JOURNAL OF VIROLOGY, June 2006, p. 5308–5320 Vol. 80, No. 11
0022-538X/06/$08.00+0 doi:10.1128/JVI.02460-05


# Characterization of the Early Steps of Hepatitis C Virus Infection by Using Luciferase Reporter Viruses

George Koutsoudakis, Artur Kaul, Eike Steinmann, Stephanie Kallis, Volker Lohmann, Thomas Pietschmann,* and Ralf Bartenschlager*

*Department of Molecular Virology, University of Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany*

Received 22 November 2005/Accepted 18 February 2006

**The lack of an efficient system to produce hepatitis C virus (HCV) particles has impeded the analysis of the HCV life cycle. Recently, we along with others demonstrated that transfection of Huh7 hepatoma cells with a novel HCV isolate (JFH1) yields infectious viruses. To facilitate studies of HCV replication, we generated JFH1-based bicistronic luciferase reporter virus genomes. We found that RNA replication of the reporter construct was only slightly attenuated and that virus titers produced were only three- to fivefold lower compared to the parental virus, making these reporter viruses an ideal tool for quantitative analyses of HCV infections. To expand the scope of the system, we created two chimeric JFH1 luciferase reporter viruses with structural proteins from the Con1 (genotype 1b) and J6CF (genotype 2a) strains. Using these and the authentic JFH1 reporter viruses, we analyzed the early steps of the HCV life cycle. Our data show that the mode of virus entry is conserved between these isolates and involves CD81 as a key receptor for pH-dependent virus entry. Competition studies and time course experiments suggest that interactions of HCV with cell surface-resident glycosaminoglycans aid in efficient infection of Huh7 cells and that CD81 acts during a postattachment step. The reporter viruses described here should be instrumental for investigating the viral life cycle and for the development of HCV inhibitors.**

Hepatitis C virus (HCV) is a positive-strand RNA virus that belongs to the family *Flaviviridae* (49). Its genome of about 9.6 kb is composed of the 5′ nontranslated region (NTR), an open reading frame encoding a large polyprotein, and the 3′ NTR (2). In the N-terminal region, the polyprotein is processed by cellular proteases (signal peptidase and signal peptide peptidase) to yield the structural proteins core and envelope protein 1 and 2 (E1 and E2) that are required for virus particle formation and infection of host cells. The hydrophobic p7 protein, which is believed to exert ion channel activity (17, 33), is located at the junction of structural and nonstructural (NS) proteins. NS2 serves as protease that in conjunction with NS3 mediates cleavage at the NS2-3 position, whereas the NS3-4A protease complex conducts all downstream cleavages. An ordered replicase complex likely consisting of NS3 to NS5B proteins and cellular polypeptides copies the viral positive strand into a negative-strand intermediate serving as a template for the synthesis of excess progeny RNA genomes. Due to the absence of a 3′-5′ exonuclease proofreading activity of the NS5B RNA-dependent RNA polymerase, HCV RNA replication is error prone, causing a high degree of sequence variability, which contributes to immune evasion and may facilitate persistence in the infected host. Based on phylogenetic analyses, isolates are classified into six genotypes (GTs) which differ by more than 30% at the nucleotide level (43). Together these genotypes make up the genus *Hepacivirinae*.

* Corresponding author. Mailing address: Department of Molecular Virology, University of Heidelberg, Im Neuenheimer Feld 345, Heidelberg 69120, Germany. Fax: 49 6221 564570. Phone for T. Pietschmann: 49 6221 566449. E-mail for T. Pietschmann: thomas_pietschmann@med.uni-heidelberg.de. Phone for R. Bartenschlager: 49 6221 564569. E-mail for R. Bartenschlager: ralf_bartenschlager@med.uni-heidelberg.de.

In regard to the early phase of the HCV life cycle, various systems were developed to identify candidate receptors. Employing serum-derived HCV particles, which are known to associate with low density lipoproteins (45, 46), evidence was provided that the low density lipoprotein receptor is involved in HCV entry (1, 52). CD81 and the human scavenger receptor class B type I (SR-B1) were identified as putative receptors based on their interaction with recombinant soluble E2 protein (36, 40). Likewise, binding of E2 to two C-type lectins (DC-SIGN and L-SIGN) was recognized, and it was proposed that this interaction plays a role in virus infection (16, 28, 37). Moreover, experiments with insect cell-derived virus-like particles and soluble E2 point to an involvement of specific cell surface heparan sulfate proteoglycans in the attachment of HCV (3). Finally, a role of the asialoglycoprotein receptor in binding and entry of HCV structural proteins purified from insect cells was described (39).

The development of retroviral pseudo-particles (HCVpp), carrying unmodified and functional HCV glycoproteins on the surface of retroviral core particles, and transducing reporter genes allowed for the first time reliable and convenient quantification of HCV glycoprotein-dependent infection, thus providing the opportunity to study the functional relevance of the described receptor candidates (4, 20). Utilizing this assay, it was shown that L-SIGN and DC-SIGN are capable of capturing HCVpp and transmitting it to neighboring cells with the appropriate entry receptors. In light of the expression of L-SIGN in endothelial cells in liver sinusoids, this mechanism was proposed to facilitate transinfection of endothelium-proximal hepatocytes (10, 27). Antibody competition experiments and RNA interference studies confirmed a role of SR-BI in the entry of HCVpp (4, 23), and there is ample evidence from different laboratories that implicates CD81 as a key component of the HCV receptor complex (4, 11, 20, 23, 29, 55).

Recently, we along with others have shown that transfection of Huh7 hepatoma cells with the JFH1 genome yields virus particles infectious both in vivo and in vitro (51, 56). In addition, chimeric constructs, consisting of the core to NS2 coding region of the J6CF (GT2a) or the Con1 (GT1b) isolate, and the remaining genome segments derived from JFH1 replicated efficiently and produced infectious particles (24; T. Pietschmann, A. Karl, G. Kontsondakis, A. Shaviuskaya, S. Vallis, E. Steinmann, K. Abid, F. Negro, M. Dreux, F.-L. Cosset, and R. Bartenschlager, unpublished data). This novel system now allows characterization of the early steps of HCV infection in cell culture by using authentic virus particles. To facilitate these studies we have constructed bicistronic genomes encoding a firefly luciferase reporter and shown that these genomes also support production of infectious particles (51). In this study we performed a detailed characterization of these reporter viruses, constructed several chimeras, and used this system to study the early steps of HCV infection.

## MATERIALS AND METHODS

**Cell culture.** All experiments described in this study were performed by using Huh7-Lunet cells. These cells originally carried a selectable luciferase HCV replicon, were cured by treatment with an HCV-specific inhibitor, and are characterized by high permissiveness for HCV RNA replication (15). A possible effect of ammonium chloride ($NH_4Cl$) and concanamycin A (ConA) on HCV replication was assessed using Huh7-Lunet replicon cells, which carry a JFH1-based selectable subgenomic luciferase replicon (Luc-ubi-neo-SG-JFH1; V. Lohmann, unpublished) analogous in design to a replicon used to generate a Con1-based indicator cell line (50). Cells were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (DMEM complete).

**Plasmid construction.** The plasmids pFK-Luc-JFH1 and pFK-Luc-JFH1/ΔE1-E2 were described recently (51) and encode bicistronic reporter constructs of the full-length JFH1 genome (GenBank accession number AB047639) or a variant thereof with an in-frame deletion of 351 amino acids encompassing most of the coding region of E1 and E2 (amino acids 217 to 567) (Fig. 1A). In both cases and in all other constructs used in this study, the HCV polyprotein-coding region is located in the second cistron and is expressed via an internal ribosome entry site (IRES) element derived from the encephalomyocarditis virus (EMCV). The first cistron contains the firefly luciferase reporter gene fused via an AscI site to the JFH1-derived 5′ NTR and the first 16 codons of the JFH1 core coding region (nucleotides 1 to 390). To allow simple generation of RNA transcripts with authentic 5′ and 3′ ends, the NTRs are flanked by a T7 promoter and by the genomic ribozyme of hepatitis delta virus, respectively. Constructs pFK-Luc-Jc1 and pFK-Luc-Con1 encode chimeric HCV polyproteins which consist of codons 1 to 846 derived from J6/CF (GenBank accession number AF177036 [53]) or, respectively, codons 1 to 842 from Con1 (GenBank accession number AJ238799 [26]) combined with codons 847 to 3033 of JFH1. As a consequence, in these chimeras core, E1, E2, p7, and the amino terminal 33 amino acids of NS2 are derived from J6CF or Con1, whereas the C-terminal part of NS2 and the remaining proteins are from JFH1. The choice of this particular junction was based on a mapping analysis of different crossover sites between Con1 and JFH1, which will be described elsewhere (Pietschmann, Karl, Kontsondakis, Shaviuskaya, Vallis, Steinmann, Abid, Negro, Dreux, Cosset, and Bartenschlager, unpublished).

**In vitro transcription.** Plasmid DNA was extracted with phenol and chloroform, precipitated with ethanol, and dissolved in RNase-free water. In vitro transcription reaction mixtures contained 80 mM HEPES (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol (DTT), a 3.125 mM concentration of each nucleoside triphosphate, 1 U of RNasin (Promega, Mannheim, Germany) per μl, 0.1 μg of plasmid DNA/μl, and 0.6 U of T7 RNA polymerase (Promega) per μl. After 2 h at 37°C, an additional 0.3 U of T7 RNA polymerase/μl was added, and the reaction mixture was incubated for another 2 h. Transcription was terminated by the addition of 1.2 U of RNase-free DNase (Promega) per μg of plasmid DNA and a 30-min incubation at 37°C. After extraction with acidic phenol and chloroform, RNA was precipitated with isopropanol and dissolved in RNase-free water. The concentration was determined by measurement of the optical density at 260 nm, and the RNA integrity was checked by denaturing agarose gel electrophoresis.

**Electroporation and luciferase assays.** Single-cell suspensions of Huh7-Lunet cells were prepared by trypsinization of monolayers and subsequent resuspension with DMEM complete. Cells were washed with phosphate-buffered saline (PBS), counted, and resuspended at $10^7$ cells per ml in Cytomix (48) containing 2 mM ATP and 5 mM glutathione. Unless otherwise stated, 10 μg of in vitro transcribed RNA was mixed with 400 μl of the cell suspension by pipetting and then electroporated and immediately transferred to 20 ml of complete DMEM. Subsequently, the cells were seeded at a density of $4.16 \times 10^4$ cells/cm$^2$, which corresponds to 2 ml of the cell suspension per well of a six-well plate. Electroporation conditions were 960 μF and 270 V with a Gene Pulser system (Bio-Rad, Munich, Germany) and a cuvette with a gap width of 0.4 cm (Bio-Rad). For assaying the luciferase activity, cells were washed once with PBS, lysed directly on the plate with 1 ml of ice-cold lysis buffer (0.1% Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT; pH 7.8), and frozen. Upon thawing, lysates were resuspended by pipetting, and 100 μl was mixed with 360 μl of assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 2 mM ATP, 15 mM $K_2PO_4$, pH 7.8) and, after the addition of 200 μl of a luciferin solution (200 μM luciferin, 25 mM glycylglycine, pH 8.0), measured in a luminometer (Lumat LB9507; Berthold, Freiburg, Germany) for 20 s. All luciferase assays were done at least in duplicate measurements.

**Preparation of total RNA and Northern blot analysis.** Total RNA was prepared by a single-step isolation method as described previously (9). Three micrograms of total RNA was mixed with glyoxal, dimethyl sulfoxide, and sodium phosphate buffer (pH 7.0) at a final concentration of 5.9%, 50%, and 10 mM, respectively, and denatured for 1 h at 50°C. Samples were separated by denaturing agarose gel electrophoresis, and RNA was transferred to positively charged nylon membranes (Hybond-N+; Amersham Biosciences, Freiburg, Germany) with 50 mM NaOH, and cross-linked by UV irradiation. Positive-strand HCV RNA was detected by hybridization with a $^{32}$P-labeled negative-sense riboprobe complementary to nucleotides 6273 to 9678 of the HCV JFH1 isolate. Hybridization with a β-actin-specific riboprobe was used to monitor equal sample loading in each lane of the gel.

**Preparation of virus stocks.** Huh7-Lunet cells were transfected by electroporation with reporter constructs as described above. Culture fluid of transfected cells was harvested and cleared by passing through 0.45-μm-pore-size filters. In the case of Luc-JFH1 and Luc-Con1, medium was harvested 72 to 96 h after transfection, whereas maximal yield for Luc-Jc1 was obtained at 48 h. Virus preparations were used directly or stored at 4°C or −80°C. We noted that each cycle of freeze and thaw results in a twofold reduction of infectivity. As the virus is stable at 4°C for at least several days, for short-term storage virus preparations were kept at this temperature.

**Preparation of retroviral pseudo-particles.** Human immunodeficiency virus (HIV)-based pseudotypes bearing vesicular stomatitis virus glycoproteins (VSV-G) or amphotropic murine leukemia virus envelope protein (A-MLV) were generated by calcium phosphate-based cotransfection of 293T cells (12). Briefly, $2.5 \times 10^6$ 293T cells were seeded in 10-cm diameter plates 1 day before transfection with 2.7 μg of envelope protein expression construct (pczVSV-G [21] or pHIT456 [7]), 8.1 μg of HIV-Gag-Pol expression construct (pCMVΔR8.74 [13]), and 8.1 μg of a firefly luciferase transducing retroviral vector by using CalPhos transfection reagents (Becton Dickinson, Heidelberg, Germany). The retroviral vector employed is a derivate of pHR′-CMV-GFP (57) (where CMV is cytomegalovirus and GFP is green fluorescent protein), which was generated by replacing the GFP gene in the parental vector by the gene encoding firefly luciferase. The medium was replaced 8 h after transfection. Supernatants containing the pseudo-particles were harvested 48 h later, cleared by passage through 0.45-μm-pore-size filters, and used for infection assays. Luciferase activity in the infected cells was measured 72 h after inoculation as described above. Envelope protein expression plasmids and HIV-based vectors were kindly provided by D. Lindemann (Technical University Dresden, Germany) and D. Trono (Swiss Institute of Technology Lausanne, Switzerland).

**Indirect immunofluorescence.** Cells were seeded on glass coverslips in 24-well plates at a density of $2 \times 10^4$ per well 24 h before infection, followed by inoculation with 250 μl of filtered cell culture supernatant. After 48 h, cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. Immunostaining of NS3 was performed according to standard protocols by using a rabbit polyclonal serum directed against the helicase domain at a dilution of 1:1,000 in PBS supplemented with 5% normal goat serum. Bound primary antibodies were detected using goat antibodies conjugated to Alexa-Fluor 546 at a dilution of 1:1,000 in PBS with 5% normal goat serum. DNA was stained with DAPI (4′,6′-diamidino-2-phenylindole) (Molecular Probes, Karlsruhe, Germany). To determine infectivity titers, virus preparations were

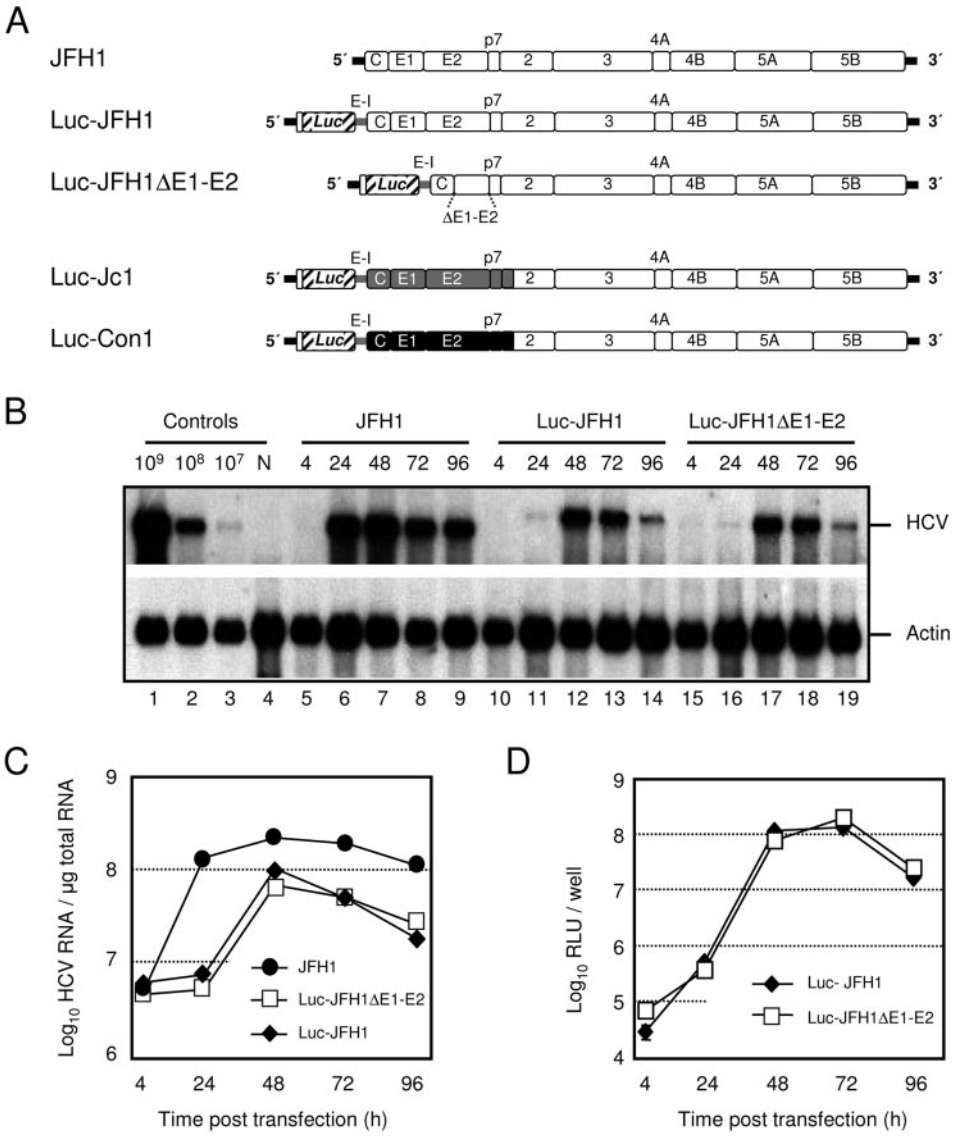


FIG. 1. Genetic organization and replication of JFH1 and JFH1-derived luciferase reporter constructs. (A) A schematic representation of parental JFH1 genome is given at the top, and derivates with the reporter gene used in this study are shown below. The 5′ and 3′ NTR are drawn as thick black lines, and the JFH1 polyprotein is depicted as an open box; individual proteins are separated by vertical lines. In the case of reporter constructs, viral proteins are expressed via an internal EMCV IRES (gray bar). Note that Luc-JFH1/ΔE1-E2 carries a large in frame deletion in the coding region for E1-E2, which is known to inactivate virus particle formation (51). The luciferase gene (hatched) is fused in frame to the 16 N-terminal residues of the JFH1 core coding region (white). Chimeric genomes are depicted with J6CF (GT 2a) and Con1 (GT 1b) proteins in gray and black boxes, respectively. The junction between the different isolates is located within NS2. (B) RNA replication of JFH1, Luc-JFH1, and Luc-JFH1/ΔE1-E2 as determined by Northern hybridization. In vitro transcribed RNAs of given constructs were transfected into Huh7-Lunet cells that were harvested at the time points specified above the panel. Total RNA was prepared and analyzed with an HCV-specific probe. Equal sample loading was monitored by detection of β-actin RNA (bottom). As controls, total RNA of naive Huh7-Lunet cells (N) and serial dilutions of in vitro transcribed RNAs spiked into total RNA of naïve cells were loaded in parallel (lanes 1 to 4). (C) Quantification of the Northern blot shown in panel B by phosphor imaging. The amount of HCV RNA/μg of total RNA was calculated by comparison with the controls loaded in parallel. Values were corrected for differences in loading by using β-actin signals. (D) Luciferase activity in transiently transfected Huh7-Lunet cells. Transfected cells were lysed at given time points, and the amount of luciferase activity was determined and is given in relative light units (RLU) per well (each point is the average value of duplicate wells; the error bars show standard deviations of the means).

serially diluted 10-fold and used to inoculate Huh7-Lunet cells. The number of HCV antigen-positive cell foci obtained at a given dilution per inoculated well was used to calculate the tissue culture infectious units.

**Luciferase infection assays.** For standard infection assays, cells were seeded at a density of $4 \times 10^4$ per well of a 12-well plate 24 h prior to inoculation with 500 μl of virus preparation. We noted that maximum infectivity can be achieved when cells are inoculated for 4 h at 37°C. Therefore, unless otherwise stated, cells were inoculated for 4 h at this temperature and lysed in 350 μl of lysis buffer 72 h later. Luciferase activity was determined as described above. For the experiments analyzing the kinetics of HCV entry (see Fig. 9), we used six-well plates and inoculated with 1 ml of virus preparation in order to improve the sensitivity of the assay.

**Quantitative detection of HCV core protein.** HCV core protein was quantified using the Trak C Core (Ortho Clinical Diagnostics, Neckargemünd, Germany)

enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer. When intracellular core expression was analyzed, cells were lysed in ice-cold PBS supplemented with 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.1 μg/ml aprotinin. Lysates were cleared at 20,000 × *g* for 10 min and measured at a dilution of 1:10 (or higher) in PBS. Cell culture medium was filtered through 0.45-μm-pore-size filters and either directly used for ELISA or diluted with DMEM complete medium prior to measurement.

**Antibodies and sources of inhibitors of HCV entry.** Heparin sodium salt from bovine lung (Calbiochem, Darmstadt, Germany), heparan sulfate sodium salt from bovine kidney (Sigma-Aldrich, Munich, Germany), chondroitin sulfate A sodium salt from bovine trachea (Sigma-Aldrich), chondroitin sulfate B sodium salt from porcine intestinal mucosa (Sigma-Aldrich), and chondroitin sulfate C sodium salt from shark cartilage (Sigma-Aldrich) were used in infection assays at concentrations given in the text. JS-81 (Becton Dickinson), 1.3.3.22 (Ancell Immunology Research Products, Bayport, MN), and WM15 (Becton Dickinson) are isotype-matched (immunoglobulin G1) mouse monoclonal antibodies directed against CD81 (JS-81 and 1.3.3.22) and CD13 (WM15) and were used as given in the text.

**Treatment of target cells with glycosaminoglycans (GAG) lyases.** Huh7-Lunet cells were washed with PBS and then incubated with a buffer consisting of 20 mM Tris-HCl pH 6.8, 50 mM NaCl, 4 mM $CaCl_2$, 0.01% bovine serum albumin (BSA) (mock treated) or the same buffer containing 0.5 U/ml heparinase I (Sigma-Aldrich), or heparinase III (Sigma-Aldrich) or chondroitinase ABC (Sigma-Aldrich). After incubation for 1 h at 37°C, cells were washed three times with PBS and then inoculated with 500 μl of reporter virus preparation for 1 h. Forty-eight hours later, cells were lysed in 350 μl of lysis buffer, and luciferase activity was measured as described above.

## RESULTS

**Characterization of the JFH1 luciferase reporter virus system.** In the first set of experiments, we compared the parental JFH1 genome to its reporter gene bearing derivate with respect to RNA replication in transfected cells (Fig. 1). A reporter construct with partially deleted E1 and E2 genes was analyzed in parallel and served as a negative control for nonspecific release of particles and nonspecific transduction of the reporter gene (51). In this and all subsequent transfection and infection assays, we used Huh7-Lunet cells, which were originally obtained by curing Huh7 cells carrying a selectable subgenomic luciferase replicon and which support high-level HCV RNA replication (15). In the case of JFH1, HCV RNA amplified rapidly and reached almost peak levels as early as 24 h after transfection (Fig. 1B and C). Highest copy numbers of RNA ($2 \times 10^8$ copies/μg of total RNA) were observed at 48 h posttransfection and declined slightly at later time points. In contrast, both reporter constructs displayed somewhat attenuated replication with a clearly delayed kinetics of RNA accumulation, lower peak copy numbers (0.8 to $1 \times 10^8$ copies/μg of total RNA), and roughly fivefold lower HCV RNA at 96 h after transfection. In the case of the reporter constructs, we monitored replication by measuring the amount of luciferase activity in parallel (Fig. 1D). Similar to what we have described previously (22, 25), we found an excellent correlation between reporter activity and RNA copy number as determined by Northern blotting. Between 4 and 48 h posttransfection, reporter activity increased by about 1,000-fold and subsequently decreased at 96 h to levels about 5- to 10-fold lower. Together these data indicate that insertion of the heterologous sequences (luciferase gene and EMCV IRES) into the JFH1 genome reduced HCV RNA replication only about two- to fivefold. Moreover, since luciferase activity correlated well with RNA abundance, it can be used to measure viral RNA replication with high sensitivity and accuracy.

Next, we assessed the ability of the reporter construct Luc-JFH1 to release infectious particles and the possibility to quantify their infectivity by using reporter assays. To this end, Huh7-Lunet cells were transfected with the parental JFH1, the Luc-JFH1, and the Luc-JFH1ΔE1-E2 genomes, and release of virus particles and infectivity into the culture fluid were quantified up to 96 h posttransfection by using a core-specific ELISA, immunofluorescence-based virus titrations, and reporter assays where applicable (Fig. 2A). Equivalent transfec-

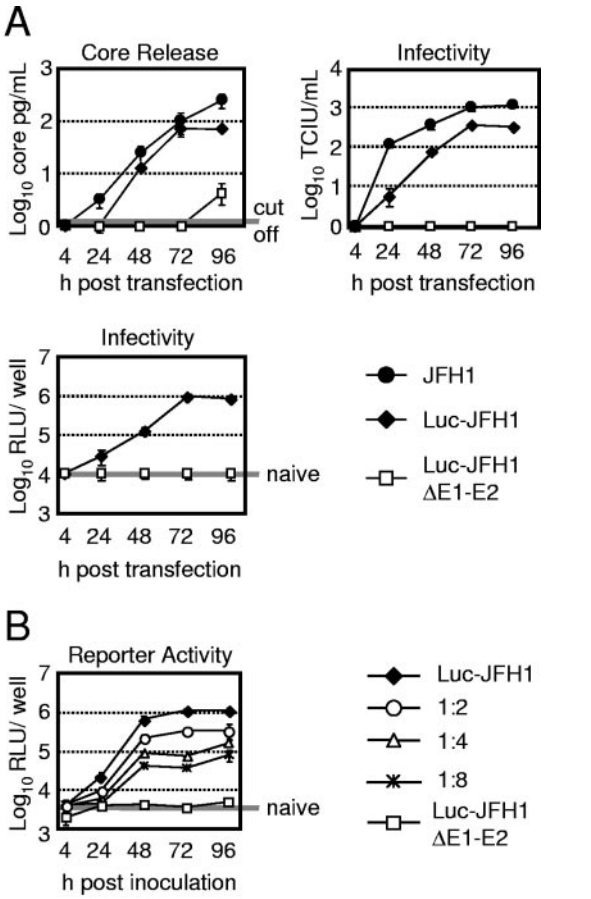


FIG. 2. Release of core protein and infectivity upon transfection of JFH1 constructs into Huh7-Lunet cells. (A) In vitro transcribed RNAs of JFH1 (black circles), Luc-JFH1 (black diamonds), and Luc-JFH1ΔE1-E2 (open squares) were transfected into Huh7-Lunet cells, and cell-free culture fluids were collected at 4 h to 96 h posttransfection. The amount of core protein present in the respective culture medium was determined employing a core-specific immunoassay (upper left graph). In parallel, supernatants were used to inoculate naïve Huh7-Lunet cells, and infectivity associated with the respective samples was quantified by using an immunofluorescence-based limiting dilution assay (upper right graph) and (where applicable) by assessing the amount of reporter activity present in target cells 72 h postinoculation (lower left graph). The gray lines indicate the background of the reporter assay from naïve Huh7-Lunet cells and the detection limit of the core ELISA, respectively. Mean values of two experiments are given; error bars represent standard errors of the means. (B) Huh7-Lunet cells were inoculated with serial dilutions of Luc-JFH1-containing medium or with undiluted Luc-JFH1/ΔE1-E2 culture fluid and lysed at the indicated time points postinoculation to quantify luciferase activity. Mean values of two independent wells are given (error bars show standard deviations). The gray line indicates the background of the assay determined by measuring mock-infected cells.

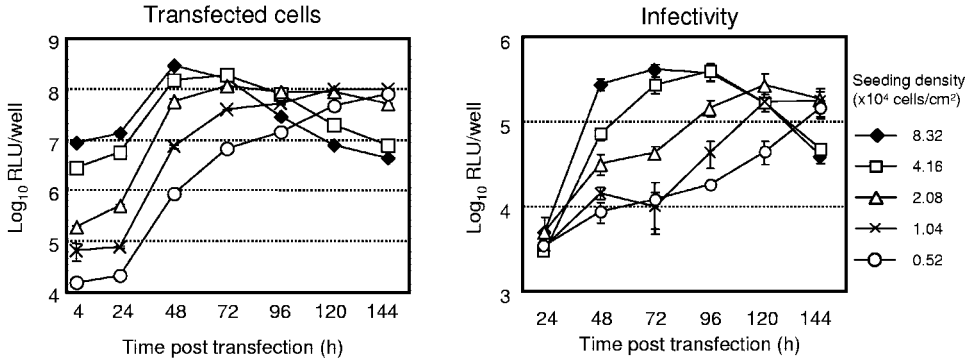


FIG. 3. Influence of cell growth on replication and release of infectious Luc-JFH1 virus particles. Huh7-Lunet cells were transfected with Luc-JFH1 RNA and seeded into multiple culture dishes with the given cell density ($8.32 \times 10^4$ to $0.52 \times 10^4$ cells/cm$^2$). Luciferase activity in transfected cells was determined at the indicated time points posttransfection (left). Cell-free supernatants from cells seeded with the given densities were collected 24 to 144 h after transfection and used to inoculate naïve Huh7-Lunet cells. Luciferase activity in the inoculated cells was determined 72 h later (right). Mean values of two independent wells measured are given (error bars represent the standard errors of the means).

tion efficiency was monitored by measuring intracellular core expression 4 h posttransfection (data not shown). For all constructs, at this time point neither infectivity nor release of core protein above the cutoff value of the ELISA (1.5 pg/ml) was detected (Fig. 2A, upper left and right graphs). Accordingly, transduction of luciferase reporter activity to cells inoculated with these supernatants was not observed (Fig. 2A, lower left graph). As expected, transfection of Luc-JFH1ΔE1-E2 did not yield infectious virus particles. Correspondingly, core protein release was below the detection limit for most of the time course except for 96 h posttransfection, when some low-level nonspecific core release that was not associated with infectivity was measured. Importantly, Luc-JFH1ΔE1-E2-conditioned culture medium did not transfer reporter activity to inoculated cells, demonstrating that nonspecific transduction does not occur (Fig. 2A, lower left graph). In contrast, cells transfected with the parental JFH1 genome and Luc-JFH1 produced easily detectable levels of infectious virus particles, with a peak of infectivity (ca. 1,000 and 350 tissue culture infectious units/ml, respectively) associated with the culture fluid collected 72 to 96 h posttransfection (Fig. 2A). Similar to the delayed kinetics of RNA replication displayed by Luc-JFH1 (Fig. 1), release of infectivity and core protein was somewhat slower and less efficient compared to the parental genome. Importantly, the infectious titer of Luc-JFH1 as measured by the immunofluorescence-based limiting dilution assay correlated well with the amount of reporter activity observed 72 h postinoculation with the respective Luc-JFH1-conditioned culture fluid.

To further corroborate this correlation, we inoculated Huh7-Lunet cells with serial dilutions of Luc-JFH1 reporter viruses and monitored the accumulation of reporter activity over time in the inoculated cells (Fig. 2B). Comparable to the kinetics in transfected cells, reporter activity rapidly increased, reaching a plateau about 200-fold above background at 48 h postinoculation in the case of undiluted virus (Fig. 2B). As expected, the magnitude of reporter activity correlated well with the amount of inoculum, demonstrating that it reflects the infectious titer in a quantitative manner. Since peak luciferase activity in the infected cells was generally reached at the latest by 72 h postinfection, in all subsequent assays, cells were lysed at this time point to quantitatively assess the infection. When we continued to culture reporter virus-infected cells, reporter activity did not significantly increase over time but was maintained at least for five passages (18 days), indicating that the reporter is stably sustained for this period of time (data not shown).

It has been reported previously that in Huh7 cells the efficiency of RNA replication attained by subgenomic Con1-derived replicons is tightly linked to cell growth. While maximum activity is observed in exponentially growing cells, HCV copy numbers rapidly decline when cells reach confluence (18, 35). In order to identify optimal conditions for production of high-titer reporter virus stocks, we investigated if and to what extent this may affect production of infectious virus particles. To this end, we transfected Huh7-Lunet cells with Luc-JFH1 RNA and seeded the cells at different densities into parallel culture dishes (Fig. 3). Subsequently, we harvested cells and supernatants at regular intervals up to 144 h posttransfection and measured replication in transfected cells by luciferase assays (Fig. 3, left graph). Moreover, we determined the quantity of infectious virus particles present in the culture supernatant at a given time point by inoculating naïve Huh7-Lunet cells and assessing luciferase expression 72 h later (Fig. 3, right graph). Four hours posttransfection, reporter activity varied roughly according to the number of cells seeded. Irrespective of the cell density, we observed an approximately twofold increase of luciferase activity between 4 and 24 h. The steepest rise of reporter activity occurred between 24 and 48 h posttransfection, indicating that during this phase RNA replication was most efficient. When cells were seeded at high density ($8.32 \times 10^4$ and $4.16 \times 10^4$ cells/cm$^2$), peak luciferase activity was reached already 48 and 72 h after transfection, respectively, and declined rapidly thereafter. In contrast, in cells seeded at lower density, reporter activity reached a plateau at 72 h, which was maintained until 144 h ($2.08 \times 10^4$ cells/cm$^2$); reporter activity rising until 120 and 144 h postseeding when cells were seeded at a density of $1.04 \times 10^4$ and $0.52 \times 10^4$ cells/cm$^2$, respectively. Not surprisingly, the different seeding conditions markedly affected the kinetics and efficiency of release of infectious virus. In all cases, no infectivity could be detected in the culture fluid harvested 24 h after transfection, whereas at 48 h it was readily measurable. At this time point, toward the

end of the most efficient period of intracellular RNA replication, the virus titers differed by about 20-fold, depending on the seeding conditions. Highest titers were generally reached 24 h subsequent to the respective intracellular peak of reporter activity, and highest overall titers could be obtained by seeding cells at high density ($8.32 \times 10^4$ and $4.16 \times 10^4$ cells/cm$^2$). Together, these data indicate that Luc-JFH1 replicates efficiently in subconfluent Huh7-Lunet cells and that the efficiency of RNA replication decreases when transfected cells reach confluence. Moreover, the amount of infectivity present in the culture fluid is closely linked to the number of virus-producing cells and intracellular RNA replication level within a given cell, and as a consequence maximum titers can be obtained concomitant to or shortly after cells reach confluence.

**Construction of chimeric reporter viruses.** Since production of infectious HCV particles so far is only possible with JFH1-derived sequences, initially only JFH1 virus particles could be obtained (51, 56). This limitation was recently alleviated by the development of an intragenotypic chimeric genome consisting of the core to NS2 coding region of the J6 isolate (GT 2a [53]) and the remaining parts of the JFH1 genome (24). In a recent study (Pietschmann, Karl, Kontsondakis, Shaviuskaya, Vallis, Steinmann, Abid, Negro, Dreux, Cosset, and Bartenschlager, unpublished), we generated an intergenotypic chimera between Con1 (GT 1b) and JFH1. In this construct we chose an alternative junction site located after the 33 amino-terminal residues of NS2, thus supporting much higher titers than a hybrid genome with a crossover at the C terminus of NS2. Similarly, the chimera Jc1, which is a hybrid between J6CF and JFH1, is fused via this novel site and was more efficient compared to the original J6CF/JFH1 chimera described by Lindenbach and coworkers (Pietschmann, Karl, Kontsondakis, Shaviuskaya, Vallis, Steinmann, Abid, Negro, Dreux, Cosset, and Bartenschlager, unpublished). In order to facilitate comparative analysis on virus assembly and entry with particles from different isolates (1b and 2a), we created luciferase reporter constructs of these novel chimeric genomes (Fig. 1). As shown in Fig. 4A, the chimeras Luc-Jc1 and Luc-Con1 replicated with similar efficiency and kinetics as the parental Luc-JFH1 construct. However, release of core protein and infectivity were much faster and somewhat more efficient in the case of Luc-Jc1. The genotype 1b chimera (Luc-Con1), on the other hand, displayed a kinetics of core release and infectivity very similar to the parental Luc-JFH1 reporter virus (Fig. 4B and C). Yet while the quantity of core release attained with Luc-Con1 was higher than with Luc-JFH1, the yield of infectivity was lower, suggesting that the genotype 1b-derived virus particles have a lower specific infectivity. Overall, for each construct the level of core protein present in the supernatant correlated well with the amount of infectivity, demonstrating that core protein release is a good marker for release of infectious particles. In summary, these data establish that neither the heterologous structural proteins nor the chimeric NS2 negatively affected replication of JFH1-based reporter virus constructs. Furthermore, this experiment shows that the structural proteins from divergent isolates display very different properties in regard to the efficiency and kinetics of assembly, release of particles, and their specific infectivity.

**CD81 is a key receptor for GT 1b and 2a virus particles.** To further validate the different reporter viruses and to assess if GT 2a and GT 1b particles deviate with respect to the requirements for productive entry into target cells, we investigated the early steps of HCV infection of Huh7-Lunet cells. Therefore, in a first set of experiments we sought to determine whether

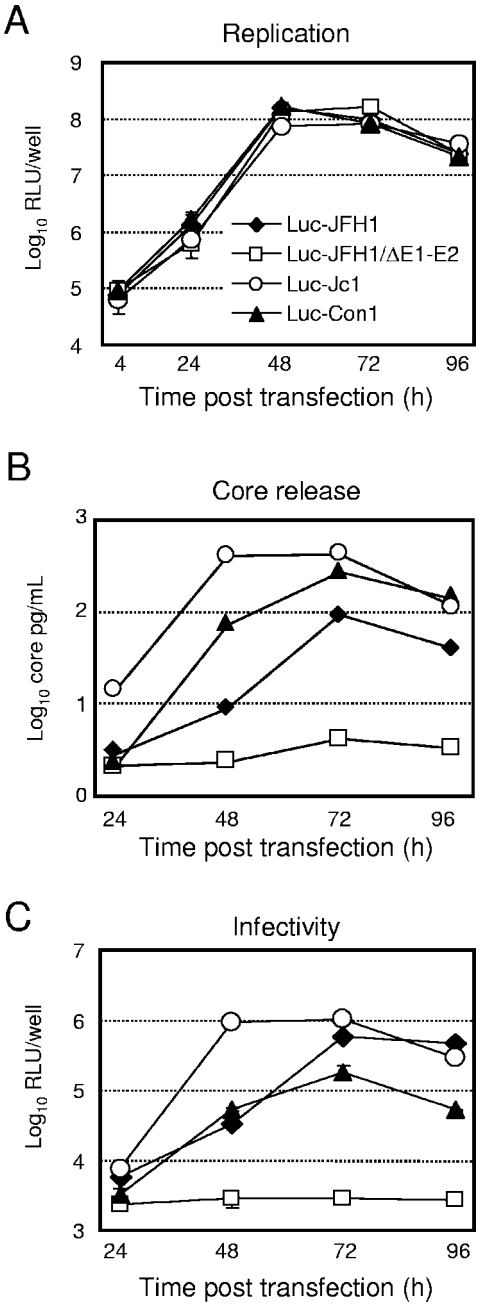


FIG. 4. Replication and production of infectious virus particles upon transfection of chimeric reporter viruses. (A) Replication kinetics of given HCV genomes and the deletion mutant as determined by luciferase assay measured at various time points posttransfection. Release of core protein (B) and infectivity into the supernatant (C) were quantified by using a core-specific immunoassay and by inoculating naïve Huh7-Lunet cells and subsequent luciferase assays, respectively. ELISAs were performed in singleton; for reporter assays, two independent wells were analyzed, and mean values are given (error bars represent the standard errors of the means).

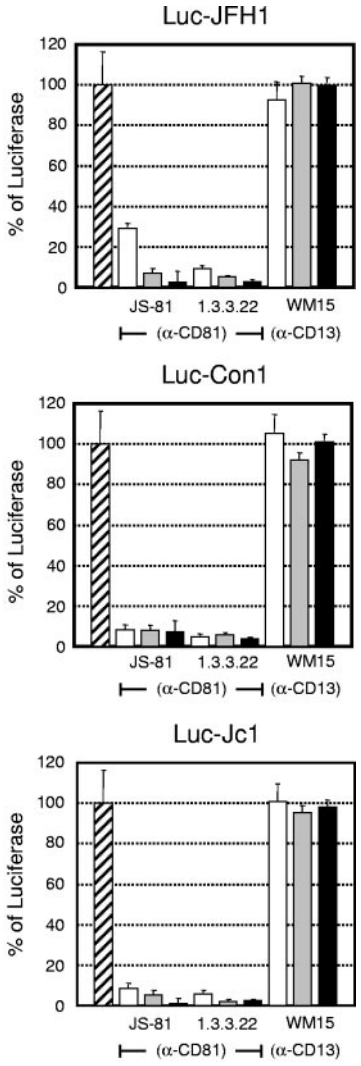


FIG. 5. Neutralization of genotype 1b and 2a HCV infection by different CD81-specific antibodies. Huh7-Lunet cells were infected with reporter viruses in the presence of increasing amounts of CD81-specific (JS-81 and 1.3.3.22) antibodies or isotype-matched control antibodies (WM15) directed against CD13, an irrelevant surface molecule on Huh7-Lunet cells. Inoculation was terminated after 4 h by washing cells with PBS and supplementing them with fresh culture medium without antibodies. Luciferase activity was determined 72 h postinfection and is expressed relative to the amount observed in the absence of inhibitory antibodies (hatched bar). White, gray, and black bars represent infections in the presence of antibodies at a concentration of 0.08 μg/ml, 0.4 μg/ml, and 2 μg/ml, respectively. The graphs (from top to bottom) depict results obtained with Luc-JFH1, Luc-Con1, and Luc-Jc1 particles, respectively. Mean values of duplicate wells are given (error bars represent standard errors of the means).

JFH1, J6, and Con1 particles utilize CD81 to infect Huh7-Lunet cells. To this end we performed infections with all three reporter viruses in the presence of increasing amounts of two different antibodies directed against this protein. To control for a nonspecific inhibitory effect caused by an obstruction of the cell surface with bound antibodies, we performed infections with the same quantity of antibodies recognizing CD13, an irrelevant molecule expressed on the surface of Huh7-Lunet cells (data not shown). As depicted in Fig. 5, control antibodies did not influence infection, whereas both CD81-binding antibodies neutralized HCV infection with similar efficiency. Already at the lowest concentration used, luciferase activity in the inoculated cells was reduced to about 30% for Luc-JFH1 and to roughly 10% for Luc-Con1 and Luc-Jc1. For all viruses used, neutralization was dose dependent, reducing luciferase expression to 10% or less at the highest concentration of antibodies (2 μg/ml).

**Interactions with cell surface-exposed GAGs contribute to efficient HCV infection.** Although it is clear that CD81 binds E2 and serum-derived HCV particles (14, 36), it is currently not known if and to what extent this interaction contributes to the attachment of authentic virus particles to CD81-expressing cells. Experiments with HCVpp and patient sera provided evidence that it may serve as a coreceptor rather than a primary attachment receptor (11). On the other hand, it is known that proteoglycans on the cell surface can act as primary attachment sites for viruses (42). As these structures vary greatly with respect to their composition and quantity among different cell types and tissues, high-affinity interactions between specific GAGs and viral surface molecules may be important determinants for viral tropism. To investigate if interactions with certain GAGs on the surface of Huh7-Lunet target cells may contribute to productive infection with HCV, we conducted a series of infection assays in the presence of various types of soluble GAGs as competitive inhibitors. The compounds tested differ with respect to polysaccharide composition and degree of sulfatation, with heparin displaying the highest number of sulfate groups (2.4 per disaccharide unit). As summarized in Table 1, we observed a moderate dose-dependent inhibition of infection by heparin, whereas neither soluble heparan sulfate nor chondroitin sulfate A, B, or C competed for productive entry of reporter viruses. All viruses tested were similarly susceptible to competitive inhibition by heparin with a 50% inhibitory concentration of approximately 50 μg/ml. Higher heparin dosage further increased the inhibitory effect up to a plateau of neutralization (80%) generally reached at a concentration of 250 μg/ml (data not shown). Dissimilar to antibodies directed against CD81, treatment of target cells prior to inoculation did not decrease infectivity, implying that heparin—in contrast to antibodies against CD81—does not act on the cell surface but may block infection by directly interacting with virus particles (Fig. 6).

To demonstrate that the observed inhibition is mediated by competition with similar molecules on the surface of target cells and to corroborate that interactions with GAGs play a role in HCV entry into Huh7-Lunet cells, we used Luc-Jc1 particles to infect Huh7-Lunet cells that had been treated with GAG lyases of different specificity (Fig. 7). We observed a moderate albeit reproducible decrease in the efficiency of infection when cells were treated with heparinase I and III, enzymes that specifically cleave heparan sulfate molecules, whereas treatment with chondroitinase ABC (degrading dermatan and chondroitin sulfate) did not affect Luc-Jc1 infection. Together, these data are in line with previous results (3) and suggest that an interaction of HCV with specific highly sulfated GAG moieties on the surface of Huh7-Lunet cells aids virus attachment and consequently contributes to efficient infection.

TABLE 1. Competition of various glycosaminoglycans with HCV infection[a]

| Virus | Heparin | Heparan sulfate | Chondroitin A | Chondroitin B | Chondroitin C | GAG concn (μg/ml) |
|---|---|---|---|---|---|---|
| Luc-JFH1 | 105.5 ± 8.7 | 81.1 ± 5.4 | 85.1 ± 2.7 | 83.8 ± 12.2 | 95.1 ± 5.1 | 2 |
| | 73.9 ± 10.1 | 105.1 ± 29.8 | 107.1 ± 6.6 | 104.5 ± 10.2 | 88.5 ± 2.7 | 10 |
| | 53.5 ± 10.2 | 103.9 ± 2.9 | 89.0 ± 6.9 | 101.0 ± 23.6 | 103.4 ± 12.7 | 50 |
| Luc-Jc1 | 87.5 ± 10.7 | 94.6 ± 11.7 | 98.0 ± 8.2 | 93.5 ± 2.7 | 86.4 ± 6.1 | 2 |
| | 68.6 ± 6.8 | 115.6 ± 12.0 | 85.8 ± 9.1 | 101.7 ± 32.6 | 92.0 ± 10.0 | 10 |
| | 54.3 ± 2.9 | 138.9 ± 21.9 | 105.7 ± 1.8 | 123.5 ± 15.6 | 104.9 ± 19.4 | 50 |
| Luc-Con1 | 94.8 ± 17.1 | 113.7 ± 17.2 | 87.9 ± 14.6 | 84.3 ± 3.2 | 97.8 ± 1.9 | 2 |
| | 80.1 ± 5.5 | 115.1 ± 23.4 | 99.5 ± 25.0 | 89.5 ± 24.6 | 89.4 ± 10.1 | 10 |
| | 45.8 ± 8.3 | 90.2 ± 11.9 | 117.8 ± 19.1 | 94.0 ± 19.0 | 83.0 ± 15.2 | 50 |

[a] Values represent percent luciferase (Luc) activity measured compared to infections in the absence of competitors; mean values of duplicates and the standard errors of the means are given.

**pH-dependent entry of reporter viruses.** It is well established that upon contact with the cell surface, enveloped viruses enter by two different strategies. A large number of retroviruses, for example, penetrate into the cytoplasm of the host cell directly at the plasma membrane by a fusion mechanism triggered through the interaction of their envelope proteins with their cognate receptors. In contrast, cell surface-attached influenza A viruses or VSV are taken up into endocytic vesicles, from which they escape upon acidification. In this case, the shift in pH triggers the fusion machinery and thus is an essential requirement for productive virus entry. Accordingly, viruses utilizing these divergent routes can be distinguished based on their susceptibility to drugs like ammonium chloride ($NH_4Cl$) or concanamycin A (ConA), which prevent the acidification of endosomes. In the event of HCV, it was shown that HCVpp entry with genotype 1a- and 1b-derived E1 and E2 is pH dependent (5, 20). However, it was unclear if authentic HCV particles penetrate the host cell in a pH-dependent manner and if this entry pathway is conserved between different HCV strains. Therefore, we examined the effect of treating target cells with $NH_4Cl$ and ConA on the infection with genotype 1b- and 2a-derived reporter viruses (Fig. 8). As control for pH-dependent and -independent entry, we used HIV-based pseudo-particles bearing VSV-G envelope or amphotropic murine leukemia A-MLV proteins, respectively. In pilot experiments we observed a dose-dependent inhibition of Luc-JFH1 infection by both drugs (data not shown). When we used $NH_4Cl$ and ConA at a concentration of 10 mM or 5 nM, respectively, infection with JFH1, J6, and Con1 particles was effectively blocked as demonstrated by about 5-fold and more than 10-fold reduced reporter activity, respectively. To rule out that drug treatment affected cell viability or replication of HCV RNA, we treated Huh7-Lunet cells carrying an autonomously replicating Luc reporter construct in the same manner and subsequently monitored reporter activity. As can be seen in Fig. 8B, neither drug significantly affected luciferase expression in the treated cells, thus demonstrating that the effect observed in the infection assays can be attributed to a block in virus entry.

**Kinetics of virus entry into Huh7-Lunet cells.** Based on the data presented so far, heparin, CD81-specific antibodies, and drugs blocking the acidification of endosomes emerged as potent inhibitors of HCV entry. However, these agents probably impede virus entry at different stages, likely affecting virus attachment in the case of heparin and possibly also anti-CD81 antibodies but a postbinding step in the case of ConA. Therefore, to characterize the sequence of events during HCV entry and the mode of inhibition by the different treatments, we investigated the inhibitory capacity of the different agents when administered at different intervals during the early phase of infection (Fig. 9). We used a high-titer reporter virus prep-

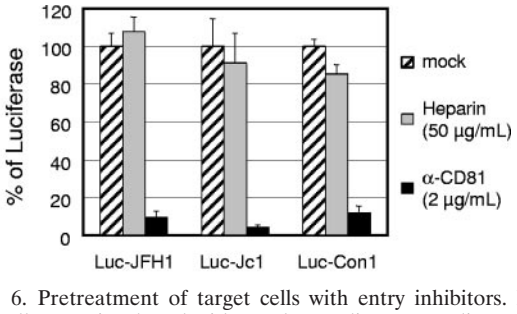


FIG. 6. Pretreatment of target cells with entry inhibitors. Huh7-Lunet cells were incubated with regular medium or medium supplemented with heparin (50 μg/ml) or antibodies against CD81 (JS-81; 2 μg/ml) for 1 h at 4°C. Subsequently, cells were washed with PBS and inoculated with reporter viruses. Infection was quantified as described in the legend of Fig. 5.

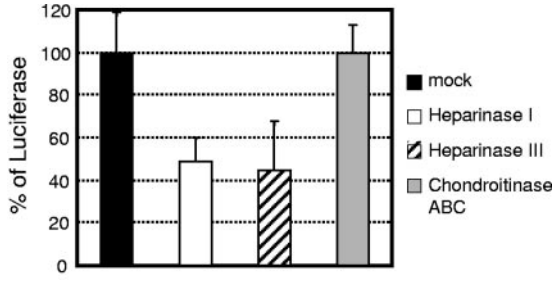


FIG. 7. Infection of Huh7-Lunet cells after pretreatment with GAG-lyases. Huh7-Lunet cells were treated with buffer alone (mock) or with buffer containing 0.5 U/ml heparinase I, heparinase III, or chondroitinase ABC for 1 h at 37°C. Subsequently, cells were washed three times with PBS and then inoculated with Luc-Jc1 for 1 h at 37°C. The efficiency of infection was determined 48 h postinoculation as described in the legend of Fig. 5.

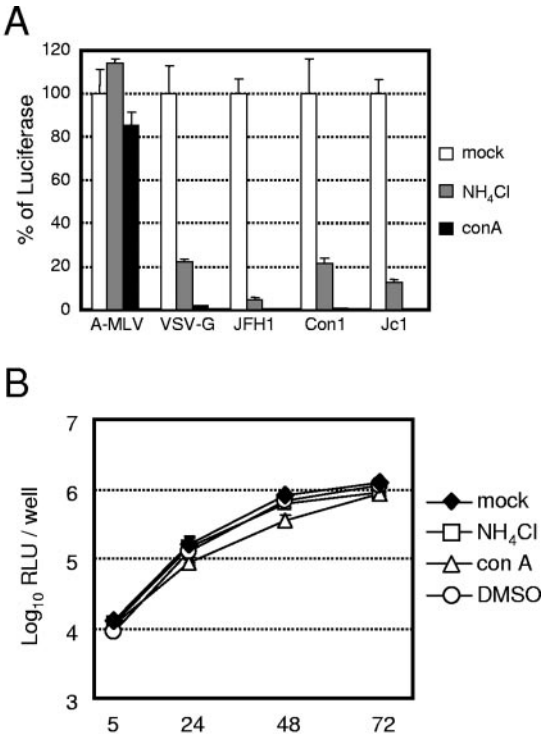


FIG. 8. Susceptibility of HCV entry to drugs inhibiting endosomal acidification. (A) Huh7-Lunet cells were mock-treated (open bars) or incubated for 1 h with medium containing 10 mM $NH_4Cl$ or 5 nM ConA (gray or black bars, respectively), washed with medium, and infected with different viruses for 4 h in the presence or absence of the drugs. As control for pH-dependent or -independent virus entry, infections with retroviral pseudo-particles bearing VSV-G or A-MLV envelope proteins, respectively, were performed in the same way. Luciferase activity was determined 72 h postinfection and is expressed relative to the values obtained in the absence of drugs. Mean values of duplicates and the standard errors of the means are given. (B) ConA and $NH_4Cl$ do not interfere with HCV RNA replication. Huh7-Lunet cells carrying selectable JFH1-based luciferase reporter replicons were seeded into replicate wells. At 24 h after seeding, cells were treated with drugs for 5 h. RNA replication was monitored by measuring reporter activity at the given time points posttreatment. Mean values of duplicates and the standard errors of the means are given.

aration of Luc-Jc1 and incubated it in the presence or absence of inhibitors with Huh7-Lunet cells for 1 h at 4°C. Under these conditions, virus attaches to the cells but does not efficiently enter, thus permitting a rather synchronous infection when the inoculum is removed and cells are shifted to 37°C. Therefore, subsequent to virus attachment, unbound virus was washed away, cells were transferred to 37°C, and inhibitors were added either directly or 1, 2, or 3 h later for an interval of 4 h as indicated in Fig. 9A. Under these conditions, we found that heparin inhibited the infection only when it was present during virus binding (Fig. 9B). Interestingly, CD81-specific antibodies blocked productive virus entry with comparable potency, irrespective of whether they were present during virus binding or if they were applied directly after virus attachment. Similarly, ConA blocked infection with maximal potency when added during virus binding and directly thereafter. When administered 1 h after transfer of the inoculated cells to 37°C, a partial reduction of luciferase activity was observed, indicating that at this time already about 50% of particles could no longer be blocked by anti-CD81 and about 35% were no longer susceptible to inhibition by ConA. Almost complete resistance to the receptor antibodies was reached about 2 h after virus attachment, while inhibition of endosomal acidification no longer affected infection when used 3 h later. The observed profiles of inhibition imply that interactions between the virus particle and cell surface-derived GAGs contribute to virus attachment; they suggest that CD81 may play a role subsequent to virus binding, and they demonstrate that HCV has passed the pH-sensitive phase during entry about 3 h postattachment.

To further distinguish the mode of action exerted by heparin and antibodies against CD81, we performed a series of infection experiments with virus inoculation at 37°C. At this temperature, virus attachment, receptor interactions, and all subsequent steps of the entry process should occur normally and proceed at the regular rate. We inoculated Huh7-Lunet cells for various durations at 37°C in the presence or absence of heparin or anti-CD81 antibodies, respectively (Fig. 9C). Subsequently, cells were washed and in all cases exposed to medium with inhibitors for 4 h before they were washed again and supplemented with normal growth medium. The efficiency of infection under the respective conditions was compared to control infections with the corresponding duration of inoculation in the absence of compound during and after inoculation. As expected, characteristic for a compound blocking virus attachment, heparin competed with productive infection only when it was present during virus inoculation (Fig. 9C, left graph). In contrast, antibodies against CD81 potently interfered with infection also when added after virus binding at 37°C (Fig. 9C, right graph). Even following an extended binding phase at 37°C, more than 60% of bound virus particles were susceptible to neutralization by anti-CD81. In summary, these data suggest that CD81 acts at a stage subsequent to initial virus binding.

## DISCUSSION

The aim of this study was to characterize the properties of bicistronic JFH1-based luciferase reporter genomes with respect to RNA replication, virus production, and infectivity and to utilize this system for the analysis of the early steps of HCV infection. We observed an excellent correlation between reporter activity and RNA copy numbers in transfected cells, and due to the robust replication of the reporter virus genome, we were able to detect already very few infection events in a highly quantitative manner. It is important that the high level of luciferase activity in cells inoculated with Luc-JFH1 reporter viruses derives from a limited number of infected cells (less than 1%). Consequently, multiple infections of target cells which may compromise the linearity of the assay are unlikely. In line with this notion, we observed linear dose dependence between the inoculum and the amount of reporter activity. Since the reporter viruses are produced from replication-competent genomes encoding the complete set of viral proteins, in principle, subsequent rounds of virus production and infection can occur. Therefore, harvesting the cells 72 h postinfection is not a strict single-round infection assay. However, taking into account the kinetics and efficiency of reporter virus production, it is unlikely that subsequent rounds of infection significantly

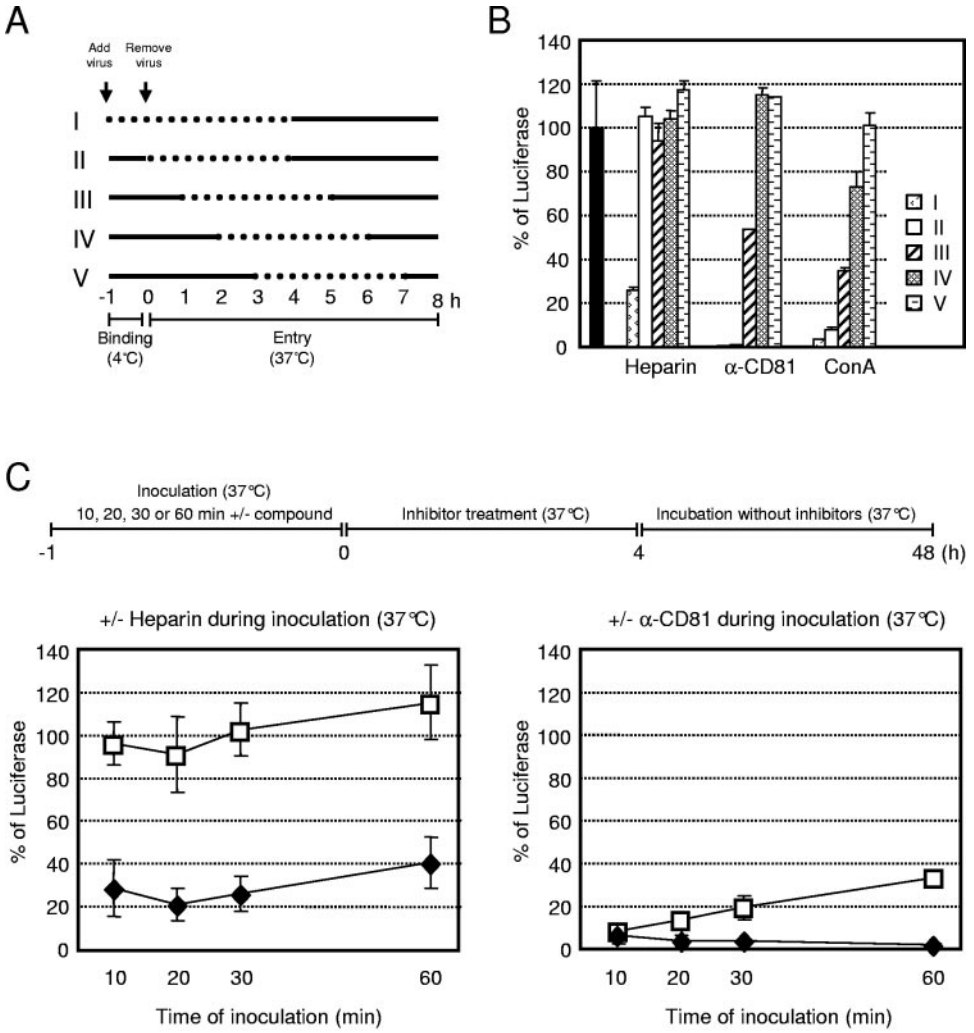


FIG. 9. Kinetics of inhibitory activity exerted by various compounds. (A) Schematic drawing of the experimental setup of the experiment depicted in panel B. Inhibition of Luc-Jc1 entry into Huh7-Lunet cells by heparin (50 μg/ml), CD81-specific antibodies (JS-81; 2 μg/ml) and ConA (5 nM) was compared using five different experimental protocols (indicated by roman numerals I through V). Virus binding to target cells was performed for 1 h at 4°C in the absence (II to IV) or in the presence (I) of compounds. Subsequently, cells were washed with PBS and shifted to 37°C to allow entry to proceed. Depending on the protocol, inhibitors were added directly or 1, 2 or 3 h thereafter (II, III, IV, and V, respectively). Dotted lines indicate the time interval during which an inhibitor was present; black arrows indicate the addition and removal of virus inoculum (B). Efficiency of infection using the compounds given below the bars and the protocols described for panel A was determined by luciferase assays 72 h postinfection and is expressed relative to infection in the absence of inhibitors (black bar). Mean values of two wells and the standard errors of the means are given. (C) A schematic drawing of the experimental setup is depicted at the top of the panel. Huh7-Lunet cells were incubated with Luc-Jc1 virus for various times at 37°C in the presence of heparin (50 μg/ml; left) or anti-CD81 antibodies (2 μg/ml; right) or in the absence of either compound (black diamonds and open squares, respectively). Subsequently, virus inocula were removed; cells were washed with PBS and supplemented with fresh culture medium containing either heparin (50 μg/ml) or anti-CD81 antibodies (2 μg/ml). Four hours later, culture medium was replaced by medium without inhibitors. The efficiency of infection was measured by luciferase assays 48 h later and is given for each time point relative to control infections performed in the same way but always without inhibitor. Mean values of two wells and the standard errors of the means are given.

contribute to the amount of reporter activity measured at this time point. This view is corroborated by our observation that during passage of Huh7-Lunet cells either transfected or infected with reporter viruses, reporter activity and the number of infected cells did not increase but stagnated and eventually declined, indicating only limited spread of reporter viruses in Huh7-Lunet cells (A. Kaul and R. Bartenschlager, unpublished observations). While this is similar to what we found for wild-type JFH1-transfected Huh7 cells (51), Zhong and coworkers recently observed rapid spread of JFH1 in transfected Huh7.5.1. cells (56), Huh7 cell clones derived by curing Huh7.5 cells carrying a subgenomic HCV replicon (30). This discrepancy indicates that Huh7 cell clones apparently differ with respect to their permissiveness for HCV spread and may in part explain the limited spread of reporter viruses in Huh7-Lunet cells. On the other hand, we noticed that the reporter virus is somewhat attenuated with respect to RNA replication and virus production compared to the parental genome, which is likely to further limit the efficiency of virus spread.

Characterizing the optimal conditions for production of re-

porter virus stocks, we observed that Luc-JFH1 replication rapidly declines when cells reach confluence. The fact that the infectious titer decreases accordingly with a delay of about 24 h indicates that there is a tight linkage between replication and production of infectious particles, and it has important practical implications for generating high-titer reporter virus stocks. It is currently not known why HCV replication is limited in confluent Huh7 cells. Besides cell death due to overconfluence, the observations that nucleoside triphosphate pools are low in resting cells (44) and that HCV IRES activity varies during the cell cycle and is lowest in the $G_0$ phase of resting cells may explain these findings (19).

In order to allow comparative analysis of HCV infection, we have constructed two further reporter viruses with Con1-derived (GT 1b) and J6-derived (GT 2a) structural proteins. Overall, we found a very similar behavior of Con1, J6CF, and JFH1 particles. This suggests that the mode of entry into Huh7-Lunet cells, which involves interactions with cell surface-exposed GAGs, CD81, and a pH-dependent step, is conserved between these isolates. As pointed out above, evidence from work with HCVpp and authentic HCV particles based on receptor competition studies using antibodies and soluble forms of the large extra cellular loop of CD81, RNA silencing of CD81, and ectopic expression in HepG2 cells, which renders them permissive for HCV, clearly indicates that CD81 is a key molecule required for productive infection by HCV (4, 20, 24, 51, 56). In contrast, however, the exact role of CD81 in the course of virus infection is as yet ill defined, and for HCV the precise sequence of events during entry is not known. The fact that HCV E2 binds CD81 together with the observation that patient-derived virus particles can be captured using the large extra cellular loop of CD81 (36) is consistent with a role of CD81 for virus attachment and may explain why reagents blocking this interaction inhibit infection with HCVpp and authentic HCV particles. However, Cormier and coworkers recently noted that binding of patient-derived HCV to human target cells was not inhibited by anti-CD81 monoclonal antibody and, moreover, demonstrated that HCVpp particles that were prebound to Huh7 cells at low temperature were fully susceptible to neutralization when anti-CD81 antibodies were added subsequent to virus binding (11). In line with these data, we observed potent inhibition of Luc-Jc1 infection irrespective of whether antibodies were added prior to, during, or after virus binding to Huh7-Lunet cells. Although we cannot rule out that a high-affinity interaction between the antibodies and CD81 may displace bound virions from the target cells, these data suggest that the interaction with CD81 may not necessarily be required for virus attachment but, rather, for a postbinding step such as routing the particle into the endocytic pathway or priming it for the pH-dependent fusion mechanism as described for avian leucosis virus (31). In that respect it is worth mentioning that recent data provided by Tscherne and coworkers are in agreement with ours, demonstrating that HCV enters Huh7 cells in a pH-dependent fashion (47). Moreover, these authors illustrate that HCV, similar to the related bovine viral diarrhea virus, requires a priming event before pH-dependent entry can occur. While it is currently not known which "signal" (e.g., receptor interaction) activates the virus to undergo pH-triggered fusion, the mode of inhibition exerted by antibodies against CD81 is, in principle, compatible with the notion that CD81 interaction serves such a function.

Besides CD81, several receptor candidates have been proposed to be essential for HCV entry. These may act in combination to mediate adhesion to target cells and could in a programmed series of steps eventually result in productive infection. Proteoglycans carrying certain types of GAGs were proposed to play a role for HCV attachment (3). While such negatively charged moieties in principle could promote particle association with the target cell predominantly via nonspecific electrostatic interactions with positively charged groups on the virus surface, susceptibility to herpes simplex virus 1 infection requires not only heparan sulfate chains but also the presence of unique monosaccharide sequences (41). Moreover, dengue virus, like HCV a member of the family *Flaviviridae*, uses a highly sulfated type of heparan sulfate as a cellular receptor (8). Similarly, Barth and colleagues recently demonstrated that heparin and liver-derived highly sulfated heparan sulfate but not other soluble GAGs inhibited attachment and entry of insect cell-derived HCV virus-like particles, suggesting that highly sulfated GAGs may be important for HCV attachment (3). Callens and colleagues, however, did not observe an interaction of detergent-solubilized E1-E2 complexes derived from HCVpp with heparin and, since HCV glycoprotein complexes derived from cell lysates bound heparin, concluded that the heparin-binding domain may no longer be accessible on mature glycoproteins on the surface of HCVpp (6). On the other hand, HCV particles can efficiently be purified from patient sera by using heparin, and GAG-binding sites within E2 have been characterized (32, 54). It is not clear if differences in glycoprotein structure between the particles are responsible for this discrepancy, but it is possible that virus particle-associated glycoprotein complexes generally have a lower affinity for heparin than cell-derived complexes. This residual affinity appears to be sufficient to purify serum-derived HCV but may be insufficient to allow detection by the pull-down assay employed for HCVpp-derived glycoprotein complexes. Nevertheless, together these data suggest that HCV can interact with highly sulfated GAGs, which may contribute to its cell adhesion and consequently to virus invasion of target cells. Our data demonstrating that heparin, but not other soluble GAGs which differ in their degree of sulfatation and the composition of disaccharide units (38), competes with productive entry of HCV virus particles into Huh7-Lunet cells are in line with this model and suggests that interactions with a specific highly sulfated type of GAG may contribute to efficient HCV infection. The finding that heparin inhibits infection only when administered during virus binding and not afterwards or when applied to the cells before the addition of virions implies that it acts via binding to positively charged residues on the surface of the virus particle, thus blocking interactions required for effective attachment. While in principle this binding could nonspecifically block interaction with any viral receptor, the observation that pretreatment of target cells with heparinase I and III reduces the efficiency of HCV infection whereas chondroitinase ABC exerts no effect lends further support to the notion that interactions with specific highly sulfated GAG residues are involved in infection. However, considering the moderate inhibition by heparin and treatment with GAG lyases, it is unlikely (at least in the case of

Huh7-Lunet cells) that highly sulfated GAGs serve as the principal attachment sites for HCV.

In summary, in this study we have characterized the properties of JFH1-based HCV luciferase reporter viruses and validated the system by applying it to dissect the early steps of HCV infection using viral particles from three different strains. Although the JFH1 isolate is certainly exceptional among other HCV isolates with respect to the extraordinary replication efficiency, in terms of virus entry, JFH1-derived particles did not deviate from Con1- or J6CF-based virus particles. This suggests that at least these strains utilize a common entry strategy. The novel reporter viruses should be a valuable instrument to further dissect HCV entry and to develop specific inhibitors.

## ACKNOWLEDGMENTS

We are grateful to Ulrike Herian for excellent technical assistance, to Heidi Barth, Thomas Baumert, Marko Lampe, Dirk Lindemann, Barbara Müller, Andreas Schulze, Didier Trono, Stephan Urban, and Takaji Wakita for reagents and advice, and to Anna Shavinskaya for critical reading of the manuscript.

This work was supported by grants from the VIRGIL European Network of Excellence on Antiviral Drug Resistance (LSHM-CT-2004-503359), the European HepCVax cluster (QLK2-CT-2002-01329), and the Bristol-Myers Squibb Foundation and by the Sonderforschungsbereich 638 (Teilprojekt A5).

## REFERENCES

1. **Agnello, V., G. Abel, M. Elfahal, G. B. Knight, and Q. X. Zhang.** 1999. Hepatitis C virus and other *Flaviviridae* viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA **96:**12766–12771.
2. **Bartenschlager, R., M. Frese, and T. Pietschmann.** 2004. Novel insights into hepatitis C virus replication and persistence. Adv. Virus Res. **63:**71–180.
3. **Barth, H., C. Schafer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. von Weizsacker, H. E. Blum, and T. F. Baumert.** 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. **278:**41003–41012.
4. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. J. Exp. Med. **197:**633–642.
5. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278:**41624–41630.
6. **Callens, N., Y. Ciczora, B. Bartosch, N. Vu-Dac, F. L. Cosset, J. M. Pawlotsky, F. Penin, and J. Dubuisson.** 2005. Basic residues in hypervariable region 1 of hepatitis C virus envelope glycoprotein e2 contribute to virus entry. J. Virol. **79:**15331–15341.
7. **Cannon, P. M., N. Kim, S. M. Kingsman, and A. J. Kingsman.** 1996. Murine leukemia virus-based Tat-inducible long terminal repeat replacement vectors: a new system for anti-human immunodeficiency virus gene therapy. J. Virol. **70:**8234–8240.
8. **Chen, Y., T. Maguire, R. E. Hileman, J. R. Fromm, J. D. Esko, R. J. Linhardt, and R. M. Marks.** 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat. Med. **3:**866–871.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
10. **Cormier, E. G., R. J. Durso, F. Tsamis, L. Boussemart, C. Manix, W. C. Olson, J. P. Gardner, and T. Dragic.** 2004. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. Proc. Natl. Acad. Sci. USA **101:**14067–14072.
11. **Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic.** 2004. CD81 is an entry coreceptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **101:**7270–7274.
12. **DuBridge, R. B., P. Tang, H. C. Hsia, P. M. Leong, J. H. Miller, and M. P. Calos.** 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. **7:**379–387.
13. **Dull, T., R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono, and L. Naldini.** 1998. A third-generation lentivirus vector with a conditional packaging system. J. Virol. **72:**8463–8471.
14. **Flint, M., C. Maidens, P. L. Loomis, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating.** 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. J. Virol. **73:**6235–6244.
15. **Friebe, P., J. Boudet, J. P. Simorre, and R. Bartenschlager.** 2005. Kissing-loop interaction in the 3′ end of the hepatitis C virus genome essential for RNA replication. J. Virol. **79:**380–392.
16. **Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson.** 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **100:**4498–4503.
17. **Griffin, S. D., L. P. Beales, D. S. Clarke, O. Worsfold, S. D. Evans, J. Jaeger, M. P. Harris, and D. J. Rowlands.** 2003. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, amantadine. FEBS Lett. **535:**34–38.
18. **Guo, J. T., V. V. Bichko, and C. Seeger.** 2001. Effect of alpha interferon on the hepatitis C virus replicon. J. Virol. **75:**8516–8523.
19. **Honda, M., S. Kaneko, E. Matsushita, K. Kobayashi, G. Abell, and S. Lemon.** 2000. Cell cycle regulation of hepatitis C virus internal ribosome entry site-directed translation. Gastroenterology **118:**152–162.
20. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100:**7271–7276.
21. **Kalajzic, I., M. L. Stover, P. Liu, Z. Kalajzic, D. W. Rowe, and A. C. Lichtler.** 2001. Use of VSV-G pseudotyped retroviral vectors to target murine osteoprogenitor cells. Virology **284:**37–45.
22. **Krieger, N., V. Lohmann, and R. Bartenschlager.** 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J. Virol. **75:**4614–4624.
23. **Lavillette, D., A. W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A. H. Patel, J. Dubuisson, J. K. Ball, and F. L. Cosset.** 2005. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatology **41:**265–274.
24. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309:**623–626.
25. **Lohmann, V., S. Hoffmann, U. Herian, F. Penin, and R. Bartenschlager.** 2003. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. J. Virol. **77:**3007–3019.
26. **Lohmann, V., F. Körner, J. O. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110–113.
27. **Lozach, P. Y., A. Amara, B. Bartosch, J. L. Virelizier, F. Arenzana-Seisdedos, F. L. Cosset, and R. Altmeyer.** 2004. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. J. Biol. Chem. **279:**32035–32045.
28. **Lozach, P. Y., H. Lortat-Jacob, A. De Lacroix De Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer.** 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. **278:**20358–20366.
29. **McKeating, J. A., L. Q. Zhang, C. Logvinoff, M. Flint, J. Zhang, J. Yu, D. Butera, D. D. Ho, L. B. Dustin, C. M. Rice, and P. Balfe.** 2004. Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner. J. Virol. **78:**8496–8505.
30. **Moradpour, D., M. J. Evans, R. Gosert, Z. Yuan, H. E. Blum, S. P. Goff, B. D. Lindenbach, and C. M. Rice.** 2004. Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. J. Virol. **78:**7400–7409.
31. **Mothes, W., A. L. Boerger, S. Narayan, J. M. Cunningham, and J. A. Young.** 2000. Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein. Cell **103:**679–689.
32. **Olenina, L. V., T. I. Kuzmina, B. N. Sobolev, T. E. Kuraeva, E. F. Kolesanova, and A. I. Archakov.** 2005. Identification of glycosaminoglycan-binding sites within hepatitis C virus envelope glycoprotein E2*. J. Viral Hepatol. **12:**584–593.
33. **Pavlovic, D., D. C. Neville, O. Argaud, B. Blumberg, R. A. Dwek, W. B. Fischer, and N. Zitzmann.** 2003. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc. Natl. Acad. Sci. USA **100:**6104–6108.
34. Reference deleted.
35. **Pietschmann, T., V. Lohmann, G. Rutter, K. Kurpanek, and R. Bartenschlager.** 2001. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J. Virol. **75:**1252–1264.
36. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science **282:**938–941.
37. **Pohlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. J. Leslie, G. Lin, A. Granelli-Piperno, R. W. Doms, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. **77:**4070–4080.
38. **Rostand, K. S., and J. D. Esko.** 1997. Microbial adherence to and invasion through proteoglycans. Infect. Immun. **65:**1–8.

39. **Saunier, B., M. Triyatni, L. Ulianich, P. Maruvada, P. Yen, and L. D. Kohn.** 2003. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. J. Virol. **77:**546–559.
40. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21:**5017–5025.
41. **Shukla, D., J. Liu, P. Blaiklock, N. W. Shworak, X. Bai, J. D. Esko, G. H. Cohen, R. J. Eisenberg, R. D. Rosenberg, and P. G. Spear.** 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell **99:**13–22.
42. **Sieczkarski, S. B., and G. R. Whittaker.** 2005. Viral entry. Curr. Top. Microbiol. Immunol. **285:**1–23.
43. **Simmonds, P., J. Bukh, C. Combet, G. Deleage, N. Enomoto, S. Feinstone, P. Halfon, G. Inchauspe, C. Kuiken, G. Maertens, M. Mizokami, D. G. Murphy, H. Okamoto, J. M. Pawlotsky, F. Penin, E. Sablon, I. Shin, L. J. Stuyver, H. J. Thiel, S. Viazov, A. J. Weiner, and A. Widell.** 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology **42:**962–973.
44. **Stuyver, L. J., T. R. McBrayer, P. M. Tharnish, A. E. Hassan, C. K. Chu, K. W. Pankiewicz, K. A. Watanabe, R. F. Schinazi, and M. J. Otto.** 2003. Dynamics of subgenomic hepatitis C virus replicon RNA levels in Huh-7 cells after exposure to nucleoside antimetabolites. J. Virol. **77:**10689–10694.
45. **Thomssen, R., S. Bonk, C. Propfe, K. H. Heermann, H. G. Kochel, and A. Uy.** 1992. Association of hepatitis C virus in human sera with beta-lipoprotein. Med. Microbiol. Immunol. **181:**293–300.
46. **Thomssen, R., S. Bonk, and A. Thiele.** 1993. Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. Med. Microbiol. Immunol. **182:**329–334.
47. **Tscherne, D. M., C. T. Jones, M. J. Evans, B. D. Lindenbach, J. A. McKeating, and C. M. Rice.** 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J. Virol. **80:**1734–1741.
48. **van den Hoff, M. J., V. M. Christoffels, W. T. Labruyere, A. F. Moorman, and W. H. Lamers.** 1995. Electrotransfection with "intracellular" buffer. Methods Mol. Biol. **48:**185–197.
49. **van Regenmortel, M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and E. B. Wickner (ed.).** 2000. Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.
50. **Vrolijk, J. M., A. Kaul, B. E. Hansen, V. Lohmann, B. L. Haagmans, S. W. Schalm, and R. Bartenschlager.** 2003. A replicon-based bioassay for the measurement of interferons in patients with chronic hepatitis C. J. Virol. Methods **110:**201–209.
51. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796.
52. **Wunschmann, S., J. D. Medh, D. Klinzmann, W. N. Schmidt, and J. T. Stapleton.** 2000. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. J. Virol. **74:**10055–10062.
53. **Yanagi, M., R. H. Purcell, S. U. Emerson, and J. Bukh.** 1999. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. Virology **262:**250–263.
54. **Zahn, A., and J. P. Allain.** 2005. Hepatitis C virus and hepatitis B virus bind to heparin: purification of largely IgG-free virions from infected plasma by heparin chromatography. J. Gen. Virol. **86:**677–685.
55. **Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating.** 2004. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. J. Virol. **78:**1448–1455.
56. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102:**9294–9299.
57. **Zufferey, R., D. Nagy, R. J. Mandel, L. Naldini, and D. Trono.** 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat. Biotechnol. **15:**871–875.