PRODUCTION OF INFECTIOUS HEPATITIS C VIRUS PARTICLES IN CELL CULTURE

This invention pertains to novel methods of producing infectious HCV Genotype 1 viruses in cell culture and is useful for screening, testing and evaluating various HCV inhibitors.

Hepatitis C virus (HCV) 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 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. This is especially true for genotype 1a HCV viral particles, despite a recent

report which details the production of low levels of infectious genotype 1a virus using HCV RNA that contains a combination of five cell culture-adaptive mutations (Yi et al., *Proc. Natl. Acad. Sci. USA* **2006** 103(7):2310-2315).

Two groups have reported the generation of genotype-1a replication system using highly permissive sublines of Huh-7 human hepatoma cells. Blight et al. (*J. Virol.* **2003** 77:3181-3190) were able to select G418 resistant colonies supporting replication of genotype 1a derived subgenomic replicons in a hyper-permissive Huh-7 subline, Huh-7.5, that was generated by curing an established G418-resistant replicon cell line of the cubgenomic Con1 replicon RNA that had been used to select it by treatment with interferon-alpha (Blight et al., *J. Virol.* **2002** 76:13001-13014). Sequence analysis of replicating HCV RNAs inside of such selected cell lines showed that the most common critical mutations were located at amino acid position 470 of NS3 (P1496L) within domain II of the NS3 helicase, and the NS5A mutation (S2204I). In another case, Grobler et al. (*J. Biol. Chem.* **2003** 278:16741-16746), used a systematic mutational approach to reach the similar conclusion that both P1496L and S2204I combination was necessary to get genotype 1a replication in a highly permissive Huh-7 subline which was selected in an independent but similar way. However, genotype-1a RNAs with these two enhanced mutations does not undergo replication in the Huh-7 cell line, indicating limited usefulness of this system.

The present invention is based on the surprising effect of using human serum to improve the production of infectious HCV genotype 1 virus particles in cell culture systems. The availability of HCV genotype 1 virus (principally associated with liver disease in most regions of the world) that can undergo the complete viral cycle in cultured cells is beneficial for the discovery and development of novel therapies for the treatment of HCV.

Accordingly, the present invention provides a method for increasing the production of HCV genotype 1 virus particles in cultured cells comprising transfecting cultured cells with a replication competent HCV genotype 1 polynucleotide that comprises the adaptive mutations, Q1067R, V1655I, K1691R, K2040R, S2204I, incubating the transfected cultured cells in the presence of 2-10% human serum, and collecting the medium from the transfected cultured cells that contains infectious HCV genotype 1 virus particles. The present invention further provides a method of screening for a HCV genotype 1 inhibitor comprising transfecting cultured cells with a replication competent HCV genotype 1 polynucleotide that comprises the adaptive

mutations, Q1067R, V1655I, K1691R, K2040R, S2204I; incubating the transfected cultured cells in the presence of 2-10% human serum; collecting the medium from the transfected cultured cells that contains infectious HCV genotype 1 virus particles; infecting native cultured cells with the infectious HCV genotype 1 virus particles in the presence or absence of a molecule being screened for HCV inhibitory activity; and measuring the level of HCV present in the infected cultured cells wherein a decrease in the level of HCV in the presence of the molecule compared to the absence of the molecule indicates that the molecule is a HCV genotype 1 inhibitor.

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.

Figure 1. Human serum does not enhance HCV replication. Rof-400c cells were transfected with *in vitro* transcribed RNA that encoded for the HCV strain H77S or a replication defective mutant (GND). At the indicated time post-transfection the intracellular RNA was purified and then used to determine the amount of HCV RNA.

Figure 2. Human serum does enhance production of infectious HCV. Rof-400c cells were transfected with *in vitro* transcribed RNA that encoded for the HCV strain H77S or a replication defective mutant (GND). At the indicated time post-transfection the medium was collected and used to infect naive cells. After three days, the intracellular RNA was purified and then used to determine the amount of HCV RNA.

Figure 3. Detection of HCV Core protein in infected cells by immunofluorescence analysis. Medium collected from cells transfected with *in vitro* transcribed RNA that encoded for the HCV strain H77S was used to infect naive cells. After four days, the expression of the HCV Core protein was analyzed by immunofluorescence.

Figure 4. Detection of HCV Core protein in infected cells by immunoperoxidase analysis. Medium collected from cells transfected with *in vitro* transcribed RNA that encoded for the HCV strain H77S was used to infect naive cells. After four days, the expression of the HCV Core protein was analyzed after immunoperoxidase staining.

Figure 5. Kinetics of infectious virus production for H77S and H77S RO-51-5B. Rof-0c cells were transfected with *in vitro* transcribed RNA that encoded for the HCV strain H77S or the chimeric stain H77S RO-51-5B. At the indicated time points, medium was collected and used to infect naive cells in order to determine the infectious virus titer which was measured as the end point dilution that resulted in 50% of the wells containing infected cells (tissue culture infected dose TCID50).

Figure 6. Potency of an NS5B inhibitor and HCV entry inhibitor against the GT 1a infectious virus. Rof-0c cells were infected with either H77S or the chimera H77S RO-51-5B. At the time of infection, the cells were treated with a serial dilution of either the NS5B inhibitor HCV-796 or the HCV entry inhibitor JS81. After three days, the intracellular RNA was purified and used to determine the amount of HCV RNA.

As used herein, the term "replication competent polynucleotide" refers to a polynucleotide that replicates when present in a cell. For instance, a complementary polynucleotide is synthesized. As used herein, the term "replicates in vitro" indicates the polynucleotide replicates in a cell that is growing in culture. The cultured cell can be one that has been selected to grow in culture, including, for instance, an immortalized or a transformed cell. Alternatively, the cultured cell can be one that has been explanted from an animal. "Replicates in vivo" indicates the polynucleotide replicates in a cell within the body of an animal, for instance a primate (including a chimpanzee) or a human. In some aspects of the present invention, replication in a cell can include the production of "infectious" virus particles, i.e., virus particles that can infect a cell and result in the production of more infectious virus particles.

A replication competent polynucleotide includes at least one adaptive mutation. As used herein, an "adaptive mutation" is a change in the amino acid sequence of the polyprotein that increases the ability of a replication competent polynucleotide to replicate compared to a replication competent polynucleotide that does not have the adaptive mutation.

One adaptive mutation that a replication competent polynucleotide referred in the present invention includes an arginine at about amino acid 1067, which is about amino acid 41 of NS3. Most clinical HCV isolates and molecularly cloned laboratory HCV strains include a glutamine at this position, thus this mutation can be referred to as Q1067R. A second adaptive mutation is

an isoleucine at about amino acid 1655, which is about amino acid 629 of NS3. Most clinical HCV isolates and molecularly cloned laboratory HCV strains include a valine at this position, thus this mutation can be referred to as V1655I. A third adaptive mutation is an arginine at about amino acid 1691, which is about amino acid 34 of NS4A. Most clinical HCV isolates and molecularly cloned laboratory HCV strains include a lysine at this position, thus this mutation can be referred to as K1691R. A fourth adaptive mutation is an arginine at about amino acid 2040, which is about amino acid 68 of NS5A. Most clinical HCV isolates and molecularly cloned laboratory HCV strains include a lysine at this position, thus this mutation can be referred to as K2040R. A fifth adaptive mutation that a replication competent polynucleotide referred in the present invention includes an isoleucine at about amino acid 2204, which is about amino acid 232 of NS5A. Most clinical HCV isolates and molecularly cloned laboratory HCV strains include a serine at this position, and this mutation has been referred to in the art as S2204I.

As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double-and single-stranded DNA and RNA. A polynucleotide may include nucleotide sequences having different functions, including for instance coding sequences, and non-coding sequences such as regulatory sequences and/or non-translated regions. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical techniques. A polynucleotide can be linear or circular in topology and can be, for example, a portion of a vector, such as an expression or cloning vector, or a fragment.

The terms "coding region" and "coding sequence" are used interchangeably and refer to a polynucleotide region that encodes a polypeptide and, when placed under the control of appropriate regulatory sequences, expresses the encoded polypeptide. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. A coding region can encode one or more polypeptides. For instance, a coding region can encode a polypeptide that is subsequently processed into two or more polypeptides. A regulatory sequence or regulatory region is a nucleotide sequence that regulates expression of a coding region to which it is operably linked. Nonlimiting examples of regulatory sequences include promoters, transcription initiation sites, translation start sites, internal ribosome entry sites, translation stop sites, and terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory sequence is "operably linked" to a coding region

when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

"Polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, polyprotein, proteinase, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. A "hepatitis C virus polyprotein" refers to a polypeptide that is post-translationally cleaved to yield more than one polypeptide.

The terms "5' non-translated RNA," "5' non-translated region," "5' untranslated region" and "5' noncoding region" are used interchangeably, and are terms of art (see Bukh et al., *Proc. Nat. Acad. Sci. USA* **1992** 89: 4942-4946). The term refers to the nucleotides that are at the 5' end of a replication competent polynucleotide.

The terms "3' non-translated RNA," "3' non-translated region," and "3' untranslated region" are used interchangeably, and are terms of art. The term refers to the nucleotides that are at the 3' end of a replication competent polynucleotide.

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.

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).

The term "HCV genotype 1 inhibitor" refers to a molecule that inhibits any function of HCV genotype-1 and may act at any step in the life cycle of the virus from initial attachment and entry to release, and may include but is not limited to an attachment inhibitor, entry inhibitor, a fusion inhibitor, a trafficking inhibitor, a replication inhibitor, a translation inhibitor, a protein processing inhibitor, or a release inhibitor. The molecule can be from a wide range and may include but is not limited to an organic molecule, a peptide, a polypeptide (for instance, an antibody), a polynucleotide (for instance an antisense oligonucleotide, siRNA, microRNA), or a combination thereof.

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.

MATERIALS AND METHODS

Cell Culture

The Rof-0 cells are a human hepatocellular carcinoma cell line derived from the Huh-7 cell line. The Rof-0 cells stably maintain a HCV genotype (GT) 1b replicon. A cell line with diminished responsiveness to interferon-α was generated by maintaining the Rof-0 cells in the presence of 400 units/ml IFN-α2a (Roferon®, Hoffmann-LaRoche Inc.) as well as G418 (Geneticin®, Invitrogen) to maintain selection of the replicon. The cell line that resulted is called Rof-400. The HCV replicon was cured from Rof-0 and Rof-400 cells by maintaining the cells in the presence of 2'-C-methyl adenosine and resulted in the cell lines Rof-0c and Rof-400c. The cell lines were cultured Dulbecco's Modified Eagle Medium (DMEM) supplemented with Glutamax™ and 100 mg/ml sodium pyruvate (Invitrogen). The medium is further supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 1% (v/v) penicillin/streptomycin.

Plasmids

A plasmid encoding the full-length GT 1a strain H77 with 5 cell culture adaptive mutations was engineered as follows. The TQ-1 plasmid, which encodes for the GT 1a H77 subgenomic replicon, and the TX-2 plasmid, which also encodes for the H77 subgenomic replicon and encodes the AsiSI and RsrII restriction sites flanking the NS5B coding sequence, were digested with the restriction enzymes AgeI and NsiI. The 6400 base pair fragment that resulted from the digest was purified. The plasmid HCV 1a H77 was digested with *AgeI* and *NsiI* and the 5100 base pair fragment that resulted was purified. The purified fragments from the TQ-1 and TX-2 digestion were separately ligated with the HCV 1a H77 digestion product resulting in the plasmid pUC HCV1a H77, which contains three adaptive mutations (K1691R, K2040R, and S2204I), and pUC HCV 1a-H77.AsiSIRsrII, which contains the same three adaptive mutations plus the AsiSI and RsrII restrictions sites used to cassette in NS5B sequences. Two additional adaptive mutations (Q1067R and V1655I) were introduced into both vectors

using the Quick Change site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). This resulted in the plasmids pUC H77S (SEQ ID NO:1) and pUC H77S.AsiSIRsrII (SEQ ID NO:2). A replication defective construct was generated by introducing a mutation in the NS5B active site (D2738N) using the Quick Change site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene) generating the construct pUC H77S GND.

A chimeric H77S virus that encodes the NS5B sequence from a clinical isolate was generated by digesting pUC H77S.AsiSIRsrII and a PCR product for the clinical isolate RO-51 NS5B sequence with AsiSI and RsrII. The fragments were ligated together resulting in the plasmid pUC H77S RO-51-5B (SEQ ID NO:3).

Virus Production

The plasmids that encode for the full-length HCV genome were linearized with the restriction enzyme *Spe*I and then treated with Mung bean nuclease. The linearized template was used in an *in vitro* RNA transcription reaction using the T7 Ribomax Express Kit (Promega) according to the manufacturer's instructions. For RNA transfection, four million Rof-0c or Rof-400c cells were electroporated with 2-10 μg of *in vitro* transcribed RNA. After electroporation, the cells were resuspended in DMEM containing either 5% (v/v) FBS or 2%-10% (v/v) human serum (HS, Bioreclamation). At the indicated time points the medium was collected, spun at 3000 RPM, and aliquoted to assay for infectious virus production.

Infectious virus assays

Medium collected from the transfected Rof-0c or Rof-400c cells was assayed for infectious virus by incubating with naive Rof-0c or Rof-400c. After incubating the naive cells for 72-96 hours, either the cellular RNA was extracted to quantify HCV RNA or the cells were fixed to analyze for expression of HCV proteins.

The presence of HCV RNA was examined after purification of total cellular RNA using the PerfectPure RNA 96 Cell Kit (5 Prime) according to the manufacturer's instructions. To quantitate the amount of HCV RNA, cDNA was amplified using either the Taqman Universal PCR mix (Applied Biosystems) or the TaqMan EZ RT-PCR kit (Applied Biosystems) with a set of primers and probe complementary to a region within the 5' untranslated region (UTR). The

primer and probe sequences are: (HCV 20F) CGACACTCCACCATAGATCACT (SEQ ID NO:4); (HCV 114R) GAGGCTGCACGACACTCATACT (SEQ ID NO:5); (HCV P43) FAM-CCCTGTGAGGAACTACTGTCTTCACGCAGA-TAMRA (SEQ ID NO:6).

The expression of HCV proteins in infected cells was examined and quantified by either an immunofluorescence assay or an immunoperoxidase assay. The cells were fixed by incubating in 2% formaldehyde for 1 hour at room temperature. Following fixation, the cells were permeabilized by a 5 minute incubation in PBS containing 0.2% TX-100 and 0.1% Na citrate. For fluorescent imaging, the permeabilized cells were blocked using 3% normal goat serum and 0.5% bovine serum albumin for 30 minutes and then stained with a mouse monoclonal antibody specific for HCV core (ab2740, Abcam) for 20 minutes. After washing, the cells were incubated with a secondary antibody (A11032, Invitrogen) for 20 minutes. The cells were mounted using 1 drop of Permafluor (Thermo Scientific) and imaged. The number of infected foci were counted in order to determine the infectious titer in focus forming units/ml.

The infectious titer could also be determined using an immunoperoxidase assay. The cells were fixed and permeabilized as described above. The cells were then blocked using the ImmPRESS Anti-Mouse Ig peroxidase Kit (MP-7402, Vector Labs) according to manufacturer's instructions. The cells were stained in block with a mouse monoclonal antibody specific to HCV core (ab2740, Abcam) for 1 hour. After washing, the cells were incubated for 30 minutes with ImmPRESS peroxidase:anti-mouse conjugate. The stained cells were visualized after a 10 minute incubation with ImmPACT DAB substrate (SK-4105, Vector Labs) followed by DAB enhancement (H-2200, Vector Labs). The infectious titer was determined as the end point dilution that resulted in 50% of the wells containing infected cells (tissue culture infected dose TCID50).

HCV infectious virus EC_{50} determinations

The sensitivity of infectious HCV to antivirals was determined using the genotype 1a strains H77S or H77S RO-51-5B. The virus stocks were generated by transfecting the full-length genome into Rof-0c cells, culturing the cells in DMEM containing 2-10% HS, and collecting the medium 7 days post-transfection. For EC_{50} determinations, the Rof-0c cells were plated at 10,000 cells per well into 96-well poly-D-lysine coated plates (BD Biosciences). Twenty-four hours post-plating, the medium was removed and 90 µl of the virus stock was

added per well. The inhibitors, at 3-fold serial dilutions, were then added. Three days post-infection, the HCV RNA was quantified as described above. The EC_{50} values were defined as the concentration at which 50% reduction in the levels of HCV RNA, as determined by quantitative RT-PCR, were observed.

RESULTS

Human serum does not affect HCV RNA replication. Studying the *in vitro* replication of an infectious GT 1a strain is currently limited by the low viral titers produced. In order to improve infectious virus production, the effect of human serum was examined. A cured Huh-7 cell line, Rof-400c, was transfected with the full-length GT 1a virus strain H77S and the cells were cultured in medium containing either 10% FBS or 10% HS. The amount of intracellular HCV RNA was determined over 5 days. Cells cultured in either HS or FBS contained a similar amount of HCV RNA through all time points tested (Fig. 1). The addition of HS to transfected cells does not appear to increase the replication of HCV RNA.

Human serum does increase the production of infectious HCV. In the same experiment described above, the effect of human serum on the production of infectious HCV particles was examined. The medium was removed every 24 hours post-transfection for five days and then inoculated onto naive cells to measure infectious virus production. The presence of infectious virus was determined by quantifying the amount of intracellular HCV RNA within naive cells after a 72 hour incubation in the presence of supernatant collected at the indicated time point. The amount of intracellular HCV RNA detected in the infected naive cells was equivalent between cells infected with supernatant collected from cells transfected either H77S or the replication-defective mutant and cultured in FBS (Fig. 2). This indicates that the amount of infectious HCV released from the cells cultured in FBS could not be differentiated from the residual HCV RNA that remained from the transfection. However, there was an increase in the amount of intracellular HCV RNA recovered from the infected naive cells that were inoculated with medium from the transfected cells cultured with HS (Fig. 2). The transfected cells cultured in HS, released infectious HCV and the amount increased throughout the five day assay. These experiments demonstrate that HS does not increase the replication of HCV RNA but does increase the production of infectious virus.

In order to verify that infectious particles were released from the transfected cells cultured in HS, naive cells were inoculated with supernatant collected at various time points and then analyzed for expression of HCV core protein. The presence of HCV core protein was confirmed in cells stained for immunofluorescence and for immunoperoxidase analysis (Fig. 3 and Fig.4). These results demonstrated that the increase in HCV RNA detected in naive cells infected with medium from transfected cells cultured with HS (Fig. 2) is a result of a productive HCV infection.

Peak production of infectious HCV. The experiments described above demonstrated that transfected cells cultured in HS released infectious particles over a five day period. In order to determine the peak time point for virus production, transfected cells were cultured in HS for up to 11 days. Medium was collected from the transfected cells and used to inoculate naive cells. The presence of infectious particles was quantified by an end-point dilution assay that determined the TCID50/ml. Rof-0c cells were transfected with H77S and at 7 days post-transfection the infectious titer peaked at approximately 6000 TCID50/ml (Fig. 5). The peak HCV infectious titer obtained from transfected cells cultured in HS was 60-fold higher than that previously reported for cells cultured in FBS (Yi et al., *Proc. Natl. Acad. Sci. USA* **2006** 103(7):2310-2315).

Generation of a NS5B chimeric virus. A NS5B cassette system has been established using the HCV replicon that facilitates the cloning and analysis of any NS5B sequence (Le Pogam *et al., J. Antimicrob. Chemother.* **2008** 61:1205-1216). The NS5B cassette has been used to analyze the phenotypic response, from a panel of NS5B isolates, to various non-nucleoside and nucleoside inhibitors. The AsiSI and RsrII restriction sites, which are utilized for cloning the NS5B sequences, were cloned into the full-length H77S genome. The consensus sequence, from a clinical isolate known to replicate within the H77 cassette replicon, was cloned into the H77S NS5B cassette resulting in the chimeric virus H77S R0-51-5B. The production of infectious virus from H77S RO-51-5B transfected cells was examined. Similar to H77S, the peak time point for infectious virus production was at day 7 although the titer of H77S RO-51-5B was decreased by 3-fold compared to H77S (Fig. 5). This data demonstrates that the NS5B cassette system can be used to generate chimeric infectious viruses.

Potency of HCV inhibitors against GT1a virus. Virus stocks were generated by collecting medium at 7 days post-transfection from cells cultured in presence of HS. The HCV

stocks were analyzed to determine if they would be sufficient to measure the potency of HCV inhibitors. Rof-0c cells were plated in a 96-well plate, infected with either H77S or H77S RO-51-5B, and then treated with either a known non-nucleoside inhibitor (HCV-796) or a known entry inhibitor (JS81). The potency of HCV-796 against infectious H77S was 32 ± 4 nM and is similar to what has been reported (Fig. 6). The potency of JS81 against H77S RO-51-5B was 139 ± 23 ng/ml and is also similar to reported data (Fig. 6). These experiments provide evidence that the GT 1a infectious virus, grown in the presence of HS, can be used to measure the potency of HCV inhibitors.

Claims

1. A method for increasing the production of infectious HCV genotype 1 virus particles in cultured cells comprising
transfecting cultured cells with a replication competent HCV genotype 1 polynucleotide that comprises the adaptive mutations, Q1067R, V1655I, K1691R, K2040R, S2204I;
incubating the transfected cultured cells in the presence of 2-10% human serum;
collecting the medium from the transfected cultured cells that contains infectious HCV genotype 1 virus particles.

2. The method of claim 1 wherein the transfected cultured cells are derived from a Huh-7 cell line.

3. The method of claim 1 or claim 2 wherein the transfected cultured cells are incubated in the presence of 10% human serum.

4. A method for increasing the production of infectious HCV genotype 1 virus particles in cultured cells comprising
transfecting cultured cells with a replication competent HCV genotype 1 polynucleotide that comprises the adaptive mutations, Q1067R, V1655I, K1691R, K2040R, S2204I, and that further comprises a NS5B polynucleotide sequence derived from a sample in a HCV genotype 1 infected subject;
incubating the transfected cultured cells in the presence of 2-10% human serum;
collecting the medium from the transfected cultured cells that contains infectious HCV genotype 1 virus particles.

5. The method of claim 4 wherein the transfected cultured cells are derived from a Huh-7 cell line.

6. The method of claim 4 or claim 5 wherein the transfected cultured cells are incubated in the presence of 10% human serum.

7. A method of screening for a HCV genotype 1 inhibitor comprising:

transfecting cultured cells with a replication competent HCV genotype-1a polynucleotide that comprises the adaptive mutations, Q1067R, V1655I, K1691R, K2040R, S2204I;
incubating the transfected cultured cells in the presence of 2-10% human serum;
collecting the medium from the transfected cultured cells that contains infectious HCV genotype 1 virus particles;
infecting native cultured cells with the infectious HCV genotype 1 virus particles in the presence or absence of a molecule being screened for HCV inhibitory activity; and
measuring the level of HCV present in the infected cultured cells wherein a decrease in the level of HCV in the presence of the molecule compared to the absence of the molecule indicates that the molecule is a HCV genotype 1 inhibitor.

8. The method of claim 7 wherein the replication competent HCV genotype 1 polynucleotide further comprises a NS5B sequence derived from a sample in a HCV genotype 1-infected subject.

9. The method of claim 7 or claim 8 wherein the transfected cultured cells and infected cultured cells are derived from a Huh-7 cell line.

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