HCV NS3 REPLICON SHUTTLE VECTORS

FIELD OF THE INVENTION

This invention pertains to novel HCV NS3 replicon shuttle vectors which are useful for screening, testing and evaluating HCV protease and helicase inhibitors.

BACKGROUND OF THE INVENTION

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 - α 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 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.

SUMMARY OF THE INVENTION

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 NS3 gene such that NS3 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 NS3 protease inhibitors and to HCV RNA helicase 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 NS3 variants and the sensitivity or resistance of these variants to drug treatment.

Accordingly, the present invention provides an HCV replicon shuttle vector comprising an HCV polynucleotide sequence wherein the HCV polynucleotide sequence comprises a polynucleotide sequence encoding a NS3 protein and unique restriction enzyme sequences flanking the 5` end and the 3` end of said polynucleotide sequence encoding the NS3 protein.

In a preferred embodiment of the invention, the unique restriction enzyme sequence at the 5` end of the polynucleotide sequence encoding the NS3 protein recognizes EcoRV and the unique restriction enzyme sequence at the 3` end of the polynucleotide sequence encoding the NS3 protein recognizes XbaI.

In a further preferred embodiment of the invention, the HCV polynucleotide sequence comprises, in order, a unique restriction enzyme sequence placed between 20 nucleotides 5` and 20 nucleotides 3` from the 5` end of a polynucleotide sequence encoding a NS3 protein; a polynucleotide sequence encoding a NS3 protein; a unique restriction enzyme sequence placed between 20 nucleotides 5` and 20 nucleotides 3` from the 3` end of a polynucleotide sequence encoding a NS3 protein; a polynucleotide sequence encoding a NS4A protein; a polynucleotide sequence encoding a NS4B protein; a polynucleotide sequence encoding a NS5A protein; and a polynucleotide sequence encoding a NS5B protein.

In still another preferred embodiment of the invention, the HCV replicon shuttle vector comprises an HCV polynucleotide selected from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:24 .

It is a second object of the present invention to provide a method for assessing the effectiveness of an HCV NS3 protease inhibitor or an HCV RNA helicase 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 NS3 protein from a plurality of HCV quasispecies present in the sample with the use of a sense-strand primer which comprises a unique restriction enzyme sequence, and an anti-sense strand primer which comprises a different unique restriction enzyme sequence, 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 NS3 protease inhibitor or the HCV RNA helicase inhibitor.

It is a third object of the present invention to provide an in vitro method for assessing the effectiveness of an HCV NS3 protease inhibitor or an HCV RNA helicase 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 NS3 protein from a plurarity of HCV quasispecies present in the sample with the use of a sense-strand primer which comprises a unique restriction enzyme sequence, and an anti-sense strand primer which comprises a different unique restriction enzyme sequence, 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 plurality 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 NS3 protease inhibitor or the HCV RNA helicase inhibitor.

In a preferred embodiment of the methods of the present invention, the restriction enzyme sequence of the sense-strand primer recognizes EcoRV and the restriction enzyme sequence of the anti-sense primer strand recognizes XbaI.

In a further preferred embodiment of the methods of the present invention, the sense-strand primer comprises a nucleotide sequence selected from SEQ ID NO:15 or SEQ ID NO: 16, and the anti-sense strand primer comprises a nucleotide selected from SEQ ID NO: 17 or SEQ ID NO:18.

In another preferred embodiment of the methods of the present invention, said PCR-amplifed HCV polynucleotide sequences encoding a NS3 protein are cloned into a HCV replicon shuttle vector comprising an HCV polynucleotide sequence wherein the shuttle vector HCV polynucleotide sequence comprises a polynucleotide sequence encoding a NS3 protein and unique restriction enzyme sequences flanking the 5` end and the 3` end of said polynucleotide sequence encoding the NS3 protein.

In a more preferred embodiment of the methods of the present invention, the unique restriction enzyme sequence at the 5` end of the polynucleotide sequence encoding the shuttle vector NS3 protein recognizes EcoRV and the unique restriction enzyme sequence at the 3` end of the polynucleotide sequence encoding the NS3 protein recognizes XbaI.

In a further preferred embodiment of the methods of the present invention, said PCR amplified HCV polynucleotide sequences encoding a NS3 protein are cloned into a HCV replicon shuttle vector comprising an HCV polynucleotide sequence wherein the shuttle vector HCV polynucleotide sequence comprises, in order: a unique restriction enzyme sequence placed between 20 nucleotides 5` and 20 nucleotides 3` from the 5` end of a polynucleotide sequence encoding a NS3 protein; a polynucleotide sequence encoding a NS3 protein; a unique restriction enzyme sequence placed between 20 nucleotides 5` and 20 nucleotides 3` from the 3` end of a polynucleotide sequence encoding a NS3 protein; a polynucleotide sequence encoding a NS4A protein; a polynucleotide sequence encoding a NS4B protein; a polynucleotide sequence encoding a NS5A protein; and a polynucleotide sequence encoding a NS5B protein.

In still another preferred embodiment of the methods of the present invention, said PCR-amplifed HCV polynucleotide sequences encoding a NS3 protein are cloned into a HCV replicon shuttle vector comprising a nucleotide sequence selected from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:24 .

The foregoing and other advantages and features of the invention, and the manner in which the same are accomplished, will become more readily apparent upon consideration of the following detailed description of the invention taken in conjunction with the accompanying examples, which illustrate exemplary embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the components of the HCV replicon shuttle vectors.

Figure 2 is a plasmid map of the Genotype-1a NS3 replicon shuttle vector, pSS-1_1a_NS3_EcoRV_XbaI_NS5B_AsiSI_RsrII (shown as its alternate name, H77 NS3 cassette).

Figure 3 is a plasmid map of the Genotype-1b NS3 replicon shuttle vector, pSC_1b_NS3_EcoRV_XbaI_NS5B_AsiSI_RsrII (shown as its alternate name, pSC FF EcoRV-XbaI-NS3 EcoRV/XbaI NS5B AsisI RsrII).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

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.

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 “EcoRV” recognizes the sequence 5’ GAT▼ATC 3’ and cuts the double-stranded DNA at the indicated nucleotides 3’ CTA▲TAG 5’ (indicated with ▼▲). The restriction enzyme “XbaI” recognizes the sequence 5’ T▼CTAGA 3’ and cuts afte the T residue on the recognition sequence. 3’ AGATC▲T 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 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.

EXAMPLES

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 NS3 replicon shuttle vectors for HCV genotype-1a and genotype-1b were derived from the HCV NS5B replicon shuttle vectors, pSS-1 and pSS-1_1a_NS5B_5`AsiSI_3`RsrII (genotype-1a), and pPI-luc/ET/SC and pSC_1b_NS5B_5`AsiSI_3`RsrII (genotype-1b), which are disclosed in the commonly owned US patent application, USSN 11/641,339, filed on December 18, 2006 by Dietrich et al., entitled "HCV Replicon Shuttle Vectors", which is incorporated by reference in full herein. The components of the replicon shuttle vectors are shown in Figure 1 and contain the HCV polynucleotide sequence from the 5`-UTR, NS3 through NS5B proteins and the 3`-UTR. The vectors also include the poliovirus internal ribosome entry site (IRES), which controls the translation of a 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 NS5B).

In order to introduce unique restriction enzyme sequences at the 5` and 3` ends of the NS3 gene sequence, intermediate vectors were created which contained the polynucleotide fragment derived from either the genotype-1a or the genotype-1b NS5B shuttle vectors and included the 3` portion of the EMCV IRES, the entire NS3, NS4A and NS4B protein coding regions, and the 5` portion of the NS5A. Mutations were introduced into the intermediate vectors using the QuickChange site-directed mutagenesis kit following the manufacturers' instructions (Stratagene, La Jolla, CA, USA). To introduce an EcoRV restriction enzyme sequence at the 5` end of the NS3 gene

and an XbaI restriction enzyme sequence at the 3` end of the NS3 gene, the following primers were used.

EcoRV sense primer	5`-TTGAAAAACACGATATCACCATGGCGCCTATTACG-3`	SEQ ID NO: 1
EcoRV anti-sense primer	5` CGTAATAGGCGCCATGGTGATATCGTGTTTTTCAA -3`	SEQ ID NO: 2
XbaI sense primer	5`-TGCATGTCGGCTGATCTAGAGGTCGTCACGAG -3`	SEQ ID NO: 3
XbaI anti-sense primer	5`- CTCGTGACGACCTCTAGATCAGCCGACATGCA -3`	SEQ ID NO: 4

Due to the presence of the EcoRV- and XbaI-recognizing restriction enzyme sequences on the firefly luciferase gene, site-directed mutagenesis was performed to remove these recognition sites. These mutations were generated also using the QuickChange site-directed mutagenesis kit and did not change the amino acid coding sequence of the firefly luciferase gene.

To generate replicon shuttle vectors with both NS3 and NS5 cassettes, the fragments containing the introduced restriction enzyme sequences were subcloned into pSS-1_1a_NS5B_5`AsiSI_3`RsrII to generate the genotype-1a NS3 shuttle vector, pSS-1_1a_NS3_EcoRV_XbaI_NS5B_AsiSI_RsrII (SEQ ID NO: 5 Figure 2) or into pSC_1b_NS5B_5`AsiSI_3`RsrII to generate the genotype-1b NS3 shuttle vector, pSC_1b_NS3_EcoRV_XbaI_NS5B_AsiSI_RsrII (SEQ ID NO: 6, Figure 3). To generate NS3 cassette only-replicon shuttle vectors, the fragments were subcloned into pSS-1 to generate pSS-1_1a_NS3_EcoRV_XbaI (SEQ ID NO:19) or into pPI-luc/ET/SC to generate pSC_1b_NS3_EcorV_XbaI (SEQ ID NO:20).

NS3 replicon shuttle vectors were also generated whereby the sequence encoding the NS3 protein were replaced by the beta-galactosidase (lacZ) coding sequence from pUC19 (GenBank Accession Number M77789). An EcoRV restriction site at the 5` end and a Xba I restriction site at the 3` end of the lacZ gene were introduced by PCR amplification. Site-directed mutagenesis was then performed to remove the internal XbaI site within the lacZ gene. LacZ vectors containing both NS3 and NS5 cassettes were generated for genotype-1a: pSS-1_1a_NS3/LacZ_EcoRV_ XbaI_NS5B_AsiSI_RsrII (SEQ ID NO:21) and for genotype-1b: pSC_1b_NS3/LacZ_EcoRV_XbaI_NS5B_AsiSI_RsrII (SEQ ID NO: 22). LacZ vectors carrying NS3 cassette-only were also generated for

genotype-1a: pSS-1_1a_NS3/LacZ_EcoRV_XbaI (SEQ ID NO:22) and for genotype-1b: pSC_1b_NS3/LacZ_EcorV_XbaI (SEQ ID NO: 23).

Replication capability of the NS3 replicon shuttle vectors was assessed using the phenotypic assay described in Example 3 and the results for the genotype-1b shuttle vector are shown on Table 1.

Table 1

	pSC_1b_NS5B_ 5`AsiSI_3`RsrII	pSC_1b_NS3_EcoRV_XbaI_ NS5B_AsiSI_RsrII
4h (RLU x10^6)	0.9	1.0
96h (RLU x10^6)	1.7	2.1

RLU represents level of firefly luciferase signal observed after 4 hours or 96 hours following transfection.

Example 2

Cloning of the NS3 PCR samples amplified from infected patients into the NS3 replicon shuttle vectors

DNA sequences encoding the NS3 protein were generated following reverse transcription of RNA from plasma obtained from patients infected with HCV and PCR-amplification of the reverse-transcribed product. Reverse transcription of RNA was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA) with random hexamer primers according to the manufacturers' protocol. The synthesized cDNA was then PCR-amplified with the Expand High-Fidelity PCR system (Roche Applied Science) 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 the first round PCR were as follows.

Genotype 1a:

Sense primer (NS2)	5`- GTAGGGGCCAGGAGATACTGCTTGG -3`	SEQ ID NO:7
Antisense primer (NS4A)	5`- GTTGACAGGCAATACGCGGCCAGAGCA -3'	SEQ ID NO:8

Genotype 1b:

Sense primer (NS2)	5`- CAGGGGGAGGGAGATACATCTGGA -3`	SEQ ID NO:9
Antisense primer (NS4A)	5`-GTTGTCAGGCAATACGCGGCCAGAGCT -3`	SEQ ID NO:10

Primers used for second round PCR were as follows:

Genotype 1a:

Sense primer (NS2)	5`- CCGATGGAATGGTCTCCAAGGGGTGGAGG -3	SEQ ID NO: 11
Antisense primer (NS4A)	5`-AGGACKCCGCCWACSAGCACCCAGGTGCA-3`	SEQ ID NO:12

Genotype 1b:

Sense primer (NS2)	5`-CCGATAGTCTTGAAGGGCAGGGGTGGCGA-3`	SEQ ID NO:13
Antisense primer (NS4A)	5`-AGGACKCCGCCWACSAGCACCCAGGTGCA-3`	SEQ ID NO:14

Sense primers used to introduce the EcoRV restriction enzyme sequence near the 5`end of the patient NS3 gene sequence were as follows.

GT1a	5`-AACACGATATCACCATGGCGCCCATCACGGCGTACGCCCAGCA -3`	SEQ ID NO: 15
GT1b	5`-AACACGATATCACCATGGCGCCTATTACGGCCTACTCCCAACAG-3`	SEQ ID NO: 16

Antisense primers used to introduce the XbaI restriction enzyme sequence near the 3` end of the patient NS3 gene sequence were as follows.

GT1a	5`- CGACCTCTAGATCGGCCGACATGCATGTCATGATG -3`	SEQ ID NO: 17
GT1b	5`- CGACCTCTAGATCAGCCGACATGCATGCCATGATG -3`	SEQ ID NO: 18

Patient NS3 DNA which were PCR amplified were then purified using Qiagen PCR Purification Columns, double digested with restriction endonucleases EcoRV and XbaI (Promega), and gel purified using Qiagen's Gel Extraction Kit.

The shuttle replicon vectors pSS-1_NS3_EcoRV_XbaI_NS5B_AsiSI_RsrII or pSC_NS3_EcoRV_XbaI_NS5B_AsiSI_RsrII were prepared by double digestion with restriction endonucleases EcoRV and XbaI (Promega). The vectors were separated from

digested insert by 1% agarose 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 DNA ligation kit (Takara Bio Inc.) for 30 minutes at 16 °C. Vector to insert ratio of 1:2 to 1:4 were routinely used. After overnight ligation, 5 µl of the reaction were transformed into 100 µl of DH5α™ Maximum Efficiency Cells (Invitrogen) and plated after 1 hour of phenotypic expression at 37C. When greater than 20% of the transformation mix was plated, sufficient number of colonies was obtained the following day.

96 individual colonies were picked to inoculate 200 µl of Terrific Broth (TB) supplemented with 50 µg/ml ampicillin. This "stock" 96-well plate was incubated overnight at 37 °C. The next day, this plate was used to prepare a replica plate. A 48 pin stamp was used to replica plate onto two LB plates supplemented with 100 µg/ml carbenicillin. The 96 individual 200 µl cultures were also transferred into 1.5 ml TB cultures supplemented with 50 µg /ml ampicillin to be used for DNA preparation. After an overnight incubation at 37 °C, the replica plate was spread with 6 mls of LB to obtain a heterogeneous pool of 96 clones, which was then used for mini-DNA preps. This DNA patient pool was then used for the subsequent Replicon Phenotypic Assay. After overnight shaking at 37°C, the 96 individual 1.5 ml TB cultures were spun down, decanted and plasmid DNA was extracted using Qiagen's Qiaprep 96 Turbo Mini-DNA Kit. These individual molecular clones, which represent the composite 96 pool used for the Replicon Phenotypic Assay, were used for sequencing reactions.

Heterogeneous clone pool plasmid DNAs were submitted to 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 NS3 sequences. One-half microgram of plasmid DNA and 8 pmole 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 µg 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 90 µl 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. Results of a study testing the effects of the NS3 protease inhibitory compound, VX-950, on the Genotype-1a template NS3 shuttle vector (designated H77 NS3) and replicons containing patient-derived NS3 are shown on Table 2. One particular sample, TN-132, which carries a V36M mutation in the NS3 gene exhibited reduced sensitivity to VX-950.

TABLE 2

NS3 V36M in GT1a clinical isolate TN132					
	H77 NS3	**TN132-1**	**TN132-2**	**TN132-6**	**2209-23**
IC50 (µM)	**0.44**	2.37	2.61	1.47	**0.63**
SEM	**0.05**	**0.76**	**0.29**	**0.20**	**0.07**

Claims

1. An HCV replicon shuttle vector comprising an HCV polynucleotide sequence wherein the HCV polynucleotide sequence comprises a polynucleotide sequence encoding a NS3 protein and unique restriction enzyme sequences flanking the 5` end and the 3` end of said polynucleotide sequence encoding the NS3 protein.

2. The HCV replicon shuttle vector of claim 1 wherein the HCV polynucleotide sequence comprises, in order:

a) a unique restriction enzyme sequence placed between 20 nucleotides 5` and 20 nucleotides 3` from the 5` end of a polynucleotide sequence encoding a NS3 protein;

b) a polynucleotide sequence encoding a NS3 protein;

c) a unique restriction enzyme sequence placed between 20 nucleotides 5` and 20 nucleotides 3` from the 3` end of a polynucleotide sequence encoding a NS3 protein;

d) a polynucleotide sequence encoding a NS4A protein;

e) a polynucleotide sequence encoding a NS4B protein;

f) a polynucleotide sequence encoding a NS5A protein; and

g) a polynucleotide sequence encoding a NS5B protein.

3. The HCV replicon shuttle vector of claim 1 or 2 wherein the unique restriction enzyme sequence at the 5` end of the polynucleotide sequence encoding the NS3 protein recognizes EcoRV and the unique restriction enzyme sequence at the 3` end of the polynucleotide sequence encoding the NS3 protein recognizes XbaI.

4. The HCV replicon shuttle vector of claim 1 to 3 comprising an HCV polynucleotide sequence selected from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24 .

5. An in vitro method for assessing the effectiveness of an HCV NS3 protease inhibitor or an HCV RNA helicase inhibitor to control an HCV infection in a subject comprising the steps of:

a) PCR-amplifying polynucleotide sequences encoding a NS3 protein from a plurality of HCV quasispecies present in a sample of the subject with the use of a sense-

strand primer which comprises a unique restriction enzyme sequence, and an anti-sense strand primer which comprises a different unique restriction enzyme sequence;

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

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

d) 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 NS3 protease inhibitor or the HCV RNA helicase inhibitor.

6. An in vitro method for assessing the effectiveness of an HCV NS3 protease inhibitor or an HCV RNA helicase inhibitor to control an HCV infection in a subject comprising the steps of:

a) PCR-amplifying polynucleotide sequences encoding a NS3 protein from a plurality of HCV quasispecies present in a sample of the subject with the use of a sense-strand primer which comprises a unique restriction enzyme sequence, and an anti-sense strand primer which comprises a different unique restriction enzyme sequence;

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

c) transforming said plasmids into cells to generate a plurality of colonies of transformed cells;

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

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

f) 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 NS3 protease inhibitor or the HCV RNA helicase inhibitor.

7. The method of claim 5 or 6 wherein the restriction enzyme sequence of the sense-strand recognizes EcoRV and the restriction enzyme sequence of the anti-sense strand recognizes XbaI.

8. The method of claim 5 to 7 wherein the sense-strand primer comprises a nucleotide sequence selected from SEQ ID NO: 15 or SEQ ID NO: 16, and the anti-sense strand primer comprises a nucleotide selected from SEQ ID NO: 17 or SEQ ID NO:18.

9. The method of claim 5 to 8 wherein the HCV replicon shuttle vector of step b) comprises the HCV replicon shuttle vector of claim 1.

10. The method of claim 5 to 8 wherein the HCV replicon shuttle vector of step b) comprises the HCV replicon shuttle vector of claim 2.

11. The method of claim 5 to 8 wherein the HCV replicon shuttle vector of step b) comprises the HCV replicon shuttle vector of claim 3.

12. The method of claim 5 to 8 wherein the HCV replicon shuttle vector of step b) comprises the HCV replicon shuttle vector of claim 4.

13. The shuttle vectors and methods substantially as hereinbefore described, especially with reference to the foregoing examples.