

CG1b•GND-transfected cell was undetectable, although SEAP activities in both culture supernatants were comparable (Fig. 1C). To test the infectivity of the cell culture-produced HCV, culture supernatants were inoculated to naïve Huh7.5.1 cells. By immunofluorescence microscopy, HCV core and NS3 proteins were detected in JFH1 supernatant infected cells. HCV core protein showed spotty perinuclear and cytoplasmic distribution, and NS3 protein showed cytoplasmic distribution (Fig. 1D). However no HCV-positive cells were detected in naïve cells incubated with culture medium from H77-, CG1b- and J6-transfected cells.

Infection of cell-cultured generated CG1b virus in a chimpanzee. JFH-1 virus generated in cell culture was shown previously to be infectious in a chimpanzee (22). To assess the *in vivo* infection of cell culture-produced CG1b virus, a naive chimpanzee was inoculated with culture medium first from mock-transfected cells (control medium) and second from pEF/CG1b-Rz/S plasmid-transfected cells (2.3×10^3 copies of HCV RNA). After inoculation of the control medium, the chimpanzee showed no signs of infection for 8 weeks. However, two weeks after inoculation of culture medium from CG1b plasmid-transfected cells, HCV RNA became positive in the chimpanzee serum and persisted up to 48 week after inoculation (Fig. 1E). The highest viral titer was 7.5×10^5 copies/mL and ALT showed a transient mild elevation. Anti-HCV was detected 40 weeks after inoculation. To demonstrate that the virus generated in cell culture did not acquire adaptive mutations in cultured cells, the entire HCV ORF from serum at 4 weeks after inoculation was sequenced. The sequence was completely identical to that of the CG1b strain that used for transfection. Culture medium of pEF/H77-Rz/S

transfected cells (1.4×10^4 copies of HCV RNA) was also inoculated into a chimpanzee who had previously recovered from HCV CG1b infection. The chimpanzee developed viremia but quickly resolved the infection (data not shown). This attenuated infection was typically observed in re-challenge experiments of chimpanzees that had recovered from a previous HCV infection (15).

Suppression of JFH1 replication by IFN- α . To test the sensitivity of HCV production to antiviral in this system, JFH1-ribozyme plasmid-transfected cells were treated with IFN- α (100 IU/ml) 3 days after transfection, and HCV core Ag and HCV RNA were measured in the culture supernatants (Fig. 2). JFH1•GND mutant that has an inactivating mutation in the NS5b RdRp site was used as a replication deficient control. IFN- α significantly suppressed the HCV core Ag level in the supernatant (4050.3 ± 310.8 to 331.3 ± 14.1 fmol/L, $p < 0.01$), to a level that is similar to the that of JFH1•GND-transfected cells (171.5 ± 42.0 fmol/L). HCV RNA titer of the supernatant was also suppressed by IFN- α ($1.92 \times 10^5 \pm 1.69 \times 10^4$ to $1.93 \times 10^4 \pm 8.25 \times 10^3$ copies/mL, $p < 0.0001$). The HCV RNA titer of JFH1•GND mutant transfected was under the detection limit. The SEAP levels were not significantly affected by IFN- α .

Density gradient analysis. To confirm HCV particle production of this system, culture medium of JFH1 expression plasmid transfected cells was concentrated, purified and subjected to iodixanol density gradient centrifugation. Distributions of HCV core Ag and HCV RNA showed similar profiles, and peaked in the fraction with a density of 1.14 g/mL (Fig. 3). The Infectivity titer of each fraction to naïve Huh7.5.1 cells was also

evaluated. The peak infectivity titer located in the fraction at a density of 1.09 g/mL. By negative stain electron microscope, the HCV particle was observed as a spherical structure measuring about 50 nm in diameter (Fig. 3, inset). The presence of the particles was detected primarily in the peak fraction of the infectivity. We have previously reported the production and secretion of HCV particles in CG1b-ribozyme plasmid transfected cells (6). The density gradient analysis and electron microscopic morphology of the particles are similar to those of the JFH1 particles described here.

Long term culture of JFH1 expressing cells. To evaluate the continuous production of HCV particles, JFH1-transfected Huh 7.5 cells were cultured and passaged for over 6 weeks. The production of HCV core Ag and secretion of HCV RNA were maintained during the observed period (Fig. 4). The highest titer of HCV core Ag and HCV RNA were observed on day 31 after transfection in this experiment: 6.35×10^4 fmol/L and 1.48×10^6 copies/mL, respectively. The SEAP production disappeared about 2 weeks after transfection, supporting the continuous replication and production of the JFH1 virus. Infectivity titers of culture medium were also determined. The peak infectivity titer of 2.50×10^3 FFU/mL was observed at 25 days after transfection, but in general the HCV core Ag, HCV RNA and infectivity titers correlated reasonably well in this experiment. However the transfected cells gradually lost the ability to support HCV replication and production beyond this time period.

Establishment of cell lines stably producing JFH1 virus. To establish stable JFH1 virus producing cells, pEF/JFH1-Rz/N plasmid was transfected into Huh7 and Huh7.5

cells. After three weeks of culture with G418 at a concentration of 1.0 mg/ml, visible colonies were identified in both transfected cell lines. A total of 23 Huh7 colonies and 26 Huh7.5 colonies were selected and screened by HCV core Ag production and indirect immunofluorescence with α -core and α -NS3 antibodies. Four Huh7 derived clones and three Huh7.5 derived clones were identified to produce HCV proteins (Table 1). Three of four Huh7 clones and one of three Huh7.5 clones were found to produce high levels of HCV core Ag and detectable HCV RNA and infectivity titers in culture medium. Among those clones, one Huh 7.5 derived clone (clone 7.5-20) with the highest HCV production was followed for an extended period of time under drug selection. This clone showed continuous and stable production of HCV core Ag (2.96×10^4 to 6.20×10^4) and HCV RNA (1.03×10^6 to 4.73×10^6) in the medium up to 24 passages (Fig. 5A). Infectivity titers of culture medium were also determined in this period, and ranged 3.25×10^2 to 6.00×10^3 . To assess the sensitivity of HCV production to IFN- α , 7.5-20 cells were treated with IFN- α (100 IU/ml) and HCV core Ag and HCV RNA were measured in culture supernatants (Fig. 5B). IFN- α significantly suppressed the HCV core Ag ($2.04 \times 10^4 \pm 1.85 \times 10^3$ to $2.90 \times 10^3 \pm 1.51 \times 10^2$ fmol/L, $p < 0.005$) and HCV RNA titer ($2.52 \times 10^5 \pm 1.25 \times 10^5$ to $3.13 \times 10^3 \pm 1.26 \times 10^3$ copies/mL, $p < 0.05$) in the supernatant.

Discussion

The discovery of the JFH1 strain enabled us to develop a robust system for HCV replication and infection in culture cells (13, 22, 26). However, the JFH1 strain is unique

among the HCV strains and not necessarily representative of HCV biology (7). Studies in chimpanzees suggest that the virus is not particularly infectious *in vivo*, causing an attenuated and transient infection, which is atypical for the general behavior of HCV (22). It is indeed interesting that this virus was originally isolated from a patient with fulminant hepatitis C (7). It is likely that the fulminant hepatitis is associated more with the clinical setting of the patient than with the virus. Further studies are necessary to resolve the question of whether viral factor(s) plays a role in the severity of acute HCV infection. Currently, no other natural HCV strain has yet been shown to replicate efficiently and demonstrate robust infectivity in cell culture without adaptive mutations. While the establishment of a genotype 1a (H77 strain) infectious system in cell culture is important, introduction of several adaptive mutations are clearly required (24). These adaptive mutations have been shown to confer unusual biological properties to the viral strain *in vivo* (3). Chimeric viruses containing the structural region of other genotypes and the JFH1 nonstructural genes have been generated and showed *in vitro* infectivity (13, 14, 17), but the biological relevance of these chimeric viruses is difficult to assess.

In this study, we established a HCV particle production system with various HCV genotypes by exploiting a recently established DNA transfection cell culture system (6). By engineering two hammerhead ribozyme sequences at the both 5' and 3' ends of the HCV genomic cDNA, DNA expression plasmids of multiple HCV strains could be constructed. These plasmids also contain the SEAP gene under the control of the SV40 promoter. By measuring the SEAP activity in the culture medium, the transfection efficiency and effect of the culture conditions, such as antiviral treatment, could be

monitored. Using this system, we demonstrated that the HCV particle production of H77 strain (genotype 1a), CG1b strain (genotype 1b) and J6 strain (genotype 2a) were comparable. Viral particles resembling HCV virions were visualized by electron microscopy in CG1b- and JFH1-transfected cells (ref. 6 and Fig. 3). JFH1 strain showed substantially higher virus production; the HCV core Ag was 50 times and HCV RNA production was 1 to 2 logs higher than those of the other strains. JFH1 virus released into the culture medium was infectious in naïve Huh7 cells. Consistent with previous reports, the infectivity titer (FFU/mL) was about 3 logs lower than the HCV RNA titer (4, 13, 22, 26). Infectivity of the culture medium of other strain-transfected cells was also assessed. However, evidence of *in vitro* infection of these strains by immunofluorescence was not detected. The absence of infection is likely a result of the sensitivity limit of the detection method and a much lower replication efficiency of the other strains as compared with the JFH1 strain. The JFH1 replicates about 1 to 2 logs higher than the other strains and the infectivity titer of the JFH1 was about 10^2 FFU/mL in this experiment. Therefore it is not surprising that we could not detect infection of the other strains. Perhaps by optimizing the culture and infection conditions, using more sensitive methods to detect infection, and concentrating the virus produced in the culture medium, we could detect infection by other genotypes.

To demonstrate the infectivity of strains other than JFH1, the cell culture-generated CG1b virus was inoculated to a naïve chimpanzee and caused a typical course of infection as the infectious CG1b RNA or serum (21). Similarly, H77 virus generated in cell culture was also infectious *in vivo*. The sequence of CG1b virus replicating in the

chimpanzee was completely identical to that of the CG1b strain used for transfection. CG1b virus seems to be capable of replicating more efficiently than the JFH-1 virus in chimpanzee without adaptive mutations, although it has a lower replication efficiency *in vitro*. This observation is consistent with the contrasting effects of the described adaptive mutations on *in vitro* replication and *in vivo* productive infection (3). This discrepancy may be explained by the possibility that lower replication efficiency *in vitro* may be essential for productive infection and persistence *in vivo*.

In the iodixanol density gradient analysis, we demonstrated the co-localization of HCV RNA and core Ag proteins. Their peaks were in the identical fraction at a density of about 1.14 g/mL, which is consistent with our previous reports (6, 22). However, the peak of infectivity titer was at a less dense fraction (a density of 1.09 g/mL). This discrepancy has also been reported previously (4, 13). We could detect HCV particles in the peak infectivity fraction; about 50 nm of spherical structures resembling putative HCV were observed by electron microscopy. Thus infection-competent HCV particles probably exist mainly in the peak fraction of infectivity titer but not in the peak fraction of HCV RNA and HCV core Ag. This observation explains the difference between HCV infectivity and RNA titer of cell culture generated HCV (about 1000 fold difference). The forms of the viral RNA and protein in the peak fraction are unknown. It is possible that they represent defective viral particles and/or nucleocapsids. Further studies are needed to clarify this point. It is also interesting to note that the ratio of HCV RNA titer to HCV core Ag level is higher in these gradient fractions than that in the unfractionated

culture medium. This could be explained by the presence of nonparticulate core protein or empty nucleocapsid in the medium.

This system has the advantage in that it is based on DNA expression plasmids, is much easier to manipulate and contains a reported gene to monitor various culture conditions. It can be extended to various HCV genotypes and strains as well as generation of stable cell lines expressing replicating HCV. In the transient transfection system, robust HCV replication could be observed up to 6 weeks post-transfection (Fig. 4). It is interesting that the transfected cells eventually lost their ability to support HCV replication. It is possible that some major alterations in the biology of the infected cultured cells, such as activation of endogenous antiviral mechanisms and/or genetic/epigenetic changes, eventually occurred to shut off the viral replication. However using a selectable marker like the *neo* gene, we could develop stable JFH1 virus producing cells. We have generated one clone that supports continuous and stable high-level viral replication and production with multiple passages for an extended period of time. A recent study reported the application of a similar DNA expression system in generating JFH1-producing cell line (4). The system used the HDV ribozyme sequence at the 3' end without any ribozyme sequence at the 5' end of the JFH1 genomic cDNA. It is not clear whether the 5' end of the resulting HCV RNA contains the correct sequence. Furthermore we also tested the HDV ribozyme sequence in our DNA expression system and found it to be not as efficient in generating the cleaved product as the hammerhead ribozyme sequence we have designed (unpublished data).

The system is also applicable to antiviral testing. In both transient transfection and stable cell lines, HCV production was substantially suppressed by IFN- α (Figs. 2 and 5b). Both HCV core Ag and HCV RNA titers were suppressed to the baseline level of the JFH1-GND transfected cells. The absence of a significant change in SEAP activity in IFN- α treated cells indicates that the effect is not of a general toxicity but specific to HCV replication. Because this system represents the complete replication cycle of HCV, it could prove very useful for high-throughput antiviral screening.

In summary, we have established an efficient HCV production system with various genotypes by using DNA expression plasmids with HCV genomic cDNA flanked by self-cleaving ribozymes. HCV generated in this system showed infection both *in vitro* and *in vivo*. This system provides a valuable tool not only to study the replication and pathogenesis of HCV but also to screen for antivirals against multiple HCV strains.

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Figure legends

Fig. 1. Production and Infection of HCV produced in cell culture. (A) Construction of HCV expressing plasmid with HCV-ribozyme system. Two hammerhead ribozyme sequences were engineered at the 5' and 3' ends of HCV full-length cDNA. This system was expressed under the control of EF1 α promoter. The SEAP reporter gene was included under the control of SV40 promoter. (B) Production of HCV various strains after transfection of HCV-ribozyme plasmids. Various HCV-ribozyme plasmids were transfected into Huh7.5 cells. HCV core Ag levels and SEAP activities in the culture medium were measured at the indicated time points. HCV RNA titers in culture medium were measured at the end of follow-up time point. (C) Comparison of HCV RNA titers of CG1b and CG1b-GND plasmid-transfected cells. HCV RNA titers and SEAP activities in the culture medium were measured 5 days after transfection. (D) Infection of naïve Huh7.5.1 cells by cell culture-generated JFH-1 virus. Culture medium from JFH-1-ribozyme transfected cells were inoculated to naïve Huh7.5.1 cells. Expression of HCV core and NS3 proteins was detected by immunofluorescence using appropriate antibodies. (E) Infectivity of cell culture-generated CG1b virus in a naïve chimpanzee. Chimpanzee X140 was inoculated with culture medium of CG1b plasmid-transfected cells containing 2.3×10^3 copies of HCV RNA.

Fig. 2. Inhibition of HCV replication and production by IFN- α . IFN- α (100 IU/mL) was administered to the JFH-1-ribozyme-transfected cells 3 days after transfection. HCV core Ag level, HCV RNA titer and SEAP activity in the culture supernatants were

monitored at the indicated time points. The JFH-1•GND mutant was used as a replication deficient control.

Fig. 3. Iodixanol density gradient analysis of the cell culture-generated JFH-1 HCV. Huh7.5.1 cells were transfected with JFH-1-ribozyme plasmid and culture medium was collected and analyzed by iodixanol density gradient as described in the Materials and Methods. Fractions were collected from the bottom of the gradient, and measured for HCV core Ag, HCV RNA and infectivity titers. The HCV particles visualized by negative stain electron microscopy in the peak fraction of infectivity titer was shown in inset.

Fig. 4. Long term culture of JFH-1-ribozyme-transfected cells. Huh7.5 cells were transfected with JFH-1-ribozyme plasmid as described previously. The transfected cells were passaged 3-4 days and culture medium was collected at various time points. HCV core Ag, HCV RNA, infectivity titer and SEAP activities in the culture medium were monitored for up to 43 days after transfection.

Fig. 5. Production of HCV in the stable JFH-1-virus producing cell line. (A) Huh7.5 cells were transfected with the pEF/JFH1-Rz/N plasmid and exposed to 1 mg/mL of G418. Clones were isolated as described. The 7.5-20 clone was passaged (every 3 - 4 days) and monitored continuously for an extended period of time. The HCV core Ag, HCV RNA and infectivity titers in the culture medium at various time points were analyzed and shown. (B) Suppression of HCV replication and production in the stable JFH-1-virus producing cell line (clone 7.5-20) by IFN- α . Production of HCV core Ag and HCV

RNA in the culture medium was assessed at various time points after administration of IFN- α (100 IU/mL).

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TABLE 1. Established clones of stable JFH1 virus producing cells

Clones	Origin	HCV core Ag (fmol/L)	HCV RNA (copies/mL)	Infectivity (FFU/mL)
7-09	Huh7	112	< 3.00 x 10 ^{2*}	< 1.00 x 10 ^{1**}
7-10	Huh7	5,053	1.78 x 10 ⁵	1.50 x 10 ²
7-19	Huh7	4,438	9.72 x 10 ⁴	3.00 x 10 ¹
7-20	Huh7	1,025	4.32 x 10 ⁴	2.00 x 10 ¹
7.5-01	Huh7.5	354	< 3.00 x 10 ²	< 1.00 x 10 ¹
7.5-15	Huh7.5	158	< 3.00 x 10 ²	< 1.00 x 10 ¹
7.5-20	Huh7.5	51,397	2.32 x 10 ⁶	5.30 x 10 ²

*under the detection limit of RT-qPCR

**under the detection limit of indirect immunofluorescence

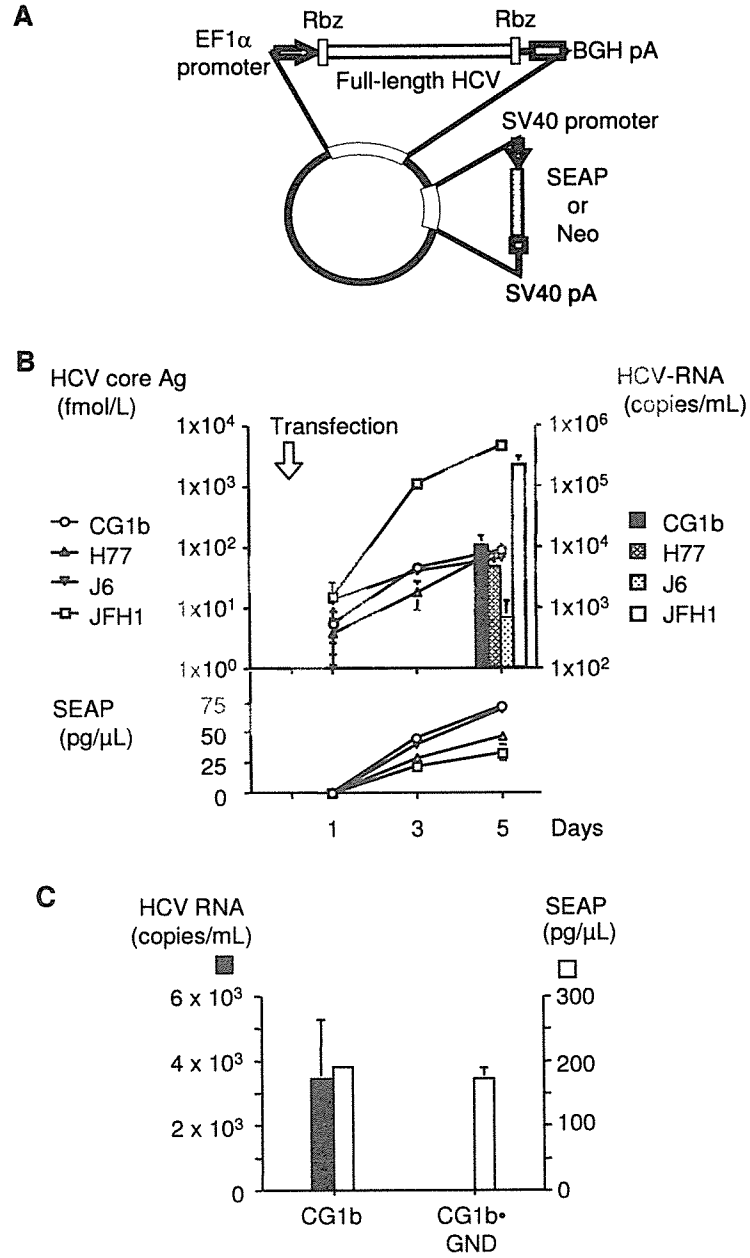


Figure 1