A novel virus culture system for hepatitis C virus

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Hepatitis C virus (HCV) is a principal cause of post-transfusion and sporadic acute hepatitis. HCV infection persists and causes chronic liver diseases, including cirrhosis and hepatocellular carcinoma. Despite HCV's importance, understanding of its life cycle has been hampered by the lack of an appropriate *in vitro* viral culture system. We isolated full-length HCV cDNA of the JFH-1 strain from a fulminant hepatitis patient, and constructed a JFH-1 subgenomic replicon that replicated efficiently in cultured cells without adaptive mutations. Full-length RNA transcripts were transfected into Huh7 cells, resulting in efficient replication of JFH-1 RNA and secretion of recombinant viral particles into the culture medium. The secreted viral particles were infectious for cultured cells and in a chimpanzee. The infectivity of these viral particles was greater for permissive cell lines than for original Huh7 cell lines. This infectious HCV system is a powerful tool for studying the HCV life cycle and for developing antiviral strategies and effective vaccines.

Hepatitis C virus (HCV) is recognized worldwide as a serious medical and public health problem, with an estimated 170 million people currently infected with the virus [1]. HCV is a principal cause of post-transfusion and sporadic acute hepatitis. After acute infection, it persists and causes chronic liver diseases, including cirrhosis and hepatocellular carcinoma, because most patients fail to clear the virus [2]. Despite HCV's importance, understanding of its life cycle has been hampered by the lack of an appropriate *in vitro* model for evaluating HCV replication. A subgenomic HCV RNA replicon system has recently been established, allowing the study of HCV replication in cultured cells [3]. Although this system is a powerful tool for the study of HCV replication mechanisms and the search for potential antiviral agents, it does not support virus particle formation. Researchers, including ourselves, have recently reported that transfection of full-length JFH-1 or chimeric J6/JFH-1 genomes into Huh7 cells results in secretion of an infectious virus (Figure 1) [4–6].

In vitro model of HCV infection

There have been many attempts to establish an *in vitro* model that mimics HCV replication and infection. There have been reports of some success using established cell lines or primary hepatocytes from humans or other susceptible animals [7–15]. Some of those researchers observed not only an increase in levels of plus-strand RNA during cultivation and the presence of minus-strand RNA (which is only produced during productive replication) but also the transmission of HCV to naïve cells [14,15]. However, those experiments suffered from poor reproducibility, and the limited susceptibility of the cells necessitated the use of a highly sensitive reverse transcription polymerase chain reaction (RT-PCR) to detect HCV replication.

HCV replicon system

Against this background, the selectable subgenomic HCV replicon system was introduced as a robust and reliable cell culture system for HCV by Bartenschlager and colleagues (University of Heidelberg, Heidelberg, Germany) [3]. A bicistronic construct was assembled using the HCV-internal ribosome entry site (IRES) to direct expression of the neomycin resistance gene, and using the encephatomyocarditis virus-IRES to direct expression of the HCV nonstructural (NS) proteins NS3, NS4A, NS4B, NS5A and NS5B. By transfecting the *in vitro*-transcribed replicon RNA into the human hepatoma cell line, Huh7, and selecting cells using neomycin sulfate (G418), researchers have achieved continuous high-level replication in transfected cells (Figure 1). Only the replicon-replicating cells (which produce neomycin phosphotransferase) can survive G418 selection and form visible colonies. This system is a novel, powerful tool for the study of HCV replication mechanisms and the search for potential antiviral agents. However, only genotype 1 replicons have been replicated using this system, and those replicons only replicated efficiently in Huh7, human hepatocyte-derived cell lines [3,16,17]. As described below, we have

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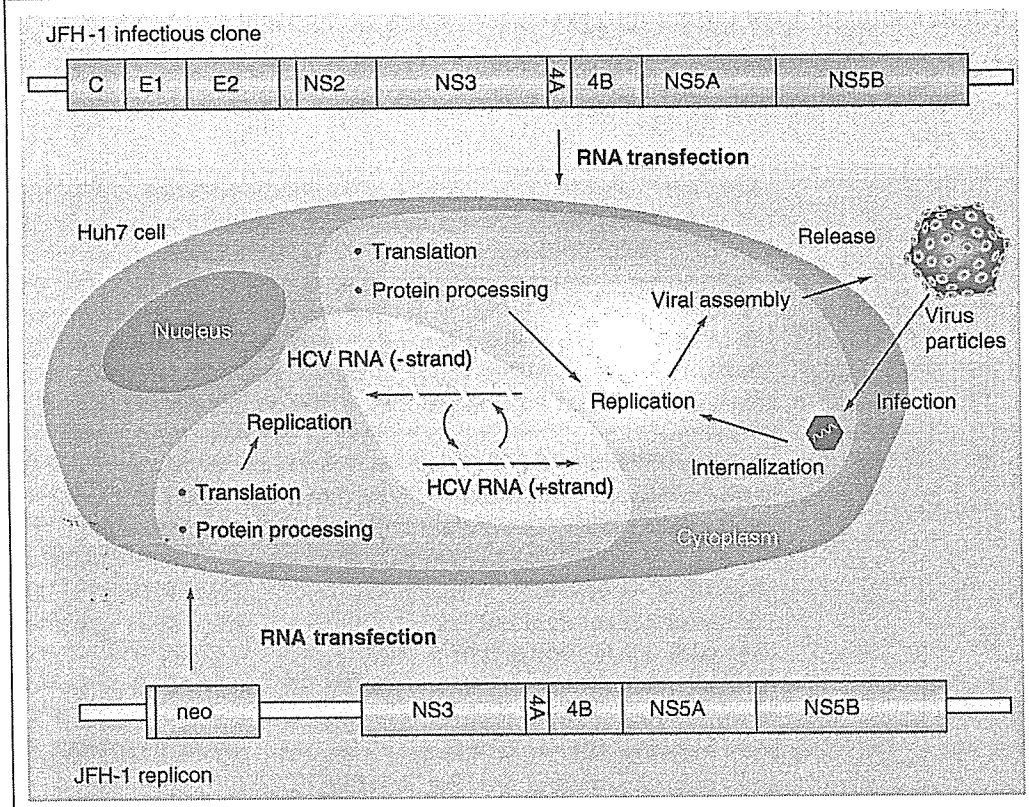
future medicine

Figure 1. Experimental system for virus replication and infection.



Virus particles are secreted by Huh7 cells transfected with full-length JFH-1 RNA. Secreted viruses are infectious for Huh7 cells. This system supports the entire HCV life cycle, including viral genome replication, virus particle formation, release and infection. The subgenomic replicon system supports analysis of viral RNA replication.
HCV: Hepatitis C virus; NS: Non-structural.

developed a different HCV replicon system using a newly isolated HCV genotype 2a strain, designated JFH-1 [18].

Isolation of JFH-1 strain

The HCV genotype 2a strain, JFH-1, was isolated from a case of fulminant hepatitis [19]. The patient was a 32-year-old man who was admitted with general fatigue, high-grade fever and liver dysfunction. There was no evidence of prior liver disease, and the patient had no history of alcohol consumption or illegal drug use. He had not received any blood transfusions or intravenous drugs, had not undergone acupuncture, or had sexual contact with a known hepatitis virus carrier in the previous 6 months. The patient had high levels of serum aspartate aminotransferase and alanine aminotransferase, a low minimum prothrombin time and stage II encephalopathy. HCV RNA was detected by RT-PCR, and the patient tested negative for anti-HCV antibodies. All other hepatitis virus markers were negative. The patient was diagnosed with HCV-associated fulminant hepatitis. The source of the HCV infection was unclear. The patient had a high level of viremia (10^5 copies/ml) at admission.

HCV was isolated from the acute-phase serum, and the entire genome was sequenced. The genome of this HCV strain (JFH-1) was analyzed for sequence homology with other HCV isolates (representative data shown in Table 1). JFH-1 belongs to genotype 2a, and its sequence shares more than 90% amino acid homology with other genotype 2a strains and approximately 70% homology with genotype 1 strains. In the phylogenetic tree, JFH-1 is located within genotype 2a, but it deviates slightly from other genotype 2a strains [19]. To determine the degree of deviation in each subgenomic region, nucleotide sequences and presumed amino acid sequences of JFH-1 subgenomic regions were compared with those of other genotype 2a strains. By calculating the ratio of genetic distances between JFH-1 and other 2a strains in each subgenomic region, it was determined that there was substantial deviation in three regions: core, NS3 and NS5A.

Table 1. Homology of nucleotide and amino acid sequences between JFH-1 and representative strains.

Clones*	HC-J6	JCH-1	JCH-2	JCH-3	JCH-4	JCH-5	JCH-6	HCV-J	H77
Nucleotide homology (%)	89.7	90.0	89.4	89.3	88.2	89.6	90.2	68.2	67.5
Amino acid homology (%)	91.9	91.8	91.7	90.7	91.0	91.4	91.7	71.0	70.7

**Accession numbers: JFH-1: AB047639; HC-J6: D00944; JCH-1–JCH-6: AB047640–AB047645; HCV-J: D90208; H77: AF009606.*

Replicon system with JFH-1

To investigate the replication capacity of JFH-1, we constructed a subgenomic JFH-1 replicon [18]. To our surprise, colony formation of JFH-1 was approximately 500-fold more efficient than that of the prototype Con-1 replicon, and 50-fold more efficient than that of a Con-1 replicon containing highly adaptive mutations. Also, this JFH-1 replicon replicated not only in Huh7 cells but also in the hepatocyte-derived cell lines, HepG2 and IMY-N9, and the non-hepatocyte-derived cell lines HeLa and 293 [20,21]. This difference may be due to the replication capacity of JFH-1. An important observation is that the JFH-1 replicon did not require an adaptive mutation to replicate in those cell lines [18,20,21]. Some clones isolated from each cell line had either no mutations or a few synonymous mutations in HCV-derived replicon regions. Full-length HCV RNA containing multiple cell-culture adaptive mutations did not produce active HCV infection [22]. Thus, it appears that high replication capacity, without the need for adaptive mutations, is an important factor in the development of an HCV infection system.

To develop a sensitive, accurate assay system for the anti-HCV effects of interferon (IFN) and ribavirin, we constructed a modified JFH-1 replicon in which the neomycin-resistance gene was replaced with a firefly luciferase gene [23]. The relative luciferase activity of this JFH-1 reporter replicon increased exponentially over the course of the experiment. However, with the replication-deficient mutant replicon (in which RNA-dependent RNA polymerase [RdRp] enzyme activity was abolished by introducing a point mutation at the Gly-Asp-Asp [GDD] motif of the RdRp gene), the relative luciferase activity did not increase over time. Using this system, we easily and accurately assessed the anti-HCV effects of IFN and ribavirin. This system is a powerful tool for screening new or candidate anti-HCV compounds. Also, this JFH-1 reporter replicon replicated efficiently in transiently transfected Huh7 cells without G418 selection.

Establishment of full-length JFH-1 cDNA

Results of studies conducted using subgenomic replicons indicate that the JFH-1 strain replicates very efficiently in Huh7 cells [18,20,21], without the need for adaptive mutations. To take advantage of this replication efficiency, we constructed full-length JFH-1 cDNA for use in assaying replication of viral RNA in transfected Huh7 cells. In chronically infected patients, circulating HCV viral genomes usually possess a wide variety of sequence deviations, dividing the genome into so-called 'quasi-species' [24]. However, monoclonality is a specific characteristic of circulating HCV in fulminant hepatitis patients [25]; the JFH-1 strain isolated from fulminant hepatitis patients was indeed monoclonal [19]. This monoclonality appears to be due to vigorous viral replication in the infected liver, and is useful for the construction of consensus full-length cDNA. JFH-1 cDNA has been cloned from RT-PCR fragments. The amino acid sequences of JFH-1 are highly conserved, and full-length cDNA encoding the JFH-1 consensus sequence is assembled easily by combining cloned PCR fragments [4,19]. To produce our full-length JFH-1 RNA, we inserted the T7 promoter sequence immediately upstream of the full-length JFH-1 cDNA sequence, and then used T7 RNA polymerase to transcribe the RNA [4].

Secretion of viral particles by viral-RNA-replicating cultured cells

Reports indicate that transfection of Huh7 cells with full-length HCV RNA of Con1 and H77 strains results in the replication of the HCV RNA [16,26]. Despite the efficient HCV RNA replication and HCV protein expression in those studies, the researchers did not observe viral particle formation or secretion. Accumulating evidence suggests that loss of hyperphosphorylation induces high-level RNA replication, but loses viral particle formation [22]. When synthesized full-length JFH-1 RNA was transfected into

naive Huh7 cells, viral RNA replication and viral protein expression were observed in the transfected cells [4]. To determine whether viral particles were secreted from cells transfected with full-length JFH-1 RNA, we first assayed for HCV core protein in a culture medium of those cells using a highly sensitive immunoassay [27]. The cells transfected with full-length JFH-1 RNA secreted HCV core protein. We also synthesized RNA from the pJFH-1/ΔE1-E2 mutant, which lacks most of the E1 and E2 regions encoding envelope proteins of HCV. Transfection with pJFH-1/ΔE1-E2 produced a level of viral RNA replication similar to that observed for full-length JFH-1 RNA transfection, but the amount of HCV core protein secreted into culture medium was much lower for pJFH-1/ΔE1-E2 than for full-length JFH-1 [4]. Next, culture supernatant of cells transfected with full-length JFH-1 RNA was analyzed by sucrose density gradient assay. Viral RNA and all structural proteins (core, E1 and E2) were detected in fractions with a density of approximately 1.15–1.17 g/ml [4], suggesting formation and secretion of complete viral particles. Viral particles secreted into the culture medium were visualized with immunoelectron microscopy by Bartenschlager and colleagues using an anti-E2 monoclonal antibody. Viral particles were spherical, with an outer diameter of approximately 55 nm [4]. These findings clearly indicate that HCV viral particles were formed in Huh7 cells transfected with full-length JFH-1 RNA, and were secreted into the culture medium.

Infectivity of recombinant viral particles

As described above, there have been many attempts to establish an *in vitro* HCV infection system, but most have not achieved productive HCV infection and replication in cultured cells. We tested the infectivity of viral particles secreted from cells transfected with full-length JFH-1 RNA. We first used double-chambered culture plates with a polyester membrane (pore size: 0.45 μm) separating the inner and outer chambers. The virus particles are smaller than the membrane's pores, and thus can diffuse across the membrane and populate both chambers. On day one, cells transfected with full-length JFH-1 RNA were placed in the inner chamber, and naïve Huh7 cells were seeded in the outer chamber. A number of days later, the cells in the outer chamber were assayed for infection with secreted virus particles, using anti-HCV antibodies; very few cells were positive for infection. We then collected the culture medium of RNA-transfected Huh7 cells, cleared it by low-speed centrifugation, and filtered it through a disk filter. Cleared virus in this medium was concentrated by ultrafiltration, and the medium was then used to inoculate new Huh7 cells seeded in a culture plate. These inoculated cells were then cultured for another 48 h in complete medium. After this culturing, the cells were fixed and immunostained using anti-HCV antibodies. The infection efficiency of the concentrated culture medium was only approximately 0.5% with standard Huh7 cells [4]. We also analyzed the kinetics of HCV RNA titer and core protein concentration in the inoculated cells. Only a small percentage of the total HCV RNA and core protein in the inoculate was adsorbed by inoculated cells. Compared with levels of HCV RNA and core protein observed in the infected cells 1 h after inoculation, the levels had decreased 12 h after inoculation, and had increased 24 h after inoculation. These data indicate that the viral particles were infectious for Huh7 cells, although at low efficiency [4].

To confirm the infectivity of the viral particles, we tested for neutralization of the infectivity. CD81 has been identified as a candidate HCV receptor molecule [28]. Anti-CD81 antibody decreased the infectivity of the culture supernatant for naive Huh7 cells by more than 90%. This is consistent with the previous finding that pseudotype virus infection was inhibited by anti-CD81 antibodies [29], and indicates that infectivity of the secreted viral particles depends on a CD81-specific pathway. The molecules that have been found to play roles in HCV infection include heparan sulfate proteoglycans, scavenger receptor class-B type-I, liver/lymph node-specific ICAM-3 grabbing nonintegrin (L-SIGN) and dendritic cell-SIGN (DC-SIGN) [30–35]. Further study is required to characterize the mechanisms of HCV infection in detail. Antibodies that inhibit HCV infection have been isolated from the sera of patients chronically infected with HCV [4]. Such antibodies could be very useful for the development of prophylaxis vaccines for HCV infection; however, it is not clear why both virus particles and neutralizing antibodies are present in patient sera.

Viral particles secreted by cultured cells were infectious for chimpanzees

We proceeded to examine whether virus particles secreted by cultured cells are infectious *in vivo* in collaboration with Liang and

colleagues (NIH, Bethesda, MD, USA). The experimental inoculum was a culture supernatant of Huh7 cells transfected with full-length JFH-1 RNA. The control inoculum was undiluted culture supernatant from untransfected Huh7 cells. A chimpanzee was first inoculated with the control inoculum, and no infection was observed. The chimpanzee was then inoculated with experimental inoculum diluted 10^4; again, no infection developed. Next, the chimpanzee was inoculated with experimental inoculum diluted 10^3, which induced viremia. Thus, the undiluted culture supernatant of transfected cells contains virus infectious for chimpanzees, at a titer of greater than 10^3 chimpanzee infectious units/ml. The induced viremia was transient, and the viral titer was low. Furthermore, the HCV infection was cleared with no evidence of abnormal liver histology, elevation of liver-specific enzymes or HCV-specific antibody seroconversion [4]. Recently, using chimeric J6/JFH-1, Lindenbach and colleagues demonstrated long-term infections in chimpanzee. Importantly, virus recovered from chimpanzees was highly infectious in cell culture, demonstrating further the biological significance of this model system for HCV infection *in vivo* [36].

Robust HCV infection using permissive cell lines

JFH-1 was the first HCV strain found to replicate efficiently and produce infectious virus particles in cultured cells. However, the infection efficiency of JFH-1 was quite low in the first studies in which it was used; only small percentages of inoculated cells were infected [4]. This limitation was overcome by using permissive cell lines. Huh7.5, which is a cured cell line established from replicon cell lines [37], supports high levels of subgenomic replication of HCV strains Con1 and H77. Huh7.5.1 is a sub-line derived from Huh7.5. The infectivity of a J6/JFH-1 chimeric virus and JFH-1 virus in Huh7.5 and Huh7.5.1 cell lines was independently examined by Rice and colleagues (Rockefeller University, New York, NY, USA) and Chisari's group (The Scripps Research Institute, La Jolla, CA, USA). Both groups found that infectivity of both viruses was significantly greater for Huh7.5 and Huh7.5.1 cells than for standard Huh7 cells [5,6]. Almost 100% of Huh7.5 and Huh7.5.1 cells inoculated with the J6/JFH-1 virus or JFH-1 virus were infected.

Chimeric virus construction & infectious genotype 1a HCV

Chimeric HCV constructs have been produced by combining genomic sequences of JFH-1 and other HCV strains [5]. Interestingly, an intragenotypic (genotype 2a) chimeric construct containing J6CF structural genes produced infectious viruses, whereas a chimeric construct based on the H77 strain (genotype 1a) did not [5]. It is important that chimeric viruses are produced from strains that differ in their structural proteins (especially envelope proteins), to allow assessment of cross-neutralization. In a recent study, Yi and colleagues demonstrated the production of an infectious virus using the genotype 1 H77-S genome [38]. Genotype 1 HCV is representative of the most prevalent HCV genotypes causing liver disease within the USA, as well as many other countries. Thus, the ability of infectious virus derived from different genotypes will widen the scope of this system profoundly.

Replicon system with full-length JFH-1 cDNA

In previous studies, HCV replicons have been constructed using full-length HCV cDNA from strains Con1, N and H77 [16,26,39]. RNA of those replicons replicated efficiently in all established replicon cells. However, these replicon cells did not secrete viral particles. Bartenschlager and colleagues have developed a replicon construct using a luciferase reporter gene and full-length JFH-1 cDNA [4]. This replicon is particularly useful for high-throughput screening for antiviral compounds. Virus infectivity can be determined by measuring reporter luciferase activity after infection of the cells. Such a reporter virus system may also be useful for *in vivo* gene delivery into liver tissue.

Production of infectious virus by cells transfected with HCV cDNA

Continuous HCV infection is possible using some sub-lines of Huh7 cells [5,6], but RNA viruses tend to accumulate mutations in their viral genome. If infectious viral particles could be produced from cell lines containing chromosomally integrated HCV cDNA, the efficiency of virus particle propagation would be improved, because cellular DNA mutates less frequently than RNA. Cai and colleagues have constructed stable human hepatoma cell lines that contain chromosomally integrated JFH-1 HCV cDNA and constitutively produce infectious virus. These stable cells robustly produced HCV virions at a rate of up to 10^8 viral RNA copies/ml and 10^4–10^5 infectious units/ml [40].

Conclusion

A robust HCV infection system was established using the JFH-1 strain and permissive cell lines (Figure 1) [4–6]. However, it is not clear why this particular HCV strain supports efficient RNA replication and virus particle formation. It is also unclear how representative JFH-1 is of HCV strains. These issues may be clarified by constructing chimeric viruses containing elements of JFH-1 and other strains, and also by isolating additional HCV strains from other fulminant hepatitis patients. It has been reported that the structural and NS2 regions of JFH-1 can be replaced by the corresponding regions of the J6CF strain without affecting RNA replication or virus particle formation [5]. Thus, it is clear that the region responsible for efficient JFH-1 replication is located in the nonstructural region downstream from NS3. Further analysis is required to determine which NS protein(s) is important for the phenotype of JFH-1. In the present study, JFH-1 virus secreted from cultured cells was infectious for a chimpanzee. Transient viremia was observed in the inoculated chimpanzee, but no liver pathology was found in the biopsy specimen. Further animal studies are required to characterize the pathogenesis of JFH-1.

Future perspective

JFH-1 was the first HCV strain found to infect cells in tissue culture. Full virus culture experiments can be performed using JFH-1 and permissive cell lines. This infectious HCV system allows the study of many steps of the HCV life cycle, including virus entry, replication, virus particle formation and virus secretion. Every step is a potential target for antiviral drugs and vaccines. The present study using JFH-1 indicates that culturing of HCV depends on both the replication capacity of the virus strain and the permissiveness of the culture cells. If more permissive cell lines are developed, it may become possible for every HCV strain to be recovered and cultured.

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Executive summary

- Hepatitis C virus (HCV) infection causes chronic liver diseases and is a worldwide health problem.
- Detailed analysis of the HCV life cycle has been hampered by a lack of efficient viral culture systems.
- The JFH-1 strain was isolated from a fulminant hepatitis patient. Full-length JFH-1 RNA replicated efficiently in transfected Huh7 cells, which secreted recombinant viral particles into culture medium. Secreted viral particles were infectious for cultured cells and a chimpanzee.
- This JFH-1 infectious HCV system is a powerful tool for the study of the HCV life cycle and for the development of antiviral strategies and effective vaccines.

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Chapter 16

Development of an Infectious HCV Cell Culture System

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ABSTRACT

Hepatitis C virus (HCV) infection causes chronic liver diseases and is a health problem worldwide. Despite the increasing demand for knowledge on viral replication and pathogenesis, detailed examinations of the viral life cycle have been hampered by the lack of efficient viral culture systems, owing in part to its narrow host range. We isolated full-length HCV clone, JFH-1strain, from a fulminant hepatitis C patient. The JFH-1strain fit into the cluster of genotype 2a with notable deviations in the 5'-untranslated region (5'UTR), core, NS3 and NS5A regions, and monoclonality of the hyper-variable region sequence. The JFH-1 subgenomic replicon replicated efficiently in a variety of cell lines without acquiring adaptive mutations in its genome. Transfection of *in vitro* transcribed full-length RNA into Huh7 cells, efficient replication of JFH-1 RNA and secretion of recombinant viral particles into culture medium. Importantly, secreted viral particles were infectious for both cultured cells and a chimpanzee. Furthermore, infectivity for cultured cells was improved by using permissive cell lines. This infectious HCV system provides for the first time a powerful tool to study the full viral life cycle, to construct anti-viral strategies and to develop effective vaccines.

INTRODUCTION

Efforts to understand the viral life cycle of hepatitis C virus (HCV) and to identify effective antiviral agents have been hampered by the lack of an efficient cell culture system for this virus. Many attempts to develop a system for HCV infection and replication in cell culture have already been undertaken; in fact, some advances have been reported (Bertolini et al., 1993; Ito et al., 1996; Mizutani et al., 1996; Iacovacci et al., 1997; Fournier et al., 1998; Rumin et al., 1999; Ito et al., 2001; Zhao et al., 2002; Zhu et al., 2003). However, the viral replication efficiencies reported in these studies were modest, requiring detection by a reverse transcription polymerase chain reaction (RT-PCR). We hypothesized that the replication ability of HCV may differ among HCV clones. We therefore isolated an HCV clone, JFH-1, from a fulminant hepatitis patient with HCV (Kato et al., 2001). JFH-1-derived subgenomic replicon proved capable of higher replicative capacity in a variety of cell lines, and production of infectious HCV particles in Huh7 cells.

A CASE OF FULMINANT HEPATITIS ASSOCIATED WITH HCV

In 1999, we obtained sera from a fulminant hepatitis patient (Kato et al., 2001). The 32-year-old male patient was admitted with general fatigue, high-grade fever, and liver dysfunction. No evidence of prior liver disease was found, and the patient had no history of drug or alcohol consumption. In the previous 6 months, he had not received any blood transfusions, taken any drugs intravenously, undergone acupuncture, nor had sexual contact with a known hepatitis virus carrier. This patient showed high levels of serum aspartate aminotransferase and alanine aminotransferase, low levels of the minimum prothrombin time value, and displayed stage II encephalopathy. HCV RNA was detected by RT-PCR, and anti-HCV antibody was negative. All other hepatitis viral markers, anti-HAV antibodies (IgG and IgM), hepatitis B virus (HBV) markers (HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc and HBV-DNA), and GB virus-C RNA, were negative. Therefore, he was diagnosed as having HCV-associated fulminant hepatitis. The infection route of HCV was obscure. The patient showed high levels of viremia, 10^5 copies/ml at admission and 10^4 copies/ml 25 days later. However, HCV was undetectable at 65 days after admission, at which point anti-HCV antibody was positive. At 75 days after admission, his condition improved and he was discharged from the hospital.

To investigate the role of strain-specific viral characteristics of HCV in fulminant hepatitis, we isolated HCV RNA from the acute phase serum of this patient, amplified it by RT-PCR, and determined the sequence of its entire genome.

SEQUENCE ANALYSIS OF JFH-1

The HCV clone isolated from the fulminant hepatitis patient, designated JFH-1, was determined to be of genotype 2a. To compare genomic characteristics, we also determined the entire genomic sequences of 6 HCV genotype 2a clones isolated from 6 chronic hepatitis patients (JCH-1 to -6). JFH-1 is 9,678 nucleotides (nt) in length with a long open reading frame spanning nt 341-9439 coding for 3033 amino acids (aa). Clones isolated from 6 chronic hepatitis patients, JCH-1 to -6, comprised 9681, 9677, 9678, 9676, 9691, and 9686 nt, respectively, and encoded either 3032 or 3033 aa. In phylogenetic analysis, JFH-1 clustered with other genotype 2a clones, but showed slight deviation from clones isolated from chronic hepatitis patients, JCH-1 to -6, and HC-J6 (prototype of HCV genotype 2a, accession number is D00944) (Fig. 1). To determine the degree of deviation in each subgenomic region or entire genome, the ratios of mean genetic distances [ratio = mean genetic distance between JFH-1 and other 2a strains (JCH-1 to -6 and HC-J6) in each subgenomic region or entire genome / mean genetic distance among all 2a strains in each subgenomic region or entire genome] were calculated. For nucleotide analysis of the entire genomes, the mean genetic distances between JFH-1 and other 2a strains (JCH-1 to -6 and HC-J6) and among all 2a strains were calculated to be 0.1136 ± 0.0073 and

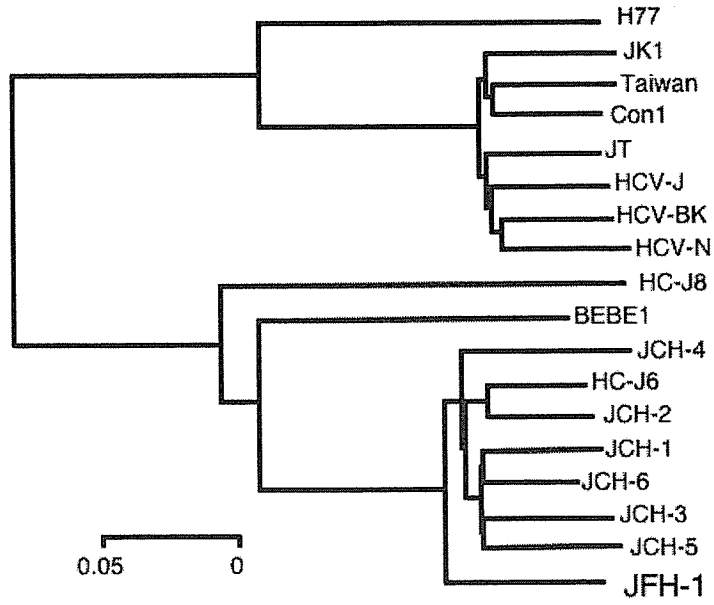

Fig. 1. Phylogenetic tree based on the entire HCV genome of for JFH-1, JCH-1 to -6 and representative strains for which the entire genome has been reported. The number of nucleotide substitutions per site at each position was estimated by the six-parameter method, and a phylogenetic tree was drawn using the neighbor-joining method. The length of the horizontal bars indicates the number of nucleotide substitutions per site.

0.0969±0.0140, respectively, with the ratio of mean genetic distances representing the deviation of clone JFH-1 among genotype 2a clones being 1.173 (Table 1). Among analyses of each subgenomic region, the 5'UTR showed the greatest ratio of mean genetic distances, 1.387, and was identified as the region with the greatest deviation. For amino acids, mean genetic distances of the entire genome between JFH-1 and other 2a strains (JCH-1 to -6 and HC-J6) and among all 2a strains were 0.0918±0.0052 and 0.0716±0.0139, respectively, giving a ratio of mean genetic distances of 1.282. Analyses of each the subgenomic region revealed greater diversity for core, nonstructural (NS) 3, and NS5A, with mean genetic distance ratios of 1.560, 1.464, and 1.596, respectively. The complexity of HCV infection in the fulminant hepatitis patient was also assessed by determining the distribution of quasispecies in the hyper-variable region (HVR). Sequences of the 20 amplified clones of the envelope (E) 2 region were determined and the frequencies of these sequences were examined at the two time points at days 1 and 23 after admission. In the early point of acute phase (day 1 after admission), 17/20 HVR sequences were identical and those of the other 3 clones showed a difference of only one aa substitution. At the later time point of the acute phase (day 23 after admission), the HCV clones were identical (20/20,). These data suggest that the HCV in this patient showed lower complexity than that of the general viral population. Monoclonality of the viral population has also been reported for another case of HCV related

Table 1. Ratios of mean genetic distance for each subgenomic region.

Region	Nucleotide	Amino Acid
5'UTR	1.387	NA**
Core	1.251	1.560
E1	0.986	0.940
E2	1.107	1.066
NS2	1.243	1.298
NS3	1.168	1.464
NS4A	1.249	1.044
NS4B	1.178	1.223
NS5A	1.222	1.596
NS5B	1.213	1.208
3'UTR	0.989	NA
Entire Genome	1.173	1.282

*Ratios were calculated using the mean genetic distances [ratio = mean genetic distance between JFH-1 and other 2a strains (JCH-1 to -6 and HC-J6) in each subgenomic region or entire genome / mean genetic distance among all 2a strains in each subgenomic region or entire genome] (Kato et al., 2001).
**NA, not available

fulminant hepatitis (Farci et al., 1996). Thus, we speculated that monoclonality of the viral population is related to the development of fulminant hepatitis and that the JFH-1 clone, especially in the 5' UTR, core, NS3 and NS5A regions, has some specific viral characteristics related to fulminant hepatitis.

PREFERENTIAL PROCESSING FOR CORE PROTEIN OF JFH-1

Among the subgenomic regions of JFH-1, 5'UTR, core, NS3 and NS5A were identified as deviated regions. Among these regions, core protein is known to form the viral particle and also to regulate multiple functions in host cells (Moriya et al., 1998; Ray et al., 2001; Watashi et al., 2003; see Chapter 3). During virus assembly, the core protein undergoes two consecutive membrane-dependent cleavages, and it develops into two forms, p23 and p21 (Liu et al., 1997). The p21 core protein is cleaved from the endoplasmic reticulum-bound p23 core protein or the longer precursor polyprotein by host signal peptide peptidase (McLauchlan et al., 2002). The p21 core protein was predominantly observed in patient serum containing native viral particles (Yasui et al., 1998). Thus, the p21 core protein is the mature and stable form that accumulates in the cell and eventually constitutes the viral capsid. We investigated the differences in p21 core protein production between JFH-1 and the other genotype 2a clones isolated from chronic hepatitis patients (JCH-1 to -5) (Kato et al., 2003a). Using the core or core-E1 expression vector of JFH-1 and JCH-1, we found that JFH-1 could preferentially produce the p21 core protein in

both *in vitro* translation assay and cell transfection assay with Huh7, HepG2 and HeLa cells. Similar results were also obtained when comparing JFH-1 with the other clones (JCH-2 to -5) isolated from chronic hepatitis patients. Investigations with chimeric constructs revealed that differences in core protein processing depend on the c-terminal region of the core protein. We identified 4 aa substitutions in this region of the core protein between JFH-1 and the other clones isolated from chronic hepatitis patients. Through experiments with mutation-introduced constructs, all 4 of these aa of JFH-1 were found to be responsible for the preferential production of p21 core protein. Based on these findings, we suspected that JFH-1 may be able to preferentially produce viral particles over other HCV clones.

REPLICATION CAPACITY OF JFH-1 AS A SUBGENOMIC REPLICON

To investigate the function of the NS region of JFH-1, we constructed a subgenomic replicon system using this clone. The HCV subgenomic replicon system has enabled us to mimic HCV replication in Huh7 cells, and has been used as a tool in the study of the mechanism of HCV replication (Lohmann et al., 1999; see Chapter 11). JFH-1 showed higher colony formation efficiency that was approximately 500-fold more efficient than the prototype Con-1 replicon and 50-fold more efficient than the Con-1/NK5.1 replicon, which contains highly adaptive mutations (Kato et al., 2003b). Furthermore, the JFH-1 replicon could replicate efficiently not only in Huh7 cells, but also in other hepatocyte-derived cell lines, HepG2 and IMY-N9 cells (Date et al., 2004), and non- hepatocyte derived cell lines, HeLa and 293 cells (Kato et al., 2005). This result may be attributed to the replication proficiency of JFH-1. Importantly, the JFH-1 replicon did not require an adaptive mutation in order to replicate in these cell lines. Most clones isolated from each of these cell lines showed no or a few aa mutations in the HCV-derived replicon regions. Previously, Bukh et al. (2002) demonstrated that HCV infection could not be achieved with full-length HCV RNA containing multiple cell-culture adaptive mutations. Thus, the higher replication capacity and the absence of adaptive mutations of JFH-1 may be important for developing an infectious HCV system.

CONSTRUCTION OF FULL-LENGTH JFH-1 cDNA

Based on results obtained using subgenomic replicons, we found that the JFH-1 strain replicates very efficiently in Huh7 cells, as shown not only by colony formation assay with G418 selection, but also by transient replication assay (Kato et al., 2003a; 2003b; Date et al., 2004; Kato et al., 2005). This suggests that the JFH-1 genome can replicate autonomously in Huh7 cells without the help of G418 selection pressure and the development of adaptive mutations. Taking advantage of the efficiency of the JFH-1 strain replication capacity, we planned to test the replication of a full-length JFH-1 clone in Huh7 cells.

Monoclonality is one of the specific characteristics of HCV strains in fulminant hepatitis, and the JFH-1 strain, as confirmed by isolations made from other patients (Farci et al., 1996; Kato et al., 2001). This characteristic is also advantageous in the construction of consensus clones in the production of full-length cDNA because, usually, HCV possess a wide variety of mutations called quasispecies (Martell et al., 1992). Thus, it was necessary to inject 10 different clonal mixtures into a chimpanzee to establish the first infectious clone for chimpanzee (Kolykhalov et al., 1997). On the other hand, JFH-1 cDNA was cloned from RT-PCR fragments and, although some sequence diversity was present, the aa sequences were highly conserved and full-length HCV cDNA encoding the JFH-1 strain consensus sequence was easily assembled by connecting the cloned PCR fragments (Kato et al., 2001; Wakita et al., 2005). The T7 promoter sequence was inserted just upstream of the full-length JFH-1 cDNA sequence, and full-length synthetic JFH-1 RNA was transcribed from pJFH-1 by T7 RNA polymerase.

REPLICATION OF FULL-LENGTH JFH-1 RNA IN Huh7 CELLS

We first transfected *in vitro* transcribed full-length JFH-1 RNA into naïve Huh7 cells, which is the original cell line used for subgenomic replicon studies. As we expected, full-length JFH-1 RNA replicated efficiently in the transfected cells, as determined by Northern blot analysis (Wakita et al., 2005). Viral proteins produced from replicated RNA were demonstrated by immunofluorescence and Western blot analyses. Transfection of replication incompetent mutant RNA transcribed from pJFH1/GND, in which GDD catalytic motif of NS5B was mutated to GND, into Huh7 cells, however, did not lead to viral replication or protein production.

We expected to achieve replication of full-length RNA in transfected Huh7 cells because full-length genotype1b RNA with adaptive mutation had been reported to replicate in Huh7 cells and subgenomic replicons of the JFH-1 strain had been shown to produce more colonies in Huh7 cells than genotype1b replicons (Ikeda et al., 2002; Pietschmann et al., 2002; Kato et al., 2003b). However, it was difficult to predict viral particle formation and secretion because these had not been achieved by full-length HCV RNA transfection, even though RNA replication was observed in the transfected Huh7 cells (Ikeda et al., 2002; Pietschmann et al., 2002). To determine whether the viral particles were formed and secreted into the culture medium from the full-length JFH-1 RNA transfected cells, we performed several biological assays. First, we analyzed the density of secreted viral proteins and viral RNA by sucrose density gradient. It has been reported that the supernatant of full-length replicon RNA replicating cells of the Con1 strain secrete viral RNA into culture medium; however, the density of viral RNA was found to have a very similar culture medium density as that from subgenomic RNA replicating cells (Pietschmann et al., 2002). We thus first analyzed an aliquot of culture supernatant from full-length JFH-1 RNA transfected cells by sucrose density gradient. Following

ultracentrifugation, 16 fractions were obtained from the bottom of the tube. Both viral core protein and RNA were quantified using sensitive core ELISA (Aoyagi et al., 1999) and RT-PCR with real-time detection, respectively (Takeuchi et al., 1999). Interestingly, both core protein and RNA peaks occurred in the same fraction (around 1.17 g/ml), a density greater than the one where subgenomic replicon cells usually segregate. Next, we determined RNase sensitivity of these peaks, as the viral RNA genome packed in the particles should be protected from RNase digestion in culture. Culture medium from the transfected cells were RNase digested, followed by density centrifugation. The profile analysis of the density peaks revealed that RNase digestion did not change the density gradient distribution of both RNA and core protein, indicating the viral genome was protected from nuclease digestion (Wakita et al., 2005).

Next, we confirmed whether the envelope proteins were incorporated into secreted viral particles. If the viral particles are properly and completely formed and secreted, viral genome and core protein form a nucleocapsid and are surrounded by envelope proteins (E1 and E2 proteins). To assay envelope proteins, we treated culture medium with detergent to strip the envelope components from the viral particles. Viral envelope usually comprises cellular membrane components such as lipids, making the density of envelope lighter than that of the inner nucleocapsids. We found that both core protein and RNA peak fractions became heavier (around 1.25 g/ml), indicating the removal of the lighter envelope components by the detergent treatment. Furthermore, we demonstrated the incorporation of both E1 and E2 proteins by Western blot analysis of peak fractions of viral RNA after density gradient centrifugation. We collected approximately 2.5 litter of culture medium from transfected cell cultures. Culture medium was concentrated by ultrafiltration and then by ultracentrifugation, and was then fractionated by sucrose density gradient. Each collected fraction (counted from the bottom of the centrifuge tube) was further concentrated by ultrafiltration. Concentrated fractions were separated by SDS-PAGE and then transferred onto PVDF membrane. Core, E1 and E2 proteins were detected on each fraction using specific antibodies. Thus, all the components of the viral particle were detected in the same density gradient fraction, suggesting proper viral particle formation and secretion. Finally, viral particles secreted into the culture medium were visualized by immuno-electron microscope analysis using anti-E2 monoclonal antibody. Viral particles were shown to be spherical, with an outer diameter of about 55 nm (Wakita et al., 2005).

INFECTIVITY OF SECRETED VIRAL PARTICLES FROM JFH-1 TRANSFECTED CELLS

After having confirmed the presence of secreted viral particles, we were interested in the infectivity level of secreted viral particles. We used double-chambered culture plates equipped with polyester membrane (0.45 μm pore size) separating the inner

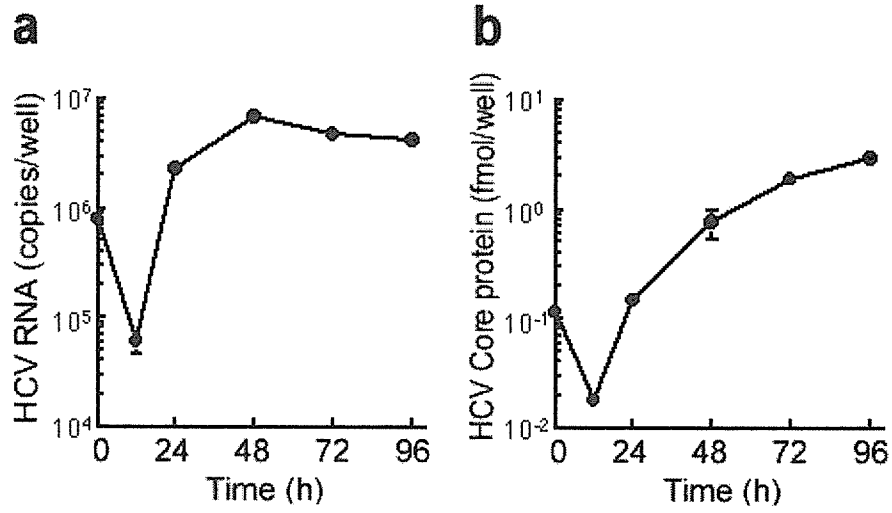

Fig. 2. HCV RNA replication (a) and core protein production (b) in infected Huh7 cells. Culture medium was collected from full-length JFH1 RNA-transfected cells and concentrated by ultrafiltration. Naïve Huh7 were seeded 24 h before infection. Filtered (0.45-μm pore size) culture media was inoculated for 2 h with periodic rocking. After inoculation, cells were washed with PBS and were cultured in complete culture medium for another 12, 24, 48, 72, and 96 h. Experiments were performed in triplicate, and mean titers (closed circles) SD (bars) are shown.

and outer chambers. Thus, substances smaller than this pore size, such as virus particles, can diffuse across the membrane and populate both chambers. Full-length JFH-1 RNA transfected cells were transferred to the inner chamber and naïve Huh7 cells were seeded in the outer chamber. A few days after the start of the experiment, naïve Huh7 cells in the outer chamber were stained with anti-HCV antibodies to confirm infection by secreted virus particles. To our surprise, a few cells were positively stained, although at very low frequency. To confirm that infection occurred for naïve Huh7 cells, we collected culture medium of transfected Huh7 cells, which was subsequently cleared by low speed centrifugation and filtered through a disk filter (0.45 μm-pore size). Naïve Huh7 cells were inoculated with the cleared free virus in a culture plate for 3 hours. Inoculated cells were then washed with PBS and cultured for another 48 h in complete medium. To increase infection efficiency, culture medium was concentrated by ultrafiltration. Inoculation of concentrated culture medium increased the numbers of infected cells, however, the efficiency was still low at around 0.5% with Huh7 cells. Inoculated cells were harvested after infection, and HCV RNA titer was determined by PCR with real time detection (Fig. 2a). Only 1% of inoculated HCV RNA was adsorbed by inoculated cells and HCV RNA copies in the infected cells were further decreased within 12 hours after inoculation. However, RNA titer in the infected cells increased at 24 hours after inoculation. Core protein expression measured in infected cells by sensitive ELISA showed a decrease within 12 hours after inoculation and an increase at 24 hours after infection (Fig. 2b). These data clearly showed that viral particles were infectious for Huh7 cells, although at low efficiency (Wakita et al., 2005).

NEUTRALIZATION OF JFH-1 INFECTIVITY BY CD81 ANTIBODY AND PATIENT SERA

CD81 has been identified as an E2 protein binding protein (Pileri et al., 1998). We thus tested the effectiveness of anti-CD81 antibody for inhibiting infectivity of culture supernatant for naïve Huh7 cells. Naïve Huh7 cells were treated with 10 μg/ml of anti-CD81 antibody at room temperature and then washed with PBS followed by inoculation with culture medium from transfected cells (Wakita et al. 2005). HCV RNA titer in the inoculated cells was inhibited more than 1 log, indicating that infection by secreted viral particles is at least partially dependent on a CD81-specific pathway. Further studies will be necessary to determine whether CD81 is a sole receptor molecule involved in adsorption and internalization steps or whether other molecules are also involved.

The neutralizing activity in chronically infected patient sera has been shown by experiments using pseudotype virus harboring HCV envelope proteins (Bartosch et al., 2003; Yu et al., 2005; Logvinoff et al., 2004). We also tested some patient sera for neutralizing activity against JFH-1. To increase sensitivity of the assay, a bicistronic replicon construct containing luciferase reporter was used (Wakita et al. 2005). Indeed, patient serum tested positive for neutralization of virus infection proved to contain some neutralizing antibodies against JFH-1 (Wakita et al., 2005).

IN VIVO INFECTIVITY OF JFH-1 CULTURE MEDIUM

To further confirm authenticity of the viral particles produced in our study, *in vivo* infectivity was tested in a chimpanzee (Wakita et al., 2005). Electroporated culture supernatant was harvested from the full-length JFH-1 RNA-transfected cells and cleared by low-speed centrifugation and then passed through a 0.45-μm disk filter. Control culture medium was prepared from the cells mixed with JFH-1 RNA, but with the omission of electroporation pulse. A chimpanzee was first inoculated with undiluted control culture medium, and no infection was observed. Then, 10^4 diluted culture medium harvested from the transfected cell was used for inoculation, but again, no infection developed. Six weeks later, 10^3 diluted culture medium was inoculated in the same subject, and viremia was induced. Viral titer was low, with the highest HCV RNA titer being 2.04×10^3 copies/ml. Furthermore, HCV infection was cleared without any evidence of abnormal liver histology or elevation of liver-specific enzymes or HCV-specific antibody seroconversions (Wakita et al., 2005). Further investigation is necessary to determine whether the nonvirulent phenotype is a characteristic of the JFH-1 strain.

PERMISSIVE CELLS FOR JFH-1 INFECTION

The infection efficiency of JFH-1 was quite limited as only a small percentage of the inoculated cells appeared positive for HCV by antigen staining (Wakita et al., 2005). To increase the infection efficiency, specific cell lines derived from

Huh7 cells were analyzed by several groups. Huh7.5, which is one of the cured cell lines established from original HCV replicon cell lines, supported high levels of subgenomic HCV replication with Con1 and H77 strains (Blight et al., 2002). Huh7.5.1 is a cell line derived from Huh7.5 (Zhong et al., 2005). Indeed, infectivity of Huh7.5 or Huh7.5.1 cell line with JFH-1 was markedly increased (almost 100%) compared to standard Huh7 cells (Lindenbach et al., 2005; Zhong et al., 2005). Furthermore, Zhong and colleagues (2005) also were able to prevent *in vitro*-produced virus from infecting Huh7.5.1 using an anti-CD81 antibody, whereas Lindenbach and his coworkers (2005) accomplished this with Huh7.5 by using a soluble recombinant CD81 fragment.

SUMMARY AND CONCLUDING REMARKS

Recombinant HCV particles were produced and secreted from JFH-1 RNA-replicating cells, and the secreted viruses were infectious to both Huh7 cells and a chimpanzee (Zhong et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005). Biophysical property analysis showed that cell culture-grown virus particles have a density of about 1.15 – 1.17 g/ml, are spherical, and have an outer diameter of about 55 nm (Wakita et al., 2005). Both the density and the overall diameter of the particle are in agreement with a recent report describing the production of virus particles with a DNA-based expression system (Heller et al., 2005). Infectivity can be significantly neutralized by CD81-specific antibodies, supporting observations that CD81 plays an important role in HCV cell entry made in HCV pseudo particles (Zhong et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005; Bartosh et al., 2003; Hsu et al., 2003). Some level of neutralization was achieved with immunoglobulins in patient serum, showing that potentially protective antibodies are generated during chronic infection but that their capacity to prevent chronicity may be limited. We also observed cross-neutralization in sera from patients infected with a genotype 1 virus (Wakita et al., 2005).

Thus, JFH-1 is the first HCV strain with the capability to produce infection in tissue culture, and serves as a platform for a new generation of HCV investigations. Furthermore, the use of permissive cell lines such as Huh7.5 and Huh7.5.1 cell lines will further expedite full virus culture experiments in the laboratory. This infectious HCV system should provide opportunities to study the full HCV life cycle, including virus entry, replication, virus particle formation, and virus secretion, as well as to develop effective antivirals and vaccines.

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