 1:867-881; R. De Francesco *et al.* Approaching a new era for hepatitis C virus therapy: inhibitors of the NS3-4A serine protease and the NS5B RNA-dependent RNA polymerase, *Antiviral Res.* **2003** 58:1-16; Q. M. Wang *et al.* Hepatitis C virus encoded proteins: targets for antiviral therapy, *Drugs of the Future* **2000** 25(9):933-8-944; J. A. Wu and Z. Hong, Targeting NS5B-Dependent RNA Polymerase for Anti-HCV Chemotherapy *Cur. Drug Targ.-Inf. Dis* .**2003** 3:207-219.

Despite advances in understanding the genomic organization of the virus and the functions of viral proteins, fundamental aspects of HCV replication and pathogenesis remain unknown. A major challenge in gaining experimental access to HCV replication is the lack of an efficient cell culture system that allows production of infectious virus particles. Although infection of primary cell cultures and certain human cell lines has been reported, the amounts of virus produced in those systems and the levels of HCV replication have been too low to permit detailed analyses.

The construction of selectable subgenomic HCV RNAs that replicate with minimal efficiency in the human hepatoma cell line Huh-7 has been reported. Lohman et al. reported the construction of a replicon (I_{377}/NS3-3') derived from a cloned full-length HCV consensus genome (genotype 1b) by deleting the C-p7 or C-NS2 region of the protein- coding region (Lohman et al., *Science* **1999** 285: 110-113). The replicon contained the following elements: (i) the HCV 5`-UTR fused to 12 amino acids of the capsid encoding region; (ii) the neomycin phosphotransferace gene (NPTII); (iii) the IRES from encephalomyocarditis virus (EMCV), inserted downstream of the NPTII gene and which directs translation of HCV proteins NS2 or NS3 to NS5B; and (iv) the 3`-UTR. After transfection of Huh-7 cells, only those cells supporting HCV RNA replication expressed the NPTII protein and developed resistance against the drug G418. While the cell lines derived from such G418 resistant colonies contained substantial levels of replicon RNAs and viral proteins, only 1 in 10^6 transfected Huh-7 cells supported HCV replication.

Similar selectable HCV replicons were constructed based on an HCV-H genotype 1a infectious clone (Blight et al., *Science* **2000** 290:1972-74). The HCV-H derived replicons were unable to establish efficient HCV replication, suggesting that the earlier-constructed replicons of Lohmann (1999), supra, were dependent on the particular genotype 1 b consensus cDNA clone used in those experiments. Blight et al. (2000), supra, reproduced the construction of the replicon made by Lohmann et al. (1999), supra, by carrying out a PCR- based gene assembly procedure and obtained G418-resistant Huh-7 cell colonies. Independent G418-resistant cell clones were sequenced to determine whether high-level HCV replication required adaptation of the replicon to the host cell. Multiple independent adaptive mutations that cluster in the HCV nonstructural protein NS5A were identified. The mutations conferred increased replicative ability in vitro, with transduction efficiency ranging from 0.2 to 10% of transfected cells as compared to earlier-constructed replicons in the art, e.g., the I_{377}/NS3-3' replicon had a 0.0001% transduction efficiency.

There is currently one approved therapy available for the treatment of HCV infection. New direct antiviral agents (DAA) targeting the NS5A protein of the HCV virus have been recently described and showed efficacy in the clinic (Lemm J et al, *J Virol.* **2010** Jan;84 (1):482-91). Viral drug resistance is likely to occur under DAA drug pressure and methodology to study the emergence of drug resistant variants in treated patients is needed. The transient subgenomic replicon system has now been validated for the study of drug susceptibility of HCV clinical

isolates from infected patients (treatment-naive or treated) as it mimics the patient's viral diversity and allows the testing and evaluating of HCV inhibitors across diverse HCV sequences (Le Pogam et al, *J Antimicrob Chemother.* **2008** Jun;61(6):1205-16).

There is a need for developing novel NS5A HCV replicon shuttle vectors with the novel and unique feature of being able to replicate when patient-derived NS5A gene(s) is shuttled into the vectors and are therefore necessary for the monitoring of development of resistance to NS5A inhibitors in clinical trials as well as allowing the evaluation of compounds inhibitory activity across genetically diverse clinical isolates.

The present invention features the development of a novel HCV replicon shuttle vector in which unique restriction enzyme sites are introduced at the 5` and 3` ends of the NS5A gene such that NS5A sequences derived from the samples of HCV-infected patients can be cloned in the shuttle vector and the resulting replicons be evaluated for replication fitness and susceptibility to HCV inhibitors. Since an individual HCV-infected patient typically contains a genetically diverse virus population due to the high error rate of the NS5B RNA polymerase, the use of the shuttle vector of the present invention would allow the characterization of specific patient-derived NS5A variants and the sensitivity or resistance of these variants to drug treatment.

Accordingly, the present invention provides a Genotype 1a or Genotype 1b HCV replicon shuttle vector comprising an HCV polynucleotide sequence that comprises, in order, a polynucleotide sequence encoding a NS3 protein, a polynucleotide sequence encoding a NS4A protein, a polynucleotide sequence encoding a NS4B protein, a restriction enzyme sequence that recognizes BstZ171 or AscI placed between 1 nucleotide and 15 nucleotides 5` from a polynucleotide sequence encoding a NS5A protein, a polynucleotide sequence encoding a NS5A protein, a restriction enzyme sequence that recognizes AsiSI placed between 1 nucleotide and 15 nucleotides 3` from a polynucleotide sequence encoding the NS5A protein, and a polynucleotide sequence encoding a NS5B protein. In one embodiment of the invention, the polynucleotide sequence encoding the NS5A protein has been replaced with a polynucleotide sequence encoding a selection protein or a reporter protein. In another embodiment of the invention, the HCV replicon shuttle vector comprises an HCV polynucleotide sequence selected from SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:21.

A further embodiment of the present invention provides a method for assessing the effectiveness of an HCV inhibitor to control an HCV infection in a subject comprising the steps of providing a sample from the subject infected with HCV, PCR-amplifying polynucleotide sequences encoding the NS5A protein from a plurarity of HCV quasispecies present in the sample with the use of a sense-strand primer which comprises a restriction enzyme sequence that recognizes BstZ171 or AscI, and an anti-sense strand primer which comprises a restriction enzyme sequence that recognizes AsiSI, cloning said PCR-amplifed polynucleotide sequences into an HCV replicon shuttle vector to produce chimeric HCV replicon plasmids, linearizing said chimeric HCV replicon plasmids and subjecting said linearized plasmids to *in vitro* transcription to produce chimeric HCV replicon RNAs, and transfecting a Huh7 cell line with said HCV replicon RNAs and measuring replication level of said HCV replicon RNAs in the presence or absence of the HCV inhibitor.

A still further embodiment of the present invention provides a method for assessing the effectiveness of an HCV polymerase inhibitor to control an HCV infection in a subject comprising the steps of providing a sample from the subject infected with HCV, PCR-amplifying polynucleotide sequences encoding the NS5A protein from a plurarity of HCV quasispecies present in the sample with the use of a sense-strand primer which comprises a restriction enzyme sequence that recognizes BstZ171 or AscI, and an anti-sense strand primer which comprises a restriction enzyme sequence that recognizes AsiSI, cloning said PCR-amplifed polynucleotide sequences into an HCV replicon shuttle vector to produce chimeric HCV replicon plasmids, transforming said plasmids into cells to generate a plurarity of colonies of transformed cells, pooling said colonies and isolating chimeric HCV replicon plasmids from the pooled colonies, linearizing said chimeric HCV replicon plasmids, subjecting said linearized plasmids to *in vitro* transcription to produce chimeric HCV replicon RNAs, and transfecting Huh7 cell line with said HCV replicon RNAs and measuring replication level of said HCV replicon RNAs in the presence or absence of the HCV inhibitor.

Figure 1 is a schematic representation of the HCV replicon shuttle vector and the positions where the restriction enzyme sites (shown as BstZ171 and AsiSI restriction sites) have been introduced to allow the cloning of patient-derived NS5A sequences into the vector.

The term "HCV replicon" refers to a nucleic acid from the Hepatitis C virus that is capable of directing the generation of copies of itself. As used herein, the term "replicon" includes RNA as well as DNA, and hybrids thereof. For example, double-stranded DNA versions of HCV genomes can be used to generate a single-stranded RNA transcript that constitutes an HCV replicon. The HCV replicons can include full length HCV genome or HCV subgenomic contructs also referred as a “subgenomic replicon”. For example, the subgenomic replicons of HCV described herein contain most of the genes for the non-structural proteins of the virus, but are missing most of the genes coding for the structural proteins. Subgenomic replicons are capable of directing the expression of all of the viral genes necessary for the replication of the viral subgenome, replication of the sub-genomic replicon, without the production of viral particles.

A basic HCV replicon is a subgenomic construct containing an HCV 5`-untranslated (UTR) region, an HCV NS3-NS5B polyprotein encoding region, and a HCV 3`-UTR. Other nucleic acid regions can be present such as those providing for HCV NS2, structural HCV protein(s) and non-HCV sequences.

The HCV 5`-UTR region provides an internal ribosome entry site (IRES) for protein translation and elements needed for replication. The HCV 5`-UTR region includes naturally occurring HCV 5`-UTR extending about 36 nucleotides into a HCV core encoding region, and functional derivatives thereof. The 5`-UTR region can be present in different locations such as site downstream from a sequence encoding a selection protein, a reporter, protein, or an HCV polyprotein.

In addition to the HCV 5`-UTR-PC region, non-HCV IRES elements can also be present in the replicon. The non-HCV IRES elements can be present in different locations including immediately upstream the region encoding for an HCV polyprotein. Examples of non-HCV IRES elements that can be used are the EMCV IRES, poliovirus IRES, and bovine viral diarrhea virus IRES.

The HCV 3`-UTR assists HCV replication. HCV 3` UTR includes naturally occurring HCV 3`-UTR and functional derivatives thereof. Naturally occurring 3`-UTRs include a poly U tract and an additional region of about 100 nucleotides.

The NS3-NS5B polyprotein encoding region provides for a polyprotein that can be processed in a cell into different proteins. Suitable NS3-NS5B polyprotein sequences that may be part of a replicon include those present in different HCV strains and functional equivalents thereof resulting in the processing of NS3-NS5B to produce a functional replication machinery. Proper processing can be measured for by assaying, for example, NS5B RNA dependent RNA polymerase.

The ability of an NS5B protein to provide RNA polymerase activity can be measured using techniques well known in the art. The NS5B protein is rendered "non-functional" when its RNA polymerase activity is virtually non-measurable and can be accomplished by "modifying" the polynucleotide sequence of the NS5B protein through, for example, the introduction of nucleotide substitutions, insertions or deletions. The NS5A protein is rendered non-functional by modifying the polynucleotide sequence of the NS5A protein through, for example the introduction of nucleotide substitutions, insertions or deletions.

A "vector" is a piece of DNA, such as a plasmid, phage or cosmid, to which another piece of DNA segment may be attached so as to bring about the replication, expression or integration of the attached DNA segment. A "shuttle vector" refers to a vector in which a DNA segment can be inserted into or excised from a vector at specific restriction enzyme sites. The segment of DNA that is inserted into shuttle vector generally encodes a polypeptide or RNA of interest and the restriction enzyme sites are designed to ensure insertion of the DNA segment in the proper reading frame for transcription and translation.

A variety of vectors can be used to express a nucleic acid molecule. Such vectors include chromosomal, episomal, and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, including yeast artificial chromosomes, from viruses such as baculoviruses, papovaviruses such as SV40, vaccinia viruses, adenoviruses, poxviruses, pseudorabies viruses, herpes viruses, and retroviruses. Vectors may also be derived from combinations of these sources, such as those derived from plasmid and bacteriophage genetic elements, e.g., cosmids and phagemids. Appropriate cloning and expression vectors for prokaryotic and eukaryotic hosts are described in Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

A vector containing the appropriate nucleic acid molecule can be introduced into an appropriate host cell for propagation or expression using known techniques. Host cells can include bacterial cells including, but not limited to, *E. coli, Streptomyces*, and *Salmonella typhimurium*, eukaryotic cells including, but not limited to, yeast, insect cells, such as *Drosophila*, animal cells, such as Huh-7, HeLa, COS, HEK 293, MT- 2T, CEM-SS, and CHO cells, and plant cells.

Vectors generally include selectable markers that enable the selection of a subpopulation of cells that contain the recombinant vector constructs. The marker can be contained in the same vector that contains the nucleic acid molecules described herein or may be on a separate vector. Markers include tetracycline- or ampicillin-resistance genes for prokaryotic host cells and dihydrofolate reductase or neomycin resistance for eukaryotic host cells. However, any marker that provides selection for a phenotypic trait will be effective.

A "polynucleotide" or "nucleic acid molecule" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

In addition, the term "DNA molecule" refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, the term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5` to 3` direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "RNA molecule" refers to the polymeric form of ribonucleotides in its either single-stranded form or a double-stranded helix form. In discussing the structure of particular RNA molecules,

sequence may be described herein according to the normal convention of giving the sequence in the 5' to 3' direction.

The term "restriction enzyme sequence" refers to a specific double stranded-DNA sequence which is recognized and cut by bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence. The restriction enzyme "AsiSI"

recognizes the sequence 5'GCGAT▼CGC 3' and cuts the double-stranded DNA after the
3'CGC▲TAGCG 5'

T residue on the recognition sequence (shown with ▼▲). The restriction enzyme "BstZ171" recognizes the sequence 5'GTA▼TAC 3'and cuts at the indicated nucleotide
3'CAT▲ATG 3'

position. Similarly, "AscI" recognizes and cuts at 5'GG▼CGCGCC 3'
3'CCGCGC▲GG 5'.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3` terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in PCR reactions, the primer is typically 15-25 nucleotides or longer in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, i.e. to be able to anneal with the desired template strand in a manner sufficient to provide the 3`-hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence (e.g. a restriction enzyme recognition sequence) may be

attached to the 5`-end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

The terms "reporter protein" or "selection protein" as used herein mean a protein that by its presence in a cell (i.e. upon expression) can allow the cell to be distinguished from a cell that does not contain the reporter protein or selection protein. A suitable reporter protein can be detected directly or indirectly such as by a suitable assay and may include but is not limited to proteins such as β-galactosidase, firefly luciferase, alkaline phosphatase, chloramphenicol acetyltransferase (CAT), β-glucuronidase (GUS), and fluorescent protein (FP). A suitable selection protein is one whose expression is required in order for a cell to survive such as a protein that confers drug resistance (e.g. neomycin resistance). Alternatively, the selection protein is one whose expression in a cell causes the cell to die, usually upon the introduction of a signal or ligand (e.g. the Fas receptor and Fas ligand).

The term “chimeric” as used herein means a molecule of DNA that has resulted from DNA from two or more different sources that have been fused or spliced together.

As used herein, the term “quasispecies” means a collection of microvariants of a predominant HCV genome sequence (i.e. genotype), said microvariants being formed in a single infected subject or even in a single cell clone or even in a single cell clone as a result of high mutation rate during HCV replication.

The term “subject” as used herein refers to vertebrates, particular members of the mammalian species and includes, but not limited to, rodents, rabbits, shrews, and primates, the latter including humans.

The term “sample” refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of in vitro cell culture constituents (including but not limited to, conditioned

medium resulting from the growth of cultured cells, putatively viral infected cells, recombinant cells, and cell components).

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA or RNA when such DNA or RNA has been introduced inside the cell. The transforming or transfecting DNA or RNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. For example, in prokaryotes, yeast, and mammalian cells, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. In the case of an HCV replicon that transforms a mammalian cell as described in the present invention, the RNA molecule, e.g., an HCV RNA molecule, has the ability to replicate semi-autonomously. Huh-7 cells carrying the HCV replicons are detected either by the presence of a selection marker or a reporter gene present on the replicon.

A "clone" refers to a population of cells derived from a single cell or common ancestor generally by the process of mitosis.

The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

Example 1

Construction of Plasmids

The transient HCV GT-1b Con1 replicon vector (rep PI-luc/ET) was obtained from R. Bartenschlager. Briefly, it includes the poliovirus internal ribosome entry site (IRES), which controls the translation of the firefly luciferase gene. Downstream of the firefly luciferase gene, the IRES from the encephalomyocarditis virus (EMCV) controls the translation of the HCV non-structural genes (NS3, NS4A, NS4B, NS5A and NS3/4A). The repPI-luc/ET vector was modified to replace the pBR322 backbone with the pUC18 backbone to generate replicon pPI-

luc/ET/SC or pSC (SEQ ID NO:1). Replicon pSC replicated with similar levels to rep PI-luc/ET as disclosed in US Patent Publication No. US2008/0026952 by Dietrich et al., which is incorporated by reference in full herein.

The transient HCV genotype-1a H77 replicon vector pSS-1 was generated by ligating a SpeI – BsrGI fragment from pPI-luc/ET/SC which contains nucleotide sequence from the pUC18 backbone, 5`-UTR, poliovirus IRES, firefly luciferase, EMCV IRES and 75 amino acids from the 5`-end of the NS3 gene (genotype 1b-Con1) with a BsrGI – SpeI fragment from HCV genotype 1a H77 (Yanagi et al. *Proc. Natl. Acad. Sci. U.S.A.* **1997** 94:8738-8743; GenBank Accession Number AF011751) which contains the remainder of the NS3 gene through the 3`-UTR, and also the S2204I adaptive mutation in the NS5A gene (Blight et al. 2000). The full nucleotide sequence of pSS-1 is shown in SEQ ID NO:2.

Mutations were introduced into the pSS-1 and pSC vectors in order to put in unique restriction enzyme sequences at the 5` and 3` ends of the NS5A gene by using the QuickChange II site-directed mutagenesis kit following the manufacturer's instructions (Stratagene, La Jolla, CA, USA). For introducing an AscI restriction enzyme sequence (underlined) immediately upstream of the 5` end of the NS5A gene, the following primers were utilized:

Sense primer (pSS-1): 5`-ATAAGCTCGGAGTGTGGCGCGCCATGCTCC-3`
(SEQ ID NO:3)

Antisense primer (pSS-1): 5`-GGAGCATGGCGCGCCACACTCCGAGCTTAT-3`
(SEQ ID NO:4)

Sense primer (pSC): 5`-ATCAACGAGGACTGCGGCGCGCCATGCTCC-3`
(SEQ ID NO:5)

Antisense primer (pSC): 5`-GGAGCATGGCGCGCCGCAGTCCTCGTTGAT-3`
(SEQ ID NO:6)

To introduce a BstZ17I restriction enzyme sequence (underlined) also immediately upstream of the 5` end of the NS5A gene, the following primers were utilized:

Sense primer (pSS-1): 5`-ATAAGCTCGGAGTGTAGTATACCATGCTCC-3`
(SEQ ID NO:7)

Antisense primer (pSS-1): 5`-GGAGCATGGTATACTACACTCCGAGCTTAT-3`
(SEQ ID NO:8)

Sense primer (pSC): 5`-ATCAACGAGGACTGCAGTATACCATGCTCC-3`
(SEQ ID NO:9)

Antisense primer (pSC): 5`-GGAGCATGGTATACTGCAGTCCTCGTTGAT-3`
(SEQ ID NO:10)

At the 3` end of the NS5A gene, an AsiSI restriction enzyme sequence (underlined) was introduced and the following primers were utilized:

Sense primer (pSS-1): 5`-ACACGGAAGATGCGATCGCCTGCTCAATGT-3`
(SEQ ID NO:11)

Antisense primer (pSS-1): 5`-ACATTGAGCAGGCGATCGCATCTTCCGTGT-3`
(SEQ ID NO:12)

Sense primer (pSC): 5`-CTAGTGAGGACGCGATCGCCTGCTCGATGT-3`
(SEQ ID NO:13)

Antisense primer (pSC): 5`-ACATCGAGCAGGCGATCGCGTCCTCACTAG-3`
(SEQ ID NO:14)

In order to insert the NS5A sequence from patients infected with HCV genotype-1a, the genotype-1a replicon shuttle vectors, pSS-1_1a_NS5A_BstZ17I_AsiSI (SEQ ID NO:15) and pSS-1_1a_NS5A_AscI_AsiSI (SEQ ID NO:21) were generated. An alternate genotype 1a replicon shuttle vector was constructed in which the NS5A nucleotide sequence within pSS-1_1a_NS5A_BstZ17I_AsiSI was replaced by the beta-galactosidase (lacZ) coding sequence from pUC19 (GenBank Accession Number M77789). BstZ17I and AsiSI restriction enzyme sequences were introduced at the 5` end and at the 3` end of the lacZ gene, respectively, by PCR amplification and resulted in generating the shuttle vector,

pSS-1_1a_(NS5A)_lacZ_BstZ17I_AsiSI (SEQ ID NO:16). The replication capability of these shuttle vectors was assessed using the phenotypic assay described in Example 3 and was found to be similar to that of wild type pSS-1.

Example 2

Cloning of the NS5A PCR samples amplified from HCV Genotype-1a infected patients into NS5A replicon shuttle vectors

DNA sequences encoding the NS5A protein were generated following reverse transcription of RNA from plasma obtained from patients infected with HCV Genotype-1a and PCR-amplification of the reverse-transcribed product. Reverse transcription of RNA was performed using Taqman Reverse Transcription Kit (Applied Biosystems, #N808-0234) using oligo dA primers and according to the manufacturers' protocol. The synthesized cDNA was then PCR-amplified with the GC RICH PCR system (Roche Applied Science, #04 743 784 001) to ensure high fidelity and robust yields. Annealing temperatures in the range of 50-52°C were used depending on patient sample and primer combinations. The primers used for this PCR round were as follows:

Sense primer: 5`-ATAGCCTTCGCCTCCCGGGG-3` (SEQ ID NO:17)

Antisense primer: 5`-CGATGAGACGAGCTGGCTT-3` (SEQ ID NO:18)

Patient NS5A DNA were then subject to PCR using primers that introduced a BstZ17I restriction enzyme sequence at the 5` end of the NS5A gene and an AsiSI restriction enzyme sequence at the 3` end of the NS5A gene. The sense primer used to introduce the BstZ17I restriction enzyme sequence was 5`-TACCATGCTCCGGTTCTGGCT -3` (SEQ ID NO:19). The antisense primer used to introduce an AsiSI restriction enzyme sequence was 5`-CATYGAGCAGGCGATCGCATCYTCCGTGTCRGCCCCACT-3` (SEQ ID NO:20).

Patient NS5A PCR amplicons were then purified using Qiagen PCR purification Columns, digested with the restriction endonucleases BstZ17I andAsiSI The final product was gel purified using Qiagen's Gel Extraction Kit.

The replicon shuttle vectors pSS-1_1a_NS5A_BstZ17I_AsiSI or pSS-1_1a_(NS5A)_lacZ_BstZ17I_AsiSI were prepared by digestion with restriction endonucleases BstZ17I and AsiSI . The vectors were separated from digested insert by 1% agarose gel electrophoresis and gel purified using Qiagen's Gel Extraction Kit. Purified vectors were then treated with Shrimp Alkaline Phosphatase (Roche Applied Science).

Twenty-five ng of shuttle vector were ligated with the digested patient amplicons using T4 DNA ligase (Roche Applied Science) overnight at 14-16C. Vector to insert ratio of 1:2 to 1:4 were routinely used. After overnight ligation, 5 ul of the reaction were transformed into 100 ul of One Shot OmniMAX 2 T Phage-Resistant Cells (Invitrogen, Carlsbad, CA, USA) and plated after 1 hour of shaking at 37°C.

Ninety-six individual colonies were picked to inoculate 1200 ul of Terrific Broth (TB) supplemented with 50 microg/ml carbenecillin. This 96-well block was incubated overnight at 37°C with shaking. The next day, 100 microl of each culture was combined and DNA was extracted by miniprep to represent the 96-clone pool. The remaining cultures were centrifuged and used for plasmid DNA extraction using Qiaprep 96 Turbo Mini-DNA Kit (Qiagen). These individual molecular clones, which represent the individual 96 variants used for the Replicon Phenotypic Assay, were then used for sequencing.

Heterogeneous 96-clone pool plasmid DNA was submitted for sequencing to confirm the identity of patient samples and to screen for potential contaminating DNA prior to the *in vitro* transcription reaction. Individual molecular clones were submitted to sequencing for subsequent analysis in an effort to gain insight between Phenotypic Replicon Assay results and patient NS5A sequences. Fifty to one-hundred nanograms of plasmid DNA and 4 pmol of sequencing primers were routinely used.

Example 3

Replicon Phenotypic Assay

A. Preparation of *in vitro* transcribed RNA

Five micrograms of DNA plasmids were linearized by Sca I restriction enzyme (Roche). After overnight digestion at 37°C, the DNA was purified using Qiagen PCR purification kit. One microgram of linearized DNA was used for the *in vitro* transcription using T7 Mega script kit following manufacturer's protocol (Ambion). After 2 hours of incubation at 37°C, DNase treatment was performed for 30 minutes at 37°C to remove the DNA template. *In vitro* transcribed RNA was then purified using NucAway Spin Column following manufacturer's protocol (Ambion).

B. Hepatoma cell line

Cured hepatoma cell line Huh7 were cultured at 37°C in a humidified atmosphere with 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Glutamax™ and 100 mg/ml sodium pyruvate (Cat# 10569-010). The medium was further supplemented with 10 % (v/v) FBS (Cat# 16000-036) and 1 % (v/v) penicillin/streptomycin (Cat# 15140-122). All reagents were from Invitrogen/Gibco.

C. Determination of transient replicons replication level

Four million cured Huh7 cells were transfected with 5 microg of *in vitro* transcribed RNA using electroporation. Cells were then resuspended in 7.2 ml of DMEM containing 5 % FBS and plated in 96-well plate at 50000 cells/well (in 90microl final volume). Inhibitors were added 24 hours post-transfection in 3 fold dilutions at a final DMSO concentration of 1 % and firefly luciferase reporter signal was read 72 hours after addition of inhibitors using the Luciferase Assay system (Promega, cat # E1501). The IC_{50} values were assessed as the inhibitor concentration at which a 50 % reduction in the level of firefly luciferase reporter was observed as compared to the level of firefly luciferase signal without the addition of compounds.

The replication capacities of pSS-1_1a_(NS5A)_lacZ_BstZ17I_AsiSI shuttle vectors containing the NS5A gene from various patient samples were tested with the luciferase signal as the readout and compared to the replication capacity of the parent replicon, pSS-1. The results are shown on Table 1.

Table 1

Patient Isolate	Replication Capacity of replicons containing NS5A isolates
pSS-1	1
RO-183	0.31 ± 0.11
RO-185	0.07 ± 0.03
RO-186	0.15 ± 0.02
RO-187	0.12 ± 0.04
RO-190	0.15 ± 0.01
RO-191	0.19 ± 0.04
RO-192	0.13 ± 0.04
RO-194	0.25 ± 0.05
RO-196	0.09 ± 0.02
RO-197	0.11 ± 0.02
RO-198	1.96 ± 0.14
RO-199	0.18 ± 0.08
RO-203	1.06 ± 0.07
RO-204	2.20 ± 0.44
RO-205	0.16 ± 0.02
RO-208	0.23 ± 0.01
RO-209	0.07 ± 0.02
RO-212	0.42 ± 0.15

Replication capacity of 18 NS5A Genotype-1a isolates cloned in the NS5A Genotype-1a shuttle vector, pSS-1_1a_(NS5A)_lacZ_BstZ17I_AsiSI as compared to the wild-type pSS-1 vector set at a value of 1.

Claims

1. An HCV replicon shuttle vector comprising an HCV polynucleotide sequence that comprises, in order:
   (a) a polynucleotide sequence encoding a NS3 protein;
   (b) a polynucleotide sequence encoding a NS4A protein;
   (c) a polynucleotide sequence encoding a NS4B protein;
   (d) a restriction enzyme sequence that recognizes AscI or BstZ17I placed between 1 nucleotide and 15 nucleotides 5` from a polynucleotide sequence encoding a NS5A protein;
   (e) a polynucleotide sequence encoding a NS5A protein;
   (f) a restriction enzyme sequence that recognizes AsiSI placed between 1 nucleotide and 15 nucleotides 3` from a polynucleotide sequence encoding the NS5A protein; and
   (g) a polypeptide sequence encoding a NS5B protein;
   wherein said HCV polynucleotide sequence is derived from HCV genotype-1a or genotype-1b.

2. The HCV replicon shuttle vector of claim 1 comprising an HCV polynucleotide sequence selected from the group consisting of SEQ ID NO: 15 and SEQ ID NO: 21.

3. An HCV replicon shuttle vector comprising an HCV polynucleotide sequence that comprises, in order:
   (a) a polynucleotide sequence encoding a NS3 protein;
   (b) a polynucleotide sequence encoding a NS4A protein;
   (c) a polynucleotide sequence encoding a NS4B protein;
   (d) a restriction enzyme sequence that recognizes AscI or BstZ17I placed between 1 nucleotide and 15 nucleotides 5` from a polynucleotide sequence encoding a reporter protein or a selection reporter;
   (e) a polynucleotide sequence encoding a reporter protein or a selection protein;
   (f) a restriction enzyme sequence that recognizes AsiSI placed between 1 nucleotide and 15 nucleotides 3` from a polynucleotide sequence encoding a reporter protein or a selection protein; and

(g) a polynucleotide sequence encoding a NS5B protein;
wherein said HCV polynucleotide sequence is derived from HCV genotype-1a or genotype-1b.

4. The HCV replicon shuttle vector of claim 3 comprising an HCV polynucleotide sequence of SEQ ID NO: 16.

5. A method for assessing the effectiveness of an HCV inhibitor to control an HCV infection in a subject comprising the steps of:

(a) providing a sample from the subject infected with HCV;

(b) PCR-amplifying polynucleotide sequences encoding the NS5A protein and the NS5B protein from a plurarity of HCV quasispecies present in the sample with the use of a sense-strand primer which comprises a restriction enzyme sequence that recognizes AscI or BstZ17I, and an anti-sense strand primer which comprises a restriction enzyme sequence that recognizes AsiSI;

(c) cloning said PCR-amplifed polynucleotide sequences into an HCV replicon shuttle vector to produce chimeric HCV replicon plasmids;

(d) linearizing said chimeric HCV replicon plasmids and subjecting said linearized plasmids to *in vitro* transcription to produce chimeric HCV replicon RNAs; and

(e) transfecting a Huh7 cell line with said HCV replicon RNAs and measuring replication level of said HCV replicon RNAs in the presence or absence of the HCV inhibitor.

6. The method of claim 6 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 1.

7. The method of claim 5 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 2.

8. The method of claim 5 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 3.

9. The method of claim 5 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 4.

10. A method for assessing the effectiveness of an HCV inhibitor to control an HCV infection in a subject comprising the steps of:

(a) providing a sample from the subject infected with HCV;

(b) PCR-amplifying polynucleotide sequences encoding the NS5A protein from a plurarity of HCV quasispecies present in the sample with the use of a sense-strand primer comprising a nucleotide sequence of SEQ ID NO:19, and an anti-sense strand primer comprising a nucleotide sequence of SEQ ID NO: 20;

(c) cloning said PCR-amplifed polynucleotide sequences into an HCV replicon shuttle vector to produce chimeric HCV replicon plasmids;

(d) linearizing said chimeric HCV replicon plasmids and subjecting said linearized plasmids to *in vitro* transcription to produce chimeric HCV replicon RNAs; and

(e) transfecting Huh7 cell line with said HCV replicon RNAs and measuring replication level of said HCV replicon RNAs in the presence or absence of the HCV inhibitor.

11. The method of claim 10 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 1.

12. The method of claim 10 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 2.

13. The method of claim 5 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 3.

14. The method of claim 5 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 4.

15. A method for assessing the effectiveness of an HCV inhibitor to control an HCV infection in a subject comprising the steps of:

(a) providing a sample from the subject infected with HCV;

(b) PCR-amplifying polynucleotide sequences encoding the NS5A protein from a plurarity of HCV quasispecies present in the sample with the use of a sense-strand primer which comprises a restriction enzyme sequence that recognizes AscI or BstZ17I, and an anti-sense strand primer which comprises a restriction enzyme sequence that recognizes AsiSI;

(c) cloning said PCR-amplifed polynucleotide sequences into an HCV replicon shuttle vector to produce chimeric HCV replicon plasmids;

(d) transforming said plasmids into cells to generate a plurarity of colonies of transformed cells;

(e) pooling said colonies and isolating chimeric HCV replicon plasmids from the pooled colonies;

(f) linearizing said chimeric HCV replicon plasmids from step (e) and subjecting said linearized plasmids to *in vitro* transcription to produce chimeric HCV replicon RNAs; and

(g) transfecting Huh7 cell line with said HCV replicon RNAs and measuring replication level of said HCV replicon RNAs in the presence or absence of the HCV inhibitor.

16. The method of claim 15 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 1.

17. The method of claim 15 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 2.

18. The method of claim 15 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 3.

19. The method of claim 15 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 4.

20. A method for assessing the effectiveness of an HCV inhibitor to control an HCV infection in a subject comprising the steps of:

(a) providing a sample from the subject infected with HCV;

(b) PCR-amplifying polynucleotide sequences encoding the NS5A protein from a plurarity of HCV quasispecies present in the sample with the use of a sense-strand primer comprising a nucleotide sequence of SEQ ID NO:19, and an anti-sense strand primer comprising a nucleotide sequence of SEQ ID NO: 20;

(c) cloning said PCR-amplifed polynucleotide sequences into an HCV replicon shuttle vector to produce chimeric HCV replicon plasmids;

(d) transforming said plasmids into cells to generate a plurarity of colonies of transformed cells;

(e) pooling said colonies and isolating chimeric HCV replicon plasmids from the pooled colonies;

(f) linearizing said chimeric HCV replicon plasmids from step (e) and subjecting said linearized plasmids to *in vitro* transcription to produce chimeric HCV replicon RNAs; and

(g) transfecting Huh7 cell line with said HCV replicon RNAs and measuring replication level of said HCV replicon RNAs in the presence or absence of the HCV inhibitor.

21. The method of claim 20 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 1.

22. The method of claim 20 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 2.

23. The method of claim 20 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 3.

24. The method of claim 20 wherein the HCV replicon shuttle vector of step (c) comprises the HCV replicon shuttle vector of claim 4.

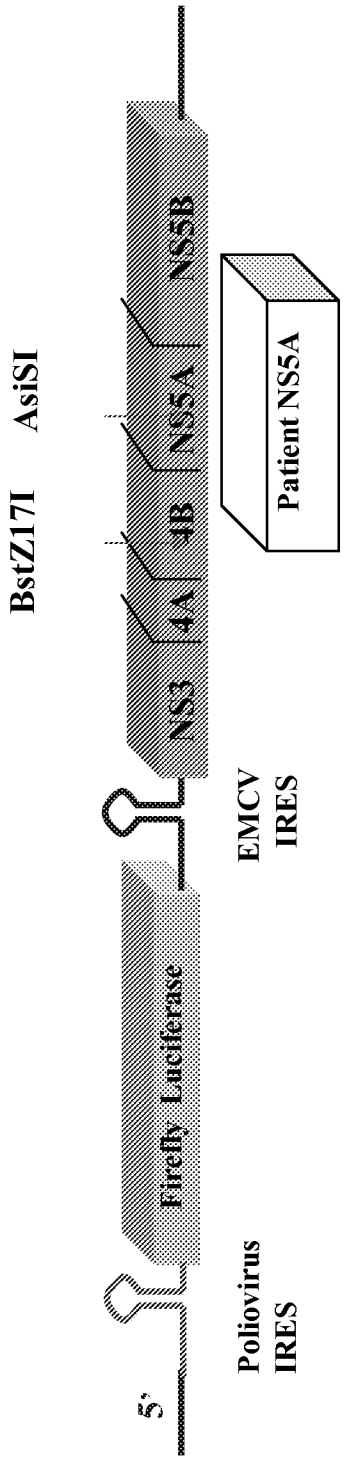
5'
Poliovirus IRES
Firefly Luciferase
EMCV IRES
NS3
4A
4B
NS5A
NS5B
Patient NS5A
BstZ17I
AsiSI

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/054664

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/86 C07K14/18 C12Q1/70
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K C12Q

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, EMBL, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRIPATHI RAKESH L ET AL: "Replication efficiency of chimeric replicon containing NS5A-5B genes derived from HCV-infected patient sera.", ANTIVIRAL RESEARCH, vol. 73, no. 1, January 2007 (2007-01), pages 40-49, XP005830363, ISSN: 0166-3542	1-4
Y	the whole document -----	5-24
Y	EP 1 801 116 A1 (HOFFMANN LA ROCHE [CH]) 27 June 2007 (2007-06-27) paragraph [0013] claims 1-24; figures 1,2; examples 1-4 -----	5-24
	-/--	

[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
28 June 2011	06/07/2011

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, Fax: (+31-70) 340-3016	Mandl, Birgit

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/054664

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEMM JULIE A ET AL: "Identification of Hepatitis C Virus NS5A Inhibitors", JOURNAL OF VIROLOGY, vol. 84, no. 1, January 2010 (2010-01), pages 482-491, XP002639563, the whole document -----	4-24
A	WO 2008/148671 A1 (HOFFMANN LA ROCHE [CH]; CHUA PONG KIAN [US]; NAJERA ISABEL [US]) 11 December 2008 (2008-12-11) the whole document -----	1-24
A	WO 2009/071488 A2 (HOFFMANN LA ROCHE [CH]; ALI SAMIR [US]; JIANG WEN-RONG [US]) 11 June 2009 (2009-06-11) the whole document -----	1-24
A	WO 03/089672 A1 (MERCK & CO INC) 30 October 2003 (2003-10-30) page 14, line 1 - line 15 example 6 -----	1-24

3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/054664

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/054664

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