Supplementary Methods

Cell culture

Huh7, HeLa and IMY-N9 cells were cultured at 37 ºC in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, under 5% CO_2 conditions. HepG2 cells were cultured in minimum essential medium containing 10% fetal bovine serum.

HCV plasmid construction

The genotype 2a clone JFH1 was isolated from a patient with fulminant hepatitis; detailed patient information has been reported previously[7,29]. Total RNA was extracted from the serum during the acute phase, and 14 fragments of HCV cDNA, covering the entire genome[7], were amplified by RT-PCR. All amplified products were purified and cloned into pGEM-T EASY™ vectors (Promega) pGEM1-258, pGEM44-486, pGEM317-849, pGEM617-1323, pGEM1141-2367, pGEM2285-3509, pGEM3471-4665, pGEM4547-5970, pGEM5883-7003, pGEM6950-8035, pGEM7984-8892, pGEM8680-9283, pGEM9231-9634, and pGEM9594-9678 (numbers of each clone indicate positions within JFH1). Based on the consensus sequence of JFH1 (accession No: AB047639), a subgenomic replicon construct of JFH1 (pSGR-JFH1; accession No: AB114136) was assembled as previously described[8]. The 5' half of the JFH1 cDNA (nt 1 to 5970) was assembled into plasmid pGEM1-5970 using the 9 plasmids containing overlapping cDNAs. An *Age*I and *EcoT22*I fragment (nt 154 to 5293) was obtained from pGEM1-5970 and inserted into pSGR-JFH1, and the resulting plasmid pJFH1 contained the full-length JFH1 cDNA downstream of the T7 RNA promoter sequence (pJFH1; Fig. 1a).

Three mutant constructs were derived from pJFH1: pJFH1/GND, with an amino acid substitution converting the GDD motif into GND and abolishing the RNA polymerase activity of NS5B[4,5]; pJFH1/ E1-E2, with an in-frame deletion of 351 amino acids (aa 217 to 567) spanning the region from E1 (aa 192 to 383) to E2 (aa 384 to 750); pJFH1/E2HA, with the HA tag sequence (YPYDVPDYA) used to replace a 9 amino acid section (aa 394 to 402) of the E2 hypervariable region. We also prepared expression vectors pEF/Core and pEF/CE2, containing Core region cDNA and Core to E2 region cDNA, respectively, in pEF1/Myc-HisB (Invitrogen) as described previously[29]. The plasmid pJ6CF, a generous gift from Dr. Jens Bukh (National Institute of Health, Bethesda, MD), is the representative HCV genotype 2a clone, and transcripts were confirmed to be infectious in chimpanzees[11]. Plasmid pJCH1 was constructed in the same manner as pJFH1, using the consensus sequence of the JCH1 strain[7] (accession No: AB047640), which was isolated from a patient with chronic hepatitis.

Plasmids pFK-Luc-JFH1 encodes a bicistronic full-length replicon. In this construct, JFH1 nucleotide residues 1 to 390 encompassing the entire 5'-UTR and the coding region of the 16 amino-terminal residues of core were fused via an *Asc*I site to the coding sequence of firefly-luciferase. The second cistron downstream of the reporter is expressed via an IRES derived from the Encephalomyocarditis virus and encodes the complete JFH1 open reading frame. To allow simple generation of in vitro transcripts with authentic 3' ends, the 3'-UTR is flanked by the genomic ribozyme derived from Hepatitis delta virus. Details of this luciferase virus system will be described elsewhere.

RNA synthesis and transfection

Plasmid pJFH1 was restricted with *Xba*I and treated with Mung Bean nuclease (New England Biolabs) to remove the 4 terminal nucleotides resulting in the correct 3'-end of the HCV cDNA. Digested plasmid DNAs were purified and used as templates for RNA synthesis. HCV RNA was synthesized *in vitro* using a MEGAscript™ T7 kit (Ambion). Synthesized RNA was treated with DNaseI (Promega) followed by acid phenol extraction to remove any remaining template DNA. Synthesized HCV RNA (10 μg) was used for electroporation. Trypsinized Huh7 cells were washed with Opti-MEM I™ reduced-serum medium (Invitrogen) and resuspended at 7.5×10^6 cells/ml with Cytomix™ buffer[5,10]. RNA was mixed with 400 μl of cell suspension and transferred into an electroporation cuvette (Thermo Hybrid). Cells were then pulsed at 260 V and 950 μF using Gene Pulser II™ apparatus (Bio-Rad). Transfected cells were immediately transferred into two 10-cm culture dishes, each containing 8 ml of culture medium.

Northern blot analysis

Total cellular RNA was isolated from harvested cells using the ISOGEN system (Nippon GENE) in accordance with the instructions of the manufacturer. Isolated RNA (4 μg) was separated on 1% agarose gels containing formaldehyde, transferred to a positively charged nylon membrane (Amersham Pharmacia), and immobilized using Stratalinker™ UV crosslinker (Stratagene). Hybridization was performed with [α-^{32}P]dCTP-labeled DNA using Rapid-Hyb™ buffer (Amersham Pharmacia). The DNA probe was synthesized from the NS3 to 5B genes of JFH1 using the Megaprime™ DNA labeling system (Amersham Pharmacia).

Western blot and immunofluorescence analysis

Cells were lysed using a radioimmune precipitation assay (RIPA) buffer containing 0.1% SDS, 0.5% NP-40, 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), and 150 mM NaCl. Protein samples were separated on 10% or12% polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Millipore). Transferred proteins were incubated with blocking buffer containing 5% non-fat dried milk (Snow Brand) in phosphate-buffered saline (PBS). HCV proteins were detected using anti-NS5A polyclonal[9], anti-NS3 polyclonal[8], and anti-core monoclonal antibodies (clone 2H9, unpublished), and peroxidase-labeled goat anti-rabbit Ig (BIOSOURCE) or sheep anti-mouse Ig (Amersham Pharmacia). Anti-E1 and anti-E2 polyclonal antibodies were raised by immunization of rabbits with synthetic peptides (E1, N'-TSSSYMVTNDC-C' and N'-SPNMAVRQPGALTQ-C'; E2, N'-GTTTVGGAVARSTN-C' and N'-CDLEDRDRSQLSPL-C') (Qiagen). Rat monoclonal anti-HA antibody (clone 3F10, Roche Diagnostic Systems) and peroxidase-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc.) were used to detect HA-tagged E2 protein produced from the JFH1/E2HA construct. Signals were detected using a chemiluminescence system (Amersham Pharmacia), with JFH1 subgenomic replicon-containing Huh7 cells as positive controls (SGR/JFH1[8], Fig. 1c) or HCV expression plasmid-transfected Huh7 cells (pEF/Core and pEF/CE2[29]).

Untransfected and transfected Huh7 cells grown on cover slips were fixed in acetone-methanol (1:1, vol/vol) for 10 min at -20 °C. Cells were then incubated in immunofluorescence assay (IF) buffer (PBS, 1% bovine serum albumin, 2.5 mM EDTA).

Anti-core monoclonal and anti-NS3 polyclonal antibodies were added at 50 μg/ml or at a dilution of 1:50 in IF buffer. After incubation for 1 h at room temperature, cells were washed, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (Cappel) in IF buffer. Cover slips were washed and mounted on glass slides using Shandon PermaFluor™ mounting solution (Thermo Electron). Cells were examined by fluorescence microscopy (Carl Zeiss).

Quantification of HCV core protein and RNA

To estimate levels of HCV Core protein in culture supernatant or cell lysate, concentrations of HCV core protein were determined. Aliquots (250 μl) of samples were assayed using a new immunoassay described previously[28]. Total RNA was isolated from harvested cells or culture media by ISOGEN (Nippon GENE). Copy numbers of HCV RNA were determined by real-time detection RT-PCR (RTD-PCR) as described previously using an ABI Prism 7700 sequence detector system (Applied Biosystems Japan)[12].

Sucrose density gradient analysis

Culture medium derived from Huh7 cells was harvested for sucrose density gradient analysis 6 days after transfection of full-length JFH1 RNA or subgenomic replicon RNA. Collected culture medium was cleared by low speed centrifugation at 2,000 rpm for 10 min, and passed through a filter with 0.45-μm pore size (Millipore). Filtered culture medium was layered on a stepwise sucrose gradient (60% to 10%, wt/vol) and centrifuged for 16 h in a SW41 or MLS50 rotor (Beckman) at 40,000 or 50,000 rpm at 4 ºC. After centrifugation, 8 or 16 fractions were harvested from the bottoms of the tubes. Core protein concentration in each

fraction was determined by an immunoassay using 100 μl of the fraction. HCV RNA titer was determined by RTD-PCR using RNA isolated from 100 μl of the fraction.

Infection of cells with secreted HCV

Seventy two h after transfection, culture medium was collected, cleared by low-speed centrifugation at 2,000 rpm for 10 min, and passed through a filter with 0.45-μm pore size (Millipore). Part of the filtered culture medium was concentrated 30-fold using an Amicon Ultra-15 device (Molecular weight cut off: 1×10^5 Da; Millipore) in accordance with the instructions of the manufacturer. Huh7, HepG2, IMY-N9 and HeLa cells were seeded 24 h before infection at a density of 5×10^4 cells/well in a 12-well plate, or at 1×10^5 cells/well in a 6-well plate. Concentrated culture medium (100 μl) was used for inoculation of cells in a well of 12- or 6-well plate for 3 h with periodic rocking. At the end of inoculation, cells were washed 3 times with PBS, followed by addition of 1 or 2 ml of complete culture medium. Replicate wells were grown for another 12, 24, 48, 72, and 96 h. At 2 days after infection, inoculated cells grown on cover slips were fixed and subsequently stained using anti-core or anti-NS5A antibodies as described above. Positively stained cells were counted by T.W. and T.D. in a blinded fashion. Amounts of HCV RNA in inoculated cells were determined by RTD-PCR.

Electron microscopy

Cell free supernatant harvested 96 h post transfection of Huh-7 cells transfected with JFH1 was concentrated via ultracentrifugation. The resulting virus suspension had a tissue culture infectious titer of approximately 10,000 infectious units per ml. As control, the same

amount of cell free culture fluid was harvested from mock transfected cells and concentrated in the same manner. Both concentrates were adsorbed for 5 min onto carbon coated formwar grids which had been glow discharged prior to use. Grids were washed on drops of PBS, fixed with 3% paraformaldehyde in PBS, and blocked in a solution of 0.8% BSA, 1% cold water fish skin gelatin (Sigma), and 20 mM Glycine in PBS. Immunogold labeling was performed with an antibody directed against E2 (CBH5) diluted 1:50 in blocking solution, and Protein A coupled to 10 nm gold particles. After extensive washing with PBS and a quick rinse with distilled water, grids were stained with a solution consisting of 9 parts 2% Methyl Cellulose and 1 part 3% Uranyl acetate (both aqueous solutions).

Patient sera

Sera from 10 different chronically infected individuals were used in this study. Collection of the sera was approved by the local ethical committee and informed consent had been obtained from all donors. Prior to use in neutralization assays, human sera were inactivated at 56°C for 1 h.

Virus neutralization assays

Target cells seeded in 12-well plates or on glass cover slips were infected with cell culture supernatants supplemented with JS-81 (BD Biosciences) or Mab46D2 (purified mouse monoclonal antibody specific for a Dengue type 2-specific virus determinant; derived from hybridoma 3H5-1, ATCC No.: HB-46) at a final concentration of 10 μg/ml (unless otherwise stated in the text) in a total volume of 500 μl. In some experiments, filtered cell culture fluid concentrated via ultrafiltration with an Amicon Ultra-15 device was used for the infection.

After 4 h at 37°C, medium was aspirated, and cells were supplemented with fresh culture medium. Seventy two hours after infection, cells were either fixed with 3% paraformaldehyde for subsequent immunofluorescence analysis or lysed for RTD-PCR or luciferase assays. Immunostaining of NS3 was performed according to standard protocols by using a rabbit polyclonal serum directed against the helicase domain at a dilution of 1:1000 in PBS supplemented with 5% normal goat serum. Bound primary antibodies were detected using goat antibodies conjugated to AlexaFluor546 at a dilution of 1:1000 in PBS with 5% normal goat serum. DNA was stained with DAPI (Molecular Probes).

For neutralization with patient sera, heat-inactivated serum was diluted with virus-containing supernatants as specified in the text. After incubation for 1 h, mixtures were added to target cells, and infection was measured as described above. Immunoglobulins (Ig) contained in human sera were purified by using a HiTrap Affinity Protein G column (Amersham Pharmacia). The flow through of the column was collected. Purified Ig eluted from the column were desalted employing a HiTrap Desalting column (Amersham Pharmacia). Protein content of the complete serum, Ig-depleted serum and purified Ig were determined by Bradford assay.

Chimpanzee experiment

The chimpanzee experiment was conducted in the Southwest Foundation for Biomedical Research, an AALAC (American Association of Laboratory Animal Care)-accredited animal facility, where the housing, maintenance and care of the chimpanzee met guidelines and requirements for the humane use of animals. To prepare inoculum for the

chimpanzee, Huh7 cells were transfected with JFH1 RNA as described above, and maintained in DMEM containing 2% human serum. Culture medium was collected after 3 days and cleared of debris by centrifugation at 10,000 rpm for 10 min. Chimpanzee X0215 was inoculated intravenously with 1 ml of culture medium collected from Huh7 cells transfected with the full-length JFH1 genome. Serum samples were tested for alanine aminotransferase (ALT) levels, anti-HCV antibodies by standard commercial anti-HCV kit (EIA2.0, Abbott Laboratories) and HCV RNA that was quantified by the Roche Amplicor Cobas Monitor II (Roche Diagnostic Systems, Branchburg, N.J.) with a lower limit of detection of 1,620 genomes/ml. Liver biopsies were collected for histological analysis.