A Novel Strategy To Develop a Robust Infectious Hepatitis C Virus Cell Culture System Directly from a Clinical Isolate

Jie Lu,^a Yu Xiang,^a Wanyin Tao,^a Qingchao Li,^a Na Wang,^a Yongfeng Gao,^a Xiaogang Xiang,^b Qing Xie,^b Jin Zhong^a

Unit of Viral Hepatitis, Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China^a; Department of Infectious Disease, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China^b

Hepatitis C virus (HCV) infection is a leading cause of chronic liver diseases. Progress in the HCV field was greatly enhanced by constructing infectious cDNA clone of JFH-1. Since then, JFH-1-based intra- and intergenotypic recombinants have been developed, and this permitted the study of vaccines and antiviral inhibitors for all genotypes. Recently, highly efficient HCV culture systems have been established by using consensus sequence-based clones. We developed a novel strategy to construct infectious HCV cDNA clone by combining functional screening of sequences directly from a genotype 2a clinical isolate (PR63) and cell culture adaptation. Using JFH-1 cDNA as the starting backbone, we sequentially replaced the JFH-1 fragments with a sequence from the pools of PR63 sequences. Through engineering adaptive mutations that improve HCV infectivity, we finally established a full-length cell culture-derived infectious clone of PR63, named PR63cc, that could efficiently produce virus particles in Huh7-derived cells, with peak titers of 1.6×10^5 focus-forming units/ml. The PR63cc could be neutralized by an anti-E2 antibody and inhibited by antiviral agents but appeared more resistant to an NS5A inhibitor than JFH-1. In summary, we developed a new approach to construct an infectious HCV cDNA clone that can produce viruses efficiently in cell culture. This approach could be applied to other viral isolates, with potential implications for individualized treatments of HCV patients.

Hepatitis C virus (HCV) infection is a heavy burden to the world health. HCV is a positive-stranded RNA virus belonging to the *Flaviviridae* family. Its genome, ~9.6 kb long, consists of an open reading frame (ORF) encoding a large polyprotein, flanked by the 5' and 3' untranslated regions (UTRs) (1). HCV has a high degree of genetic heterogeneity and can be classified into at least six major genotypes and numerous subtypes (2). The genotypes differ not only in sequence but also in response to antiviral treatment (3).

In vitro HCV culture models are indispensable for the research of HCV life cycle and antiviral therapy. Patient sera are infectious when inoculated into chimpanzees but do not result in productive infection in cell culture. It is desirable to generate an infectious cDNA clone from clinical isolates that produces infectious virions and recapitulates the entire life cycle of HCV in cell culture. JFH-1, a genotype 2a clone derived from a patient with fulminant hepatitis C, can robustly replicate in Huh7 cells (4). Taking advantage of this isolate, the production of infectious HCV particles has finally been accomplished either with JFH-1 or intragenotypic J6/JFH-1 chimera (5–7). Soon after that, inter- and intragenotypic recombinants, including 5'UTR-NS2, NS3 protease/NS4A, NS5A, and Core-NS3protease/NS4A-5A have been developed (8–18). Only recently have highly efficient HCV culture systems been established for isolates other than JFH-1, including genotype 1a (GT1a; TN), GT2a (JFH-2 and J6), and GT2b (J8, DH8, and DH10) (19–22). These studies all start with previously established HCV cDNA clones generated from consensus sequence of viral genome. The common way to make a consensus clone is to determine the HCV genome sequence from patient serum, assemble a master sequence, and synthesize it *in vitro*. However, DNA single-genome sequencing analysis has revealed enormous genetic diversity of HCV in chronically infected subjects (23), and the consensus sequence may not reflect the heterogeneous viral population that exists in HCV-infected patients. Whether a sequence resulting from the predominant nucleotide at each position exists in nature is still unknown.

No full-length HCV cell culture system has been developed directly from clinical samples. We previously succeeded in constructing infectious GT1b/JFH-1 chimeric viruses from clinical isolates through functional selection (24). From a pool of sequences directly amplified from GT1b clinical isolates, we were able to select a sequence of the region from Core to the first transmembrane domain of NS2 that can produce infectious GT1b/JFH-1 chimeric viruses (24). In the present study, we used the similar strategy to construct the infectious full-length cDNA clone of a genotype 2a clinical isolate (PR63). By combining this functional selection and cell culture adaptation, we established a full-length cell culture-derived infectious clone of PR63, named PR63cc, which efficiently produces virus particles in Huh7-derived cells with peak titers of 1.6×10^5 focus-forming units (FFU)/ml. Interestingly, PR63cc is much more resistant to an NS5A inhibitor than is JFH-1, highlighting the importance of developing cell culture model from clinical isolates.

MATERIALS AND METHODS

Source of HCV patient serum. The genotype 2a strain PR63 was isolated from a patient with chronic hepatitis C, whose serum was collected at the Department of Infectious Diseases of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The study was approved by the Ethics Committee of Shanghai Ruijin Hospital in accordance with the Helsinki Declaration, and written informed consent was obtained. HCV RNA

Received 6 October 2013 Accepted 6 November 2013

Published ahead of print 13 November 2013

Address correspondence to Jin Zhong, jzhong@sibs.ac.cn.

Y.X., W.T., and Q.L. contributed equally to this article.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JVI.02929-13>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JVI.02929-13

FIG 1 Molecular phylogenetic analysis of PR63 by the maximum-likelihood method. The analysis involved 11 nucleotide sequences (H77 accession number, AF009609; JFH-1, AB047639; HC-J6, D00944; HCV-J, D90208; Con-1, AJ238799; HCV-J8, D10988; BEBE1, D50409; J6CF, AF177036; JFH-2, AB690460; PR63 consensus, KF676351; and PR63 cell-culture derived [PR63cc], KF676352). The PR63 consensus and PR63cc sequences are marked with asterisks.

quantification and genotyping was performed as described elsewhere (24).

HCV sequence amplification and determination. Serum RNA isolation and reverse transcription were performed as previously described (24). HCV open reading frame (ORF) was divided into overlapping fragments and amplified by RT-PCR with specific primer sets (see Table S1 in the supplemental material). All amplified products were purified and sequenced directly. The 5'UTR sequence of PR63 was determined by 5'RACE (rapid amplification of cDNA ends) (14). The 3'UTR variable region and poly(U/UC) tract were amplified and cloned into pEasy-T1 (TransGen, Beijing, China) and sequenced from both directions. The extreme end (positions 9604 to 9678, 75 nucleotides [nt]) of 3'UTR sequence was directly taken from JFH-1 (AB047639). The consensus sequence of PR63 was obtained by assembling overlapping sequences and has been deposited in GenBank under accession number KF676351. To determine the cell culture-derived HCV sequence, total RNA was extracted from cells with total RNAprep Pure kit (Tiangen, Beijing, China) and sequenced as described above.

HCV transfection and infection. *In vitro*-transcribed HCV RNAs were transfected to Huh7.5.1 cells by electroporation as described previously (7). For infection, 10^5 cells per well were seeded in 12-well plates and infected at a multiplicity of infection (MOI) of ~ 0.01 and subcultured every 2 to 3 days. HCV-positive cells were detected by anti-E2 immunofluorescence staining with human anti-HCV E2 protein monoclonal antibody AR3A (25) as the primary antibody and Alexa Fluor 555 goat anti-human IgG (Invitrogen) as the secondary antibody.

Transient-transfection assay. A total of 5 μ g of synthesized luciferase-reporter replicon RNA was electroporated into Huh7.5.1 cells. Transfected cells were seeded in 48-well plates and harvested at 4, 24, 48, 72, and 96 h posttransfection. The lysates of the transfected cells were analyzed for the firefly luciferase activities (Promega).

Plasmids construction. PR63-derived fragment 1 (Core-NS3protease, nt 476 to 4111), fragment 2 (NS3helicase-NS5A, nt 4112 to 7465), and fragment 3 (NS5B, nt 7466 to 9334) were amplified from PR63 serum with specific primers listed in Table S2 in the supplemental material. Viable PR63/JFH-1 chimeras containing PR63-derived fragment 1, fragment 2, or fragment 3 were constructed by fusion of PR63 sequence with a JFH-1 backbone and selected from PR63/JFH-1 recombinant cDNA libraries. Individual viral mutations were introduced by QuikChange II XL site-directed mutagenesis kit (Stratagene). PR63-origin subgenomic replicons carrying luciferase reporter were constructed as JFH-1-SGR-Luc (26) by fusion of PR63's 5'UTR with a luciferase-neo^r gene segment and a PR63-NS3 region and then insertion into the EcoRI and SpeI sites of PR63_{FL}, PR63_{FL-N1931S}, PR63_{FL-N1931S-GND}, or PR63cc, respectively. The GND mutant abolished RNA polymerase activity of

NS5B as described previously (4). All recombinant constructs were verified by sequencing.

HCV antiviral treatment. A total of 2×10^4 Huh7.5.1 cells preseeded in 48-well plates were infected with HCV at an MOI of ~ 0.1 and treated with antivirals 24 h postinfection. The HCV RNAs were extracted from cells and determined by reverse transcription-quantitative PCR (RT-qPCR) at 72 h postinfection, expressed as the percentage of mock treatment. The dosages of IFN- α treatment are ca. 0.1 to 1,000 IU/ml. The dosages of daclatasvir treatment are 6.7 to 67,000 pM. The dosages of 2'-C-methyl-adenosine (2'CMA) treatment are 7.98 to 7,980 nM. The median 50% effective concentration (EC_{50}) was calculated as the concentration of inhibitor required for a 50% reduction in the HCV RNA level.

Neutralization with E2 antibody. Approximately 250 FFU of viruses were incubated with serial dilutions of anti-E2 monoclonal antibody for 1 h at 37°C. The virus-antibody mixture was added to 2×10^4 preseeded Huh7.5.1 cells in each well of a 48-well plate, followed by incubation for 3 days. The efficiency of infection was determined by RT-qPCR analysis. The percentage of neutralization was compared to the mock treatment.

Nucleotide sequence accession number. The nucleotide sequences reported here were deposited in GenBank under accession numbers KF676351 and KF676352.

RESULTS

Sequencing analysis of an HCV isolate from a chronic genotype 2a hepatitis C patient. We chose a genotype 2a clinical isolate to construct an infectious full-length cDNA clone. A Chinese patient was chronically infected by genotype 2a HCV (patient PR63). The serum HCV RNA titer was 3.5×10^6 copy/ml. The consensus sequence of PR63 was deduced by bulk sequencing overlapping fragments of HCV genome and was submitted to GenBank (accession number KF676351). The phylogenetic analysis of the PR63 ORF is shown in Fig. 1. The PR63 consensus sequence exhibits 89.3 and 91.2% homology at the nucleotide level with JFH-1 and J6CF (GenBank accession numbers AB047639 and AF177036), respectively, and 92 and 94.6% amino acid homology with JFH-1 and J6CF, respectively (Table 1).

Substitution of the Core-NS3protease, NS3helicase-NS5A, and NS5B regions. The previously reported HCV cell culture-derived (HCVcc) cDNA clones were all derived from a consensus sequence deduced from HCV quasiespecies existing in patient serum. These consensus sequence-based cDNA clones are generally

TABLE 1 Sequence homology between PR63, JFH-1, and J6CF^a

Region	PR63 nucleotide profile			PR63 amino acid profile		
	Length (nt)	% Identity vs strain:		Length (aa)	% Identity vs strain:	
		JFH-1	J6CF		JFH-1	J6CF
Entire genome	9,607	89.3	91.2	3,033	92.0	94.6
5'UTR	340	99.1	100	NA		
Core	573	94.2	96.0	191	94.8	99.0
E1	576	89.8	91.8	192	90.6	93.2
E2	1,101	86.8	89.0	430	91.0	87.7
p7	189	79.4	87.3	63	76.2	88.9
NS2	651	87.1	89.7	217	88.0	90.3
NS3	1,893	90.4	92.7	631	94.0	97.1
NS4A	162	90.7	90.1	54	96.3	96.3
NS4B	783	90.9	91.8	261	97.7	98.9
NS5A	1,398	87.1	91.0	466	85	93.3
NS5B	1,776	92.8	93.4	591	95.4	96.1
3'UTR	165	88.5	82.0	NA		

^a nt, nucleotides; aa, amino acids; NA, not applicable.

FIG 2 Construction of the PR63cc genome. The genomes of JFH-1 and PR63 are represented by white and gray boxes, respectively. For technical convenience, HCV ORF was divided into three fragments: fragment 1 (FspAI-SpeI) contains the Core-NS3protease region (nt 476 to 4111), fragment 2 (SpeI-RsrII) contains the NS3helicase-NS5A region (nt 4112 to 7465), and fragment 3 (RsrII-SgrAI) contains the NS5B region (nt 7466 to 9334). PR63_{FL} genome with the five adaptive mutations was designated PR63cc (for PR63 cell culture-derived). The nucleotide positions of PR63-origin sequences in each clone are indicated in parentheses. Adaptive mutations engineered are depicted by dots (●).

not infectious in cell culture without adaptive mutations, with JFH-1 as an exception. Therefore, we used a previously described screening approach (24) to construct the full-length HCVcc cDNA of PR63. Due to the technical difficulty of making a meaningful size of pool containing the 9.6-kb full-length HCV genome, our strategy was to divide the HCV ORF into three regions for the convenience of cloning: Core-NS3protease (fragment 1, FspAI-SpeI, nt 476 to 4111, or amino acid residues 46 to 1257), NS3helicase-NS5A (fragment 2, SpeI-RsrII, nt 4112 to 7465, or amino acid residues 1258 to 2375), and NS5B (fragment 3, RsrII-SgrAI, nt 7466 to 9334, or amino acid residues 2376 to 2998) and

then to construct a pool for each of these three regions, select viable clones individually, and assemble selected fragments into a full-length genome (Fig. 2).

Using the functional selection strategy that we developed previously (24), we amplified fragment 1 from PR63 serum RNA sample by RT-PCR and fused it with a JFH-1 backbone. Plasmids with the correct insertion size were pooled and separated into several groups for HCV RNA synthesis. After transfecting the RNAs into Huh7.5.1 cells, we passaged the cells and monitored the percentage of HCV-positive cells and supernatant infectivity. When a certain group produced detectable infectious particles in

the culture supernatants, we repeated the above-described experiment to identify the viable clone(s) within this group. In this way, we were able to select an infectious clone out of 21 candidates with the PR63-origin Core-NS3protease region on the JFH-1 backbone, with titers of 8.95×10^2 FFU/ml at day 9 posttransfection. Similarly, one infectious clone was screened out of 23 candidates with the viable PR63-origin NS3helicase-NS5A region, with titers of 6.1×10^3 FFU/ml at day 16 posttransfection, and three infectious clones were selected from 35 candidates with the PR63-origin NS5B region, with titers of 3.35×10^3 , 7.55×10^3 , and 4.75×10^3 FFU/ml at day 9 posttransfection, respectively.

Construction of the chimeric virus containing the Core-NS5B region of PR63. Next, we combined the three selected PR63-origin fragments to make the near full-length PR63 clone. However, the combination of the selected fragment 1, fragment 2, and either of the three clones of fragment 3 did not result in HCV replication, since no HCV-positive cell was ever detected up to 15 days posttransfection. When we only combined the selected fragment 1 and fragment 2 of PR63, HCV-positive cells were readily detected at day 4 posttransfection, although the infectivity titer was still low (data not shown).

Since the combination of selected fragment 1 and fragment 2 retained a good ability to replicate the chimeric genome, we decided to use this construct as the starting point to select a fragment 3 sequence that would result in an infectious full-length PR63 clone. To facilitate the selection, we first sought to improve the infectivity titers of the starting construct by continuously passaging virus, an approach that has been shown to enhance HCV infectivity (8–11, 15, 17–22, 24, 27, 28). The percentage of HCV-positive cells increased with the passaging of the transfected cells, a finding indicative of supernatant infectivity. The sequencing analysis of recovered viruses at day 27 posttransfection revealed two mutations: A1676S in NS4A and L2285I in NS5A. The A1676S mutation was previously discovered to promote virus production of J6 adapted virus (20). We engineered these mutations singly or in combination into PR63_{Core-5A}/JFH-1. Our results showed that A1676S by itself greatly enhanced the virus production, since the recombinant containing this mutation had titers of 8×10^3 FFU/ml at day 4 posttransfection. The L2285I change did not have any significant effect on virus production (data not shown). We therefore used adapted PR63_{Core-5A}/JFH-1 as the backbone to select a functional fragment 3 sequence (Fig. 2).

Starting from adapted PR63_{Core-5A}/JFH-1, we succeeded in obtaining one viable clone from 79 candidates of PR63-NS5B region (fragment 3), with peak titers of 6.4×10^3 FFU/ml at day 96 posttransfection. Recovered viruses had gained extra adaptive mutations: W849G and Q862P (NS2), C2432R (NS5A), and A2941T and S2955F (NS5B) (Fig. 2). Next, we engineered these five mutations together into PR63_{Core-5B}/JFH-1 to make the adapted PR63_{Core-5B}/JFH-1, which had infectivity titers of 6×10^3 FFU/ml at day 10 posttransfection.

Construction of the full-length infectious genome of PR63. Adapted PR63_{Core-5B}/JFH-1 still had JFH-1 sequences in its 5'UTR, Core N terminus, NS5B C terminus, and 3'UTR. We first replaced JFH-1's 5'UTR and the N terminus of the Core region (5'UTR-FspAI, nt 1 to 475) (Fig. 2) with the consensus PR63 sequence because this region is highly conserved. Our results showed that the replacement with the consensus PR63 sequence in this region had no effect on infectivity, since the infectivity titers of

FIG 3 Adaptive mutations enhance the infectivity of PR63cc. (A) Transfection kinetics of PR63_{FL}, PR63_{FL}-N1931S, and PR63_{FL}-D398G-N415D-N1931S-H2100R-T2941V (or PR63_{FL}-GDSRV) in Huh7.5.1 cells. The cells were electroporated with 10 μ g of *in vitro*-transcribed individual RNA, the supernatants were collected at the indicated time points, and their infectivity was determined by titration. A dashed line (10 FFU/ml) marks the detection limit of the titration assay. Means and standard deviations for two independent experiments are shown. (B) Subgenomic luciferase reporter HCV replicons containing JFH-1's NS3-3'UTR or PR63_{FL}-derived sequence of NS3-3'UTR with changes of N1931S, N1931S-GND, or N1931S-H2100R-T2941V were electroporated into Huh7.5.1 cells for transient replication assay. The luciferase activities were measured at 4, 24, 48, 72, and 96 h posttransfection in the cell lysates. The error bars were calculated from three independent experiments.

PR63_{5UTR-5B}/JFH-1 were 5×10^3 FFU/ml at day 12 posttransfection.

We then replaced the 3'UTR of the chimeric virus with the PR63 sequence. We were able to obtain one clone of the 3'UTR substituted chimeric virus, PR63_{5UTR-5B&3UTR}/JFH-1_{NS5B}, which had peak infectivity titers of 3×10^3 FFU/ml at day 65 posttransfection. Adaptive mutations were detected in recovered viruses, including P1100L (NS3), Y1388H (NS3), and N2584T (NS5B), and four changes in the variable region (A9449G, A9456G, A9460G, and T9481C) (Fig. 2). After engineering all of these mutations into PR63_{5UTR-5B&3UTR}/JFH-1_{NS5B}, the virus production was greatly enhanced with peak infectivity titers of 1.5×10^4 FFU/ml at day 5 posttransfection.

The NS5B C terminus (amino acid residues 557 to 591) was the only JFH-1's sequence on adapted PR63_{5UTR-5B&3UTR}/JFH-1_{NS5B}. We replaced this region with a clone of PR63 sequence to make the full-length genome of PR63, denoted PR63_{FL} (Fig. 2). The infectivity of PR63_{FL} could be detected as early as day 6 posttransfection and reached peak titers of 4.05×10^3 FFU/ml at day 52 posttransfection (Fig. 3A).

Adaptive mutations enhance the infectivity of PR63_{FL} virus. Next, we set out to improve the infectivity of PR63_{FL} by serial passages. The peak infectivity titers of PR63_{FL} increased to 5.1×10^3 , 2.2×10^4 , and 6×10^4 FFU/ml in the first, second, and third passages in Huh7.5.1 cells. Recovered viruses from the first passage had N1931S (NS4B) mutation, whereas viruses from the third passage had codon changes of N1931S, D398G (E2), N415D (E2), H2100R (NS5A), and T2941V (NS5B) (Fig. 2). We generated PR63_{FL}-N1931S, as well as PR63_{FL}-D398G-N415D-N1931S-H2100R-T2941V (or PR63_{FL}-GDSRV). The former had peak titers of 3.9×10^3 FFU/ml at day 18 posttransfection, while the latter spread at a much faster pace and reached infectivity titers of 1.6×10^5 FFU/ml at day 7 posttransfection (Fig. 3A). This efficient PR63 recombinant (PR63_{FL}-GDSRV) was designated PR63cc (for PR63 cell-culture derived). The sequence of PR63cc was submitted to GenBank

under accession number [KF676352](#). Phylogenetic analysis showed that PR63cc is mostly related to PR63 consensus among the sequences compared ([Fig. 1](#)).

To determine whether the adaptive mutations promote the replication fitness of PR63_{FL}, we constructed PR63-derived subgenomic replicon, which is a bicistronic RNA containing firefly luciferase expressed via the HCV's internal ribosome entry site (IRES) and HCV's NS3-3'UTR controlled by the EMCV's IRES (SGRLuc) ([26](#)). The mutation N1931S alone, or in combination with H2100R and T2941V, was introduced into the subgenomic replicon expressing NS3-3'UTR of PR63_{FL}. The mutations D398G and N415D were not studied in the subgenomic replicon experiment because they are located in the viral structural proteins of PR63. In addition, the PR63-N1931S subgenomic replicon containing a point mutation inactivating RNA-dependent RNA polymerase activity (GND) was included as a negative control. The *in vitro*-synthesized RNAs of these subgenomic replicons were electroporated into Huh7.5.1 cells, and replication was assessed by the luciferase activity at various time points after transfection ([Fig. 3B](#)). The JFH-1-SGRLuc replicated most efficiently, while the luciferase activity of replication-incompetent negative control PR63-SGRLuc-N1931S-GND gradually decreased after transfection. Among the PR63-derived subgenomic replicons, PR63_{FL-3M} (N1931S/H2100R/T2941V) displayed the greatest fitness, followed by PR63_{FL-N1931S} and PR63_{FL} in descending order of fitness. Thus, these newly emerged mutations H2100R and T2941V boosted the replication to a higher degree than N1931S.

Comparison between PR63cc sequence and the PR63 consensus sequence. Next, we analyzed the difference between PR63cc sequence and the PR63 consensus sequence. A total of 38 amino acids were identified in PR63cc that differ from the consensus sequence ([Table 2](#)). Among them, two variations (2374G and 2375P) resulted from the engineering of RsrII restriction site in the cloning process. Twenty-four changes were present in the selected subclones out of the pools constructed directly from the clinical isolate. The remaining 12 changes were adaptive mutations introduced during the passaging of virus-infected or viral RNA-transfected cells. The sequence comparison with JFH-1 or the recently established J6cc showed that eight adaptive mutations (N415D, W849G, Q862P, Y1388H, H2100R, C2432R, N2584T, and S2955F) were not seen in either JFH-1 or J6cc, suggesting they are specifically adapted for PR63cc ([Table 2](#)). One adaptive mutation, A2941V, is found in JFH-1. Interestingly, three adaptive mutations (P1100L, A1676S, and N1931S) were among the six critical adaptive changes that enhanced J6cc infectivity, implying that these adaptive mutations may be important for the *in vitro* infectivity of HCVcc.

Characterization of the PR63cc viruses. The growth kinetics of PR63cc were compared side by side with those of JFH-1 in Huh7.5.1 and Huh7 cells ([Fig. 4A and B](#)). Naive cells were infected with the viruses at an MOI of ~0.01. The culture supernatants at the various days postinfection were collected for measuring the infectivity titers. In Huh7.5.1 cells, the peak infectivity of JFH-1 appeared on day 5 postinfection, and the titers were 6×10^4 FFU/ml, whereas PR63cc had the highest infectivity titers of 3.4×10^4 FFU/ml at day 11 postinfection ([Fig. 4A](#)). In Huh7 cells, the peak infectivity of JFH-1 appeared on day 6 postinfection, and the titers were 4.5×10^3 FFU/ml, whereas PR63cc had peak infectivity titers of 8×10^3 FFU/ml at day 14 postinfection ([Fig. 4B](#)). Generally, the peak infectivity of JFH-1 and PR63cc is comparable, while PR63cc

TABLE 2 Analysis of different amino acid residues between the PR63 consensus sequence and the PR63cc genome

Position (aa) ^a	Gene	PR63 consensus	PR63cc	Origin ^b	Corresponding residue ^c		
					J6cc	JFH-1	H77
249	E1	Q	R	Subclone	Q	R	249R
250	E1	R	Q	Subclone	R	Q	250D
337	E1	A	V	Subclone	A	V	337L
384	E2	S	G	Subclone	R	G	384E
390	E2	G	A	Subclone	G	G	390G
397	E2	S	R	Subclone	G	N	397A
401	E2	N	G	Subclone	S	G	401G
402	E2	L	F	Subclone	L	V	402L
404	E2	S	D	Subclone	D	S	404T
405	E2	A	V	Subclone	M	H	405P
415	E2	N	D	Adaptation	N	N	415N
416	E2	S	T	Subclone	T	T	416T
417	E2	S	K	Subclone	N	N	417N
439	E2	T	A	Subclone	A	A	439A
453	E2	P	V	Subclone	P	P	453P
849	NS2	W	G	Adaptation	W	W	845W
862	NS2	Q	P	Adaptation	Q	Q	858H
925	NS2	M	V	Subclone	M	V	921I
1100	NS3	P	L	Adaptation	L	P	1096P
1388	NS3	Y	H	Adaptation	Y	C	1384V
1634	NS3	S	P	Subclone	S	P	1630A
1638	NS3	E	G	Subclone	E	E	1634E
1676	NS4A	A	S	Adaptation	S	A	1672A
1931	NS4B	N	S	Adaptation	S/T	N	1927N
2083	NS5A	K	R	Subclone	K	K	2079K
2100	NS5A	H	R	Adaptation	H	H	2096V
2329	NS5A	R	M	Subclone	R	R	2329K
2374	NS5A	S	G	Cloning	S	G	2374S
2375	NS5A	R	P	Cloning	Q	P	2375G
2392	NS5A	M	I	Subclone	M	M	2388M
2432	NS5A	C	R	Adaptation	C	C	2408V
2439	NS5A	V	L	Subclone	V	T	2417V
2440	NS5A	V	E	Subclone	V	V	2418V
2513	NS5B	I	V	Subclone	I	I	2491V
2584	NS5B	N	T	Adaptation	N	N	2562N
2608	NS5B	G	S	Subclone	G	G	2586G
2941	NS5B	A	V	Adaptation	A	V	2919A
2955	NS5B	S	F	Adaptation	S	S	2933S

^a The positions of the amino acid residue are given according to the JFH-1 genome ([AB047639](#)).
^b The origins of the residues were categorized as follows: subclone (from the clone we selected), adaptation (from adaptive mutations), or cloning strategy (introduced during cloning process).
^c The corresponding residues in the J6cc genome ([JQ745650](#)) or JFH-1 genome are listed for comparison. The positions and the corresponding residues in the H77 genome ([AF009606](#)) are provided as a reference.

has slower spread kinetics in both Huh7 and Huh7.5.1 cells than JFH-1.

Next, we determined the sensitivity of PR63cc infection to a previously described α -E2 neutralizing human monoclonal antibody AR3A ([25](#)). Serially diluted antibodies were incubated with PR63cc and JFH-1cc for 1 h before the inoculation to Huh7.5.1 cells. As shown in [Fig. 4C](#), PR63cc was more sensitive to the blockade of this neutralizing antibody. For JFH-1, an ~75% inhibition was observed at 1:500 dilution of E2 antibody, whereas for PR63cc, an ~95% inhibition was achieved at the same dosage.

Finally, we tested the sensitivities of PR63cc to antiviral reagents, including NS5A inhibitor (daclatasvir [[BMS-790052](#)]) ([29](#)), NS5B inhibitor (2'CMA) ([30](#)), and IFN- α . JFH-1cc- or PR63cc-infected Huh7.5.1 cells were treated with different doses of antivirals, and the HCV RNA levels in the cells were compared by RT-qPCR as the percentage of mock treatment ([Fig. 4D](#)). Both viruses responded similarly to IFN- α and 2'CMA, since the median effective concentration (EC₅₀) values were comparable between JFH-1 and PR63cc ([Fig. 4E](#)). Interestingly, the PR63cc was much more resistant to the NS5A inhibitor daclatasvir (BMS-

FIG 4 Characterization of PR63cc. (A and B) Infection kinetics of JFH-1 and PR63cc in Huh7.5.1 cells (A) and Huh7 cells (B). The cells were inoculated with viruses at an MOI of ~0.01. The infectivity was measured as described in the text. A dashed line (10 FFU/ml) indicates the detection limit of the titration assay. (C) Blockade of HCV infection by α -E2 antibody. A total of ~250 FFU of virus were incubated with serial 5-fold dilutions (1:20 to 1:2,500) of E2 monoclonal antibody for 1 h and then used to infect Huh7.5.1 cells for 3 days. The intracellular HCV RNA was quantified by RT-qPCR and is expressed as the percentage of the culture medium control. The error bars indicate the standard deviation from the mean. (D) Efficacy of NS5A, NS5B inhibitors, and IFN- α against JFH-1 and PR63cc. Antivirals were administered 24 h postinfection at the indicated doses and maintained in cell culture medium for 48 h. The NS5A inhibitor is daclatasvir (BMS-790052), and the NS5B inhibitor is 2'-C-methyl-adenosine (2'CMA). Values are means of duplicate determinations with the standard errors of the mean. (E) EC₅₀ of the antivirals against the indicated viruses. (F) Efficacy of daclatasvir against JFH-1, PR63cc, and PR63cc-NS5A-M31L. Values are means of duplicate determinations with the standard errors of the mean.

790052). The EC₅₀ of daclatasvir against PR63cc was 2,032 pM, which is 80-fold higher than its EC₅₀ against JFH-1cc (Fig. 4E).

Daclatasvir targets the first 100 amino acid residues of NS5A (29). The daclatasvir resistance profile of GT1a and GT1b replicons revealed the following changes in NS5A protein were related to resistance: L31V and Y93H in the GT1a replicon and M28T, Q30H, Q30R, L31M, L31V, and Y93C in the GT1b replicon (29). Interestingly, GT2a J6 was also more resistant to daclatasvir than JFH-1 (18, 20). PR63cc and J6cc genomes have methionine at residue 31 in NS5A, whereas JFH-1 has a leucine. Residues 28, 30, and 93 are the same for all three genomes. Therefore, the methionine at residue 31 of NS5A likely accounts for J6's and PR63cc's low responsiveness to daclatasvir. To test this hypothesis, we mutated the methionine to leucine on the PR63cc genome and obtained PR63cc-NS5A-M31L. The virus production of PR63cc-NS5A-M31L was not affected (data not shown). However, the virus became as sensitive to daclatasvir as JFH-1 (Fig. 4F), suggesting that the methionine at residue 31 of NS5A is responsible for the resistance of PR63cc to daclatasvir.

DISCUSSION

In vitro HCV culture systems greatly promote basic HCV research, preclinical drug testing, and vaccine development. The extensive

heterogeneity of HCV genotypes leading to varied response to antiviral drugs argues for an urgent need to develop more full-length culture systems. Although people have cloned a few full-length HCV genomes and confirmed their infectivity in chimpanzee models (31–36), none of them is infectious in cell culture except for JFH-1, which is the first HCV clone to support efficient virus production in Huh7 hepatoma cells. With the help of JFH-1, more full-length or nearly full-length HCV culture systems were established (MA, J6, JFH-2, J8, TN, DH8, and DH10) (15, 19–22). All of these studies require the prior establishment of a full-length cDNA clone based on the consensus sequence, the accuracy of which is heavily dependent upon the sequencing methods and the complexity of the HCV quasispecies. In the present study, we developed a new method to construct HCVcc cDNA clone directly from clinical isolates without making a consensus genome first. By sequentially replacing fragments of JFH-1 with a sequence of PR63 via functional screening, we were able to construct a new GT2a infectious clone PR63cc. PR63cc virus can propagate efficiently in Huh7.5.1 cells, with peak titers of 1.6×10^5 FFU/ml, similar to that of JFH-1.

Methodologically, our strategy has several advantages. First, there is no need to construct a full-length consensus cDNA clone, which is not easy for the 9.6-kb HCV genome. Second, the func-

tional screening can be performed without the detailed sequencing analysis of clinical isolates, as long as a large enough pool for each swapping fragment can be established. Moreover, our method can be readily applied to other clinical isolates since it does not rely on sequence homology but just on functional selection. Of course, the development of cell culture systems for other isolates, especially isolates of other genotypes, can be very difficult due to potential intergenotypic incompatibility. However, the availability of non-GT2a HCVcc clones such as H77S (37), TN, or J8 may facilitate the construction of non-GT2a HCV cell culture models by serving as the driver strains in the construction process.

We identified 38 amino acid variations between PR63cc and the PR63 consensus sequences. The majority of these variations (24 of 38) were present in the subclones selected from the pools of sequences constructed from the PR63 quasiespecies. Among them, 17 (out of 24) variations can be detected in the original PR63 serum by cloning sequencing, which are also confirmed by doublet peaks in sequencing chromatograms of bulking sequencing analysis, suggesting the sequences of subclones do exist in the PR63 serum quasiespecies, although in minority. The absence of the subclone sequences at the remaining seven positions in the sequencing analyses could be due to the very low frequencies of these sequences in the patients, although we cannot rule out the possibility that these changes may result from the PCR errors. These analyses suggest that the minor species within HCV quasiespecies can support virus production in cell culture after adaptation, highlighting the importance of functional selection in constructing an HCVcc clone. Twelve adaptive mutations were also identified in PR63cc; three of these mutations (P1100L, A1676S, and N1931S) probably help the adaptation of J6c virus (20). Whether the combination of P1100L, A1676S, and N1931S can help HCVcc adaptation in a more universal way requires further study.

Daclatasvir (BMS-790052) is a small molecule inhibitor of HCV NS5A and exhibits picomolar EC_{50} toward JFH-1 infectious viruses in cell culture (29). Our finding showed that the EC_{50} of daclatasvir to PR63cc is 2,032 pM, 80-fold higher than that of JFH-1. Specific residues in domain I of NS5A were associated with variations in sensitivity to daclatasvir between isolates of the same subtypes (18). This observation is in line with our result. The resistance of PR63cc to daclatasvir is due to the methionine residue at residue 31 of the NS5A protein, since the PR63cc-NS5A-M31L has a responsiveness to daclatasvir similar to that of JFH-1. Our results not only highlight the importance of constructing more HCVcc in antiviral development but also advance our understanding of how NS5A interacts with daclatasvir and may facilitate the development of other NS5A inhibitors.

In conclusion, we have developed a method to construct a highly efficient full-length HCV culture system through functional selection and adaptation, and this method may be applicable to the establishment of new infectious HCV clones with different genotypes.

ACKNOWLEDGMENTS

This study was supported by grants from the Chinese National 973 Program (2009CB522501), by the Chinese National Key Programs on Infectious Disease (2012ZX10002007-003; 2008ZX10002-014), by the National Natural Science Foundation of China (31170149 to J.Z.), and by the CAS/SAFEA International Partnership Program for Creative Research Teams.

We thank Yiping Li (Copenhagen University Hospital, Denmark) for

helpful discussion and valuable technical advice and Qiang Ding (Institut Pasteur of Shanghai) for manuscript revision.

The authors have no competing interests.

REFERENCES

- Bartenschlager R, Frese M, Pietschmann T. 2004. Novel insights into hepatitis C virus replication and persistence. *Adv. Virus Res.* 63:71–180. [http://dx.doi.org/10.1016/S0065-3527\(04\)63002-8](http://dx.doi.org/10.1016/S0065-3527(04)63002-8).
- Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin IT, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A. 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 42:962–973. <http://dx.doi.org/10.1002/hep.20819>.
- Feld JJ, Hoofnagle JH. 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* 436:967–972. <http://dx.doi.org/10.1038/nature04082>.
- Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T. 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 125:1808–1817. <http://dx.doi.org/10.1053/j.gastro.2003.09.023>.
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. 2005. Complete replication of hepatitis C virus in cell culture. *Science* 309:623–626. <http://dx.doi.org/10.1126/science.1114016>.
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11:791–796. <http://dx.doi.org/10.1038/nm1268>.
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 102:9294–9299. <http://dx.doi.org/10.1073/pnas.0503596102>.
- Gottwein JM, Scheel TK, Hoegh AM, Lademann JB, Eugen-Olsen J, Lisby G, Bukh J. 2007. Robust hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses. *Gastroenterology* 133:1614–1626. <http://dx.doi.org/10.1053/j.gastro.2007.08.005>.
- Gottwein JM, Scheel TK, Jensen TB, Ghanem L, Bukh J. 2011. Differential efficacy of protease inhibitors against HCV genotypes 2a, 3a, 5a, and 6a NS3/4A protease recombinant viruses. *Gastroenterology* 141:1067–1079. <http://dx.doi.org/10.1053/j.gastro.2011.06.004>.
- Gottwein JM, Scheel TK, Jensen TB, Lademann JB, Prentoe JC, Knudsen ML, Hoegh AM, Bukh J. 2009. Development and characterization of hepatitis C virus genotype 1–7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* 49:364–377. <http://dx.doi.org/10.1002/hep.22673>.
- Imhof I, Simmonds P. 2010. Development of an intergenotypic hepatitis C virus (HCV) cell culture method to assess antiviral susceptibilities and resistance development of HCV NS3 protease genes from HCV genotypes 1 to 6. *J. Virol.* 84:4597–4610. <http://dx.doi.org/10.1128/JVI.02698-09>.
- Jensen TB, Gottwein JM, Scheel TK, Hoegh AM, Eugen-Olsen J, Bukh J. 2008. Highly efficient JFH1-based cell-culture system for hepatitis C virus genotype 5a: failure of homologous neutralizing-antibody treatment to control infection. *J. Infect. Dis.* 198:1756–1765. <http://dx.doi.org/10.1086/593021>.
- Li YP, Gottwein JM, Scheel TK, Jensen TB, Bukh J. 2011. MicroRNA-122 antagonism against hepatitis C virus genotypes 1–6 and reduced efficacy by host RNA insertion or mutations in the HCV 5'UTR. *Proc. Natl. Acad. Sci. U. S. A.* 108:4991–4996. <http://dx.doi.org/10.1073/pnas.1016606108>.
- Li YP, Ramirez S, Gottwein JM, Bukh J. 2011. Non-genotype-specific role of the hepatitis C virus 5' untranslated region in virus production and in inhibition by interferon. *Virology* 421:222–234. <http://dx.doi.org/10.1016/j.virol.2011.10.002>.
- Murayama A, Kato T, Akazawa D, Sugiyama N, Date T, Masaki T, Nakamoto S, Tanaka Y, Mizokami M, Yokosuka O, Nomoto A, Wakita T. 2012. Production of infectious chimeric hepatitis C virus genotype 2b harboring minimal regions of JFH-1. *J. Virol.* 86:2143–2152. <http://dx.doi.org/10.1128/JVI.05386-11>.
- Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R.

2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc. Natl. Acad. Sci. U. S. A.* 103:7408–7413. <http://dx.doi.org/10.1073/pnas.0504877103>.
17. Scheel TK, Gottwein JM, Jensen TB, Prentoe JC, Hoegh AM, Alter HJ, Eugen-Olsen J, Bukh J. 2008. Development of JFH1-based cell culture systems for hepatitis C virus genotype 4a and evidence for cross-genotype neutralization. *Proc. Natl. Acad. Sci. U. S. A.* 105:997–1002. <http://dx.doi.org/10.1073/pnas.0711044105>.
18. Scheel TK, Gottwein JM, Mikkelsen LS, Jensen TB, Bukh J. 2011. Recombinant HCV variants with NS5A from genotypes 1–7 have different sensitivities to an NS5A inhibitor but not interferon- α . *Gastroenterology* 140:1032–1042. <http://dx.doi.org/10.1053/j.gastro.2010.11.036>.
19. Date T, Kato T, Kato J, Takahashi H, Morikawa K, Akazawa D, Murayama A, Tanaka-Kaneko K, Sata T, Tanaka Y, Mizokami M, Wakita T. 2012. Novel cell culture-adapted genotype 2a hepatitis C virus infectious clone. *J. Virol.* 86:10805–10820. <http://dx.doi.org/10.1128/JVI.07235-11>.
20. Li YP, Ramirez S, Gottwein JM, Scheel TK, Mikkelsen L, Purcell RH, Bukh J. 2012. Robust full-length hepatitis C virus genotype 2a and 2b infectious cultures using mutations identified by a systematic approach applicable to patient strains. *Proc. Natl. Acad. Sci. U. S. A.* 109:E1101–E1110. <http://dx.doi.org/10.1073/pnas.1203829109>.
21. Li YP, Ramirez S, Jensen SB, Purcell RH, Gottwein JM, Bukh J. 2012. Highly efficient full-length hepatitis C virus genotype 1 (strain TN) infectious culture system. *Proc. Natl. Acad. Sci. U. S. A.* 109:19757–19762. <http://dx.doi.org/10.1073/pnas.1218260109>.
22. Ramirez S, Li YP, Jensen SB, Pedersen J, Gottwein JM, Bukh J. 2013. Highly efficient infectious cell culture of three HCV genotype 2b strains and sensitivity to lead protease, NS5A, and polymerase inhibitors. *Hepatology*. <http://dx.doi.org/10.1002/hep.26660>.
23. Li H, Stoddard MB, Wang S, Blair LM, Giorgi EE, Parrish EH, Learn GH, Hraber P, Goepfert PA, Saag MS, Denny TN, Haynes BF, Hahn BH, Ribeiro RM, Perelson AS, Korber BT, Bhattacharya T, Shaw GM. 2012. Elucidation of hepatitis C virus transmission and early diversification by single genome sequencing. *PLoS Pathog.* 8:e1002880. <http://dx.doi.org/10.1371/journal.ppat.1002880>.
24. Lu J, Tao W, Li R, Xiang Y, Zhang N, Xiang X, Xie Q, Zhong J. 2013. Construction and characterization of infectious hepatitis C virus chimera containing structural proteins directly from genotype 1b clinical isolates. *Virology* 443:80–88. <http://dx.doi.org/10.1016/j.virol.2013.04.030>.
25. Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastaminza P, Chisari FV, Jones IM, Fox RI, Ball JK, McKeating JA, Kneteman NM, Burton DR. 2008. Broadly neutralizing antibodies protect against hepatitis C virus quasiespecies challenge. *Nat. Med.* 14:25–27. <http://dx.doi.org/10.1038/nm1698>.
26. Targett-Adams P, McLauchlan J. 2005. Development and characterization of a transient-replication assay for the genotype 2a hepatitis C virus subgenomic replicon. *J. Gen. Virol.* 86:3075–3080. <http://dx.doi.org/10.1099/vir.0.81334-0>.
27. Kaul A, Woerz I, Meuleman P, Leroux-Roels G, Bartenschlager R. 2007. Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. *J. Virol.* 81:13168–13179. <http://dx.doi.org/10.1128/JVI.01362-07>.
28. Zhong J, Gastaminza P, Chung J, Stamataki Z, Isogawa M, Cheng G, McKeating JA, Chisari FV. 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. *J. Virol.* 80:11082–11093. <http://dx.doi.org/10.1128/JVI.01307-06>.
29. Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, Serrano-Wu MH, Langley DR, Sun JH, O'Boyle DR, 2nd, Lemm JA, Wang C, Knipe JO, Chien C, Colonno RJ, Grasela DM, Meanwell NA, Hamann LG. 2010. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* 465:96–100. <http://dx.doi.org/10.1038/nature08960>.
30. Wong KA, Xu S, Martin R, Miller MD, Mo H. 2012. Tegobuvir (GS-9190) potency against HCV chimeric replicons derived from consensus NS5B sequences from genotypes 2b, 3a, 4a, 5a, and 6a. *Virology* 429:57–62. <http://dx.doi.org/10.1016/j.virol.2012.03.025>.
31. Gottwein JM, Scheel TK, Callendret B, Li YP, Eccleston HB, Engle RE, Govindarajan S, Satterfield W, Purcell RH, Walker CM, Bukh J. 2010. Novel infectious cDNA clones of hepatitis C virus genotype 3a (strain S52) and 4a (strain ED43): genetic analyses and in vivo pathogenesis studies. *J. Virol.* 84:5277–5293. <http://dx.doi.org/10.1128/JVI.02667-09>.
32. Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM. 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 277:570–574. <http://dx.doi.org/10.1126/science.277.5325.570>.
33. Yanagi M, Purcell RH, Emerson SU, Bukh J. 1997. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc. Natl. Acad. Sci. U. S. A.* 94:8738–8743. <http://dx.doi.org/10.1073/pnas.94.16.8738>.
34. Yanagi M, Purcell RH, Emerson SU, Bukh J. 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. <http://dx.doi.org/10.1006/viro.1999.9889>.
35. Yanagi M, St Claire M, Shapiro M, Emerson SU, Purcell RH, Bukh J. 1998. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. *Virology* 244:161–172. <http://dx.doi.org/10.1006/viro.1998.9092>.
36. Sakai A, Takikawa S, Thimme R, Meunier JC, Spangenberg HC, Govindarajan S, Farci P, Emerson SU, Chisari FV, Purcell RH, Bukh J. 2007. In vivo study of the HC-TN strain of hepatitis C virus recovered from a patient with fulminant hepatitis: RNA transcripts of a molecular clone (pHC-TN) are infectious in chimpanzees but not in Huh7.5 cells. *J. Virol.* 81:7208–7219. <http://dx.doi.org/10.1128/JVI.01774-06>.
37. Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc. Natl. Acad. Sci. U. S. A.* 103:2310–2315. <http://dx.doi.org/10.1073/pnas.0510727103>.