Genotype 2a Hepatitis C Virus Subgenomic Replicon Can Replicate in HepG2 and IMY-N9 cells

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Running Title: HCV Genotype 2a Replicon in HepG2 and IMY-N9
Summary

A hepatitis C virus genotype 2a subgenomic replicon, JFH-1 replicon, was previously established using the consensus sequence of clone JFH-1 from a patient with fulminant hepatitis, and in a previous report, was indicated to replicate efficiently in Huh7. Here the replication of JFH-1 replicon was tested in HepG2, a human hepatocyte-derived cell line, and in IMY-N9, a cell line developed by fusing human hepatocyte and HepG2. Following transfection with \textit{in vitro} transcribed replicon RNA and selection by cultivation with G418, colonies formed in both cell lines, although at efficiencies substantially lower than those of Huh7. The H2476L mutation identified in the Huh7 replicon in our previous study increased the colony formation efficiencies of the JFH-1 replicon in HepG2 and IMY-N9 cells. Higher amounts of replicon RNA were detected in IMY-N9 clones than in HepG2 clones by real-time detection RT-PCR, and replicon RNA replication and viral protein expression were confirmed by Northern and Western blotting in isolated clones. Sequencing of replicon RNAs revealed that mutations found in hepatitis C virus-derived regions were not identical and that 2 out of 9 HepG2 clones and 3 of 9 IMY-N9 clones had no or one synonymous mutation. This system with the JFH-1 replicon and three cell lines is useful not only for estimating the cellular factors affecting viral activity but also for clarifying the common host’s gene response.
Introduction

Hepatitis C virus (HCV), one of the plus-strand RNA viruses, is a principal agent in post-transfusion and sporadic acute hepatitis (1, 2). Infection with HCV leads to chronic liver diseases, including cirrhosis and hepatocellular carcinoma, because most patients fail to clear the virus and the persistent infection that follows (3-5). Although HCV belongs to the Flaviviridae family, and has a genome structure similar to the other flaviviruses including yellow fever, dengue and West Nile virus, efficient cell culture system or small animal infection models have not yet been established (1, 6-8). The lack of a useful system for evaluating the viral replication not only hampers the understanding of the life cycle of this virus, but also prevents the development of adequate treatment for HCV infection. In an important development, a subgenomic HCV RNA replicon system containing HCV-IRES driving neomycin-resistant (neo') gene, and EMCV-IRES driving HCV nonstructural (NS) proteins, NS3 – NS5B has been developed (9) and has enabled the assessment of HCV replication in cultured cells. Although this represents a powerful tool in the study of HCV replication mechanisms and the search for potential antiviral agents, it was constructed with a limited HCV genotype, genotype 1, and replication has been limited to the human hepatocyte-derived cell line, Huh7 (7, 10, 11).

Recently, we used a HCV genotype 2a clone from a patient with fulminant hepatitis to develop a new HCV replicon system, JFH-1 (12).
JFH-1 replicon system showed improved colony formation efficiency and robust RNA replication in Huh7. In this study, we show that JFH-1 can replicate in two other hepatocyte-derived cell lines, HepG2 and IMY-N9, an HCV-replicable cell line formed by fusing human primary cultured hepatocyte and HepG2 (13).
Experimental Procedures

Cell culture system

Huh7 cells provided by Dr. Tetsuro Suzuki (National Institute of Infectious Diseases, Tokyo, Japan) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (DMEM-10) as previously described (12). HepG2 cells maintained at the Tokyo Metropolitan Institute for Neuroscience were cultured at 37°C in minimum essential medium containing 10% fetal bovine serum (MEM-10). IMY-N9 cells developed at the Tokyo Metropolitan Institute for Neuroscience by fusing the human hepatocytes and HepG2 cells as previously described were cultured in DMEM-10 (13).

HCV genotype 2a replicon constructs

The HCV genotype 2a clone, JFH-1, was isolated from a patient with fulminant hepatitis and used to build a replicon construct as reported previously (12, 14, 15). Construct pSGR-JFH1 (accession number: AB114136) was built by inserting a segment sequentially comprised of the EcoRI restriction enzyme site, the minimal T7 RNA promoter site, the 5' un-translated region (UTR) core fragment of JFH-1 with an additional guanidine upstream of the 5' end of the HCV sequence, the neo', the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES), nonstructural proteins (NS) 3 – 3'X fragment of JFH-1, and the XbaI restriction enzyme site into the pUC19 vector (Fig. 1).
mutant construct pSGR-JFH1/GND possesses a point mutation of the GDD motif of RNA-dependent RNA polymerase in NS5B, a change of D to N in the second position of the motif (Fig. 1). Another mutant construct pSGR-JFH1/H2476L was also used, which contains a point mutation A to T at nucleotide position 6113 to change H to L at amino acid position 2476 in the NS5B region (12).

RNA synthesis

The XbaI-digested replicon constructs were further treated with mung bean nuclease (New England Biolabs, Beverly, MA, USA) to remove four nucleotides and leave the correct 3’ end of the HCV cDNA. Digested plasmid DNAs were purified and used as templates for in vitro RNA synthesis using the MEGAscript™ T7 kit (Ambion, Austin, TX, USA). Synthesized HCV subgenomic RNA was treated with DNasel (RQ1™ RNase-free DNase, Promega, Madison, WI, USA) followed by acid phenol extraction to remove any remaining template DNA.

RNA transfection

Synthesized replicon RNA (0.1 ng to 100 ng) was adjusted to 10 µg with cellular RNA isolated from untransfected cells and transfected into Huh7 as previously described (12). HepG2 and IMY-N9-cells were also transfected by electroporation using the following procedures. Between 90 ng and 9 µg of synthesized replicon RNA was adjusted to 30 µg with untransfected cellular RNA,
and 30 µg of synthesized or adjusted RNAs were transfected into HepG2 or IMY-N9 cells. Trypsinized cells were washed with Opti-MEM I™ reduced serum medium (Invitrogen, Carlsbad, CA, USA) and resuspended with Cytomix buffer at 0.9x10^6 cells/ml for HepG2 cells or at 1.8x10^6 cells/ml for IMY-N9 cells. RNA (30 µg) was mixed with 400 µl of the cell suspensions, transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK), and pulsed at 260 V and 950 µF with the Gene Pulser II™ apparatus (Bio-Rad, Hercules, CA, USA). Transfected cells were immediately transferred to 24 ml of MEM-10 or DMEM-10 and divided among 3 culture dishes (10-cm, Corning Inc., Corning, NY, USA) coated with collagen (Cellgen, Koken Co., Ltd, Tokyo, Japan). G418 (0.8 - 1.0 mg/ml) (Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 16-24 h after transfection, and culture medium supplemented with G418 was replaced twice a week. Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet.

Analysis of G418-resistant cells

G418-resistant colonies were collected and used for further analysis as cell pellets; sparsely grown colonies were independently isolated using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan) and amplified until they were 80% to 90% confluent in 10-cm culture dishes for use in nucleic acid and protein analyses. Total RNA and genomic DNA were simultaneously isolated from
amplified clones using the ISOGEN™ reagent (Nippon Gene, Tokyo, Japan).

Another portion of the cell pellet was dissolved in RIPA buffer containing 0.1% SDS for protein analysis.

Northern blot analysis

Isolated RNA aliquots (6 µg or 4 µg) were separated on a 1% agarose gel containing formaldehyde, transferred to a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia, Buckinghamshire, UK), and immobilized by Stratalinker™ UV cross linker (Stratagene, La Jolla, CA, USA). Hybridization was carried out with [α-32P] dCTP-labeled DNA probe using Rapid-Hyb™ buffer (Amersham Pharmacia). The DNA probes were synthesized from neo' and EMCV IRES genes using the Megaprime™ DNA labeling system (Amersham Pharmacia). The DNA probe of β-actin was also synthesized as a control.

Genomic DNA PCR

To detect neo' gene integration into the genomic DNA, isolated cellular genomic DNA was amplified by PCR using neo' gene-specific primers (NEO-S3, 5'-AACAAGATGGATTGCACGCA-3'; NEO-R, 5'-CGTCAAGAAGGCGATAGAAAG-3'). To confirm the integrity of the genomic DNAs isolated from replicon cells, the β-globin gene was also amplified using primers GH-20 (5'-GAAGAGCCAAGGACAGGTAC-3') GH21
(5'-GGAAAATAGACCAATAGGCAG-3') as described previously (16).

Western blot analysis of HCV proteins

The protein samples were separated on 7.5% - 15% gradient polyacrylamide gels (Biocraft, Tokyo, Japan) and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon™, Millipore, Bedford, MA, USA). Transferred proteins were incubated with blocking buffer containing 5% non-fat dry milk (Snow Brand, Sapporo, Japan) in phosphate-buffered saline (PBS). Mouse polyclonal antibody specific for NS5A protein was produced by DNA immunization with the JFH-1 NS5A expressing construct according to the method described previously (17). HCV proteins were detected using anti-NS5A mouse polyclonal antibody, and peroxidase-labeled goat anti-rabbit Ig (BIOSOURCE, Camarillo, CA, USA). Detection was carried out with a chemiluminescence system (ECL Plus™, Amersham Pharmacia) using JFH-1 replicon replicating in Huh7 cells as positive controls (12 and unpublished; shown in lanes 4-1 and C6 in Fig. 5).

Indirect immuno-fluorescence

Immuno-fluorescence analysis was performed as previously described (12). Briefly, cells grown on a cover glass were fixed in cold acetone-methanol, blocked with IF buffer (PBS, 1% bovine serum albumin, 2.5 mM EDTA), and incubated for 1 h with the above described anti-NS5A mouse antibody diluted
1:50 with IF buffer. Subsequently, the cells were washed and incubated for 1 h with a fluorescein isothiocyanate conjugated anti-mouse IgG antibody (Cappel, Durham, NC, USA) diluted 1:50 with IF buffer. Coverslips were washed and mounted on glass slides with PermaFluor™ mounting solution (Immunon, Pittsburgh, PA, USA), and cells were examined under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

RT-PCR and sequencing analysis

The cDNAs of the HCV RNA replicon were synthesized from total RNA isolated from replicon RNA transfected cells. These cDNAs were subsequently amplified with DNA polymerase (TaKaRa LA™ Taq, Takara Bio Inc., Otsu, Japan). Five separate PCR primer sets were used to amplify nt 65 - 390, nt 150 - 2959, nt 2909 - 5598, nt 5568 – 7695, and nt 7627 - 7930 of the replicon construct to cover the entire open reading frame. The sequence of each amplified DNA fragment was determined with the ABI 3100 automatic DNA sequencer (Applied Biosystems Japan, Tokyo, Japan).

Quantification of replicon RNA by real time detection RT-PCR

Copy numbers of replicon RNA in cells were determined by real-time detection RT-PCR (RTD-PCR) using the ABI Prism 7700™ sequence detector system (Applied Biosystems Japan) (18). The data were adjusted by measuring intracellular GAPDH concentration with RTD-PCR according to the
manufacturer's instruction.

Statistics

Statistical analysis was conducted with the Mann-Whitney U-test, and $p$ values of $< 0.05$ were considered significant.
Results

Replication of JFH-1 replicon in HepG2 and IMY-N9 cells

To estimate the replication ability of the JFH-1 replicons in various cells, RNA transcribed from linearized pSGR-JFH1 and negative control pSGR-JFH1/GND with a mutation in the NS5B polymerase catalytic domain preventing replication (Fig. 1), were transfected into HepG2 and IMY-N9, along with Huh7. Transfected cells were cultured for 3 weeks with G418 at a working concentration of 0.8 – 1.0 mg/ml. Three weeks later, visible colonies were observed in all three cell lines that were transfected with transcribed replicon RNA from pSGR-JFH1, although the numbers of colonies in HepG2 and IMY-N9 was substantially lower than that in Huh7 (Fig. 2). All the transfections of synthesized RNA from pSGR-JFH1/GND resulted in no visible colony formation. In our previous study, colony formation efficiency of pSGR-JFH1 RNA in Huh7 was $5.32 \times 10^4 \pm 5.02 \times 10^4$ colony forming units (CFU) / µg RNA (12). Likewise, the colony formation efficiencies in HepG2 and IMY-N9 were estimated to be $1.31 \times 10^2 \pm 0.74 \times 10^2$ and $1.88 \times 10^1 \pm 1.49 \times 10^1$ CFU / µg RNA, respectively, based on three independent assays. Then, nine colonies were cloned from pSGR-JFH1 RNA transfected HepG2 and IMY-N9 cells and cultured for further analysis.

We previously determined that a point mutation of H to L at amino acid position 2476 (H2476L) in the JFH-1 replicon increases colony formation
efficiency and enlarges colony size (12). We tested the colony formation of this JFH1/H2476L mutant replicon in HepG2 and IMY-N9 cells in this study. Colony formation efficiency of JFH1/H2476L RNA in Huh7 was $1.45 \times 10^5 \pm 2.38 \times 10^4$ CFU / µg RNA as reported previously (12, Fig. 2). Likewise, the colony formation efficiencies when using JFH1/H2476L RNA in HepG2 and IMY-N9 also improved to $3.02 \times 10^2 \pm 1.48 \times 10^2$ and $6.29 \times 10^1 \pm 4.78 \times 10^1$ CFU / µg RNA, respectively, based on 5 independent assays (Fig. 2). The average colony sizes of JFH1 replicon were estimated as $0.72 \pm 0.15$ mm$^2$ in HepG2 and $0.50 \pm 0.09$ mm$^2$ in IMY-N9 cells, respectively (Fig. 2). Similarly, the average colony sizes of JFH1/H2476L were increased to $1.50 \pm 0.30$ mm$^2$ in HepG2 and $1.35 \pm 0.56$ mm$^2$ in IMY-N9 cells, respectively (Fig. 2).

Detection of replicon RNA

To estimate the size of the replicating replicon RNA, Northern blot analysis was performed with 9 clones of each cell line, HepG2 and IMY-N9. In all of the clones, replicon RNA with the expected size of approximately 8 kb was detected using neo$^-$ and EMCV IRES probes (Fig. 3). The amount of replicon RNA in IMY-N9 was higher than that in HepG2, although it varied among the clones.

To rule out the possibility that G418 resistance resulted from integration of the neo$^-$ gene into the cellular genome, the integrated neo$^-$ gene was estimated by PCR with genomic DNA isolated from each cell clone. However,
the \textit{neo}$^\prime$ gene was not detected in any clones of either cell line, HepG2 or IMY-N9 (Fig. 4). To confirm the integrity of isolated genomic DNA from replicon cells, the \(\beta\)-globin gene was also amplified as described previously (16), and positive signals were present in all DNA samples (Fig. 4). Thus, each DNA sample was shown to be sufficiently intact to allow amplification of an endogenous or integrated gene. These results demonstrated that the G418 resistance in JFH-1 replicon RNA-transfected HepG2 and IMY-N9 cells was not due to \textit{neo}$^\prime$ gene integration but replication of JFH-1 replicon RNA.

Detection of HCV protein expression

Expression of HCV non-structural proteins in replicon RNA-transfected HepG2 and IMY-N9 cells was detected by Western blotting using the mouse polyclonal antibody specific for HCV NS5A. NS5A protein, primarily about 56 kDa in size, was detected by HCV-specific polyclonal antibody in JFH-1 replicon RNA-transfected IMY-N9 clones and in HepG2 clones (Fig. 5A). However, signals of these proteins in IMY-N9 clones were stronger than those in HepG2 clones, and additional smaller bands were observed in IMY-N9 clones (Fig. 5B).

HCV antigens were also detected in JFH-1 replicon RNA-transfected HepG2 and IMY-N9 clones using NS5A-specific polyclonal antibody (Fig. 6). Fine reticular and granular cytoplasmic staining was observed in both replicon RNA-transfected cell clones, although no signal was detected in un-transfected parental cells.
Sequence analysis of cloned replicon cells

To estimate the adaptive mutations in HepG2 and IMY-N9, replicon RNA isolated from each clone was amplified by RT-PCR and sequenced directly. Copy numbers of replicating RNA in clones were also determined by RTD-PCR, and compared with the data in Huh7. Based on multiple measurements, the mean copy number of replicating RNA in Huh7 was estimated to be $2.59 \times 10^7 \pm 1.97 \times 10^7$ copies / µg RNA (unpublished data).

Of the 9 HepG2 clones, seven clones had 1 – 3 non-synonymous mutations, and 2 clones had no or one synonymous mutation in the HCV-derived region of the replicon (Table 1). Most of the non-synonymous mutations were concentrated in NS5B (8 / 11 non-synonymous mutations). Copy numbers of HepG2 clone replicon RNA ranged from $6.67 \times 10^5 - 2.55 \times 10^7$ copies / µg RNA, with an average of $6.32 \times 10^6 \pm 7.74 \times 10^6$ copies / µg RNA. These replicon titers in clones were almost in agreement with data from Northern blotting. Copy numbers of replicon RNA in clones with no or one synonymous mutation were at the same level as in clones with non-synonymous mutations.

Of the 9 IMY-N9 clones, three clones had no mutations in the HCV-derived region (Table 2). Six clones had one or two synonymous mutations, and the affected regions were distributed randomly over the several regions. Copy numbers of replicon RNA in IMY-N9 clones ranged from $1.71 \times 10^7 - 9.36 \times 10^7$ copies / µg RNA, with the average being $3.98 \times 10^7 \pm 2.78 \times 10^7$. 


copies / µg RNA. This was significantly higher than in HepG2 cells ($p < 0.005$), and exceeded that in Huh7. As in HepG2, the replicon RNA titers in all the cloned IMY-N9 replicon cells were similar regardless of whether the replicon RNA contained these mutations. This indicates that the JFH-1 replicon can replicate efficiently without any amino acid mutations in HepG2 and IMY-N9 cells.
Discussion

The HCV replicon system is the only system able to mimic HCV replication in cultured cells, and it is utilized to assess antiviral drugs or to clarify the alteration of gene expression in host cells during HCV replication. However, the limited availability of isolate genotype 1 and culture cell line Huh7 posed a limitation to the investigations viral and host characteristics in nature because it is questionable whether the data from these limited conditions reflect the general situation. Thus, studies with clones of multiple genotypes in multiple cell lines were required. We previously demonstrated that the genotype 2a replicon could efficiently replicate in Huh7, and here we showed that it can also replicate in other hepatocyte-derived cells, HepG2 and IMY-N9 cells.

Sufficiently replicable cell culture systems or infection models with small animals are essential for understanding the viral life cycle. With regard to HCV, many studies on the infection or replication system in culture cells have been undertaken (7, 13, 19-25). For example, primary human hepatocytes can support HCV replication (21, 24, 25), but this susceptibility is limited to 90 days (25). Thus, it is conceivable that some important cellular factors that support the HCV replication may be present only in differentiated human hepatocytes. As an HCV replication system with culture cells, IMY-N9 cells were developed by fusing human primary cultured hepatocytes and HepG2 (13) and can support HCV replication at a higher level than the parental HepG2. However, HCV
replication in these culture cells seems to be insufficient, as replicated RNA could only be detected by RT-PCR. Thus, we exploited the IMY-N9 cell line for the HCV replicon system, and obtained evidence of HCV replicon replication not only by RT-PCR but also by Northern blotting. After transfection of JFH-1 replicon RNA and selection with G418, visible colonies were observed in IMY-N9 and HepG2 as in Huh7 (Fig. 2). No replicon DNA integration was detected by genomic PCR analysis (Fig. 4). The colony forming efficiencies were better for HepG2 than for IMY-N9, although both were substantially lower than that of Huh7. The reasons for these differences are unclear, but one possible explanation is the difference in transfection efficiency. In fact, the transfection efficiency of IMY-N9 was lower than that of HepG2 and Huh7 (data not shown).

On the other hand, the ability of IMY-N9 to support HCV replication seemed to be higher than that of HepG2 or even that of Huh7. The amount of replicating replicon RNA was higher in IMY-N9 clones than in HepG2 clones in Northern blotting (Fig. 3). Likewise, the mean replicon titer was higher in IMY-N9 clones than in HepG2 or Huh7. In Western blotting, the expression levels of HCV antigens were also stronger in IMY-N9 clones than in HepG2 clones (Fig. 5). Thus, IMY-N9 may contain some yet unidentified cellular factors, which are advantageous for supporting HCV replication and which may be acquired by fusing with human primary hepatocytes. In Western blotting, additional smaller bands were observed in IMY-N9 clones using anti-NS5A. They may result from degraded proteins because of instability of HCV-related proteins in IMY-N9.
Another possibility is that the incomplete translated proteins may be related to the robust replication of replicon RNA in IMY-N9 cells.

Blight et al. reported that RNA replication can only be detected in a subpopulation of Huh7 cells and that self-replicating subgenomic RNA could be eliminated from Huh7 clones by prolonged treatment with alpha interferon (26). For cells from which self-replicating subgenomic RNA was eliminated, a higher proportion could support HCV replication. Selection of subclones by interferon treatment may be an especially valuable step in the process of achieving increased HCV replication efficiency of HepG2 and IMY-N9 cells. Thus, interferon sensitiveness of JFH-1 replicon in Huh7, HepG2 and IMY-N9 cells should be evaluated to establish such subclones.

In our previous study, the JFH-1 replicon was found to replicate in Huh7 cells without non-synonymous mutation in the HCV-derived region, although the RNA titer of the replicon was lower than that of clones with mutations (12). The H2476L mutation found in Huh7 replicon cells was recognized as an adaptive mutation since the replicons containing this mutation had increased colony formation efficiency and colony size (12). JFH1/H2476L mutant replicon was tested to form colonies in HepG2 and IMY-N9 cells in this study and found to also increase colony formation efficiency and colony size (Fig. 2). Thus, H2476L also functions as an adaptive mutation in HepG2 and IMY-N9 cells. It should be determined that a portion of the mutations found in HepG2 and IMY-N9 replicon clones may also function as adaptive mutations.
In this study, 2 of 9 HepG2 clones and 3 of 9 IMY-N9 clones had no or one synonymous mutation in the HCV-derived region (Tables 1 and 2). However, unlike in Huh7, the RNA titer in these clones was not lower than that of clones with mutations in HepG2 and IMY-N9 cell lines. Therefore, adaptive mutation might not be needed in the JFH-1 replicon in order to replicate efficiently. Based on sequence analysis of isolated clones in HepG2, mutated amino acids were concentrated in the NS5B region. On the other hand, there were fewer mutated amino acids in IMY-N9, than in HepG2, and they were distributed randomly. These differences may be due to differences in cellular factors between HepG2 and IMY-N9. In HepG2, mutations of NS5B in the JFH-1 replicon may enhance the colony forming ability or replication capacity. In IMY-N9, replicon RNA was found to replicate efficiently using their native genome, and mutations are not introduced as frequently. However, the effect of these mutations in HepG2 and IMY-N9 replicon clones should be determined by examining the replication of mutations introduced in replicon constructs.

Although many attempts have been made to establish the replicon system with other human hepatocyte-derived cell lines, these efforts have been unsuccessful, with the exception of Huh7. Our investigation revealed that incorporating the HCV replicon system in two other hepatocyte-derived cell lines, HepG2 and IMY-N9, is possible. The major factor of this accomplishment is the difference in the replicon clone used. The HCV clone used to produce the JFH-1 replicon was of genotype 2a and was isolated from a fulminant hepatitis...
patient. This replicon clone has potent replication ability beyond that previously reported in a clone that had adaptive mutations in its genome. Furthermore, it can replicate in Huh7 without any adaptive mutations. To estimate the tissue tropisms of HCV using this robust replicable replicon, further trials in non hepatocyte-derived cells should be undertaken. Recently, replication of HCV genotype 1b replicon in HeLa and mouse hepatoma cells has been reported (27). However, RNA replication was only observed when cellular RNA isolated from Huh7 replicon cells was transfected, and the replicon isolated from these clones contained several adaptive mutations. It is informative to assess whether the JFH-1 replicon can replicate in these cell lines without mutations.

The full-length RNA replicon with isolates Con1 and HCV-N has already been investigated (28, 29). However, no evidence of HCV particle assembly and release from Huh7 has been observed. Thus, Huh7 may be devoid of the factors associated with these steps. By using the full-length replicon with JFH-1 clone in HepG2 or IMY-N9 cells, HCV particle assembly and release may be accomplished, but further investigation is required.

In summary, HCV genotype 2a replicon could replicate in HepG2 and IMY-N9 cells. Studies using this replicon system will shed light on understanding the mechanisms of HCV replication, host-viral interaction, and anti-viral drug evaluation.
References


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Figure legends

Fig. 1. **Structure of the subgenomic HCV RNA replicon construct, pSGR-JFH1 (upper), pSGR-JFH1/H2476L (middle) and pSGR-JFH1/GND (lower).**

Open reading frames (thick boxes) are flanked by un-translated regions (thin boxes). *EcoRI* and *XbaI* indicate positions of the respective restriction sites. A T7 RNA promoter is located upstream of the 5′ end of the replicon construct. An adaptive mutant replicon in Huh7, pSGR-JFH1/H2476L, was constructed by introducing a point mutation of A to T at nucleotide position 6113 to change H to L at amino acid position 2476 in the NS5B region (12). GDD is the motif of HCV NS5B, RNA-dependent RNA polymerase. pSGR-JFH1/GND was constructed as a negative control by inducing a point mutation changing GDD to GND.

Fig. 2. **Colony formation of JFH-1 HCV subgenomic RNA replicon in Huh7, HepG2 and IMY-N9 cell lines.**

Transcribed RNAs from pSGR-JFH1, pSGR-JFH1/H2476L and pSGR-JFH1/GND were transfected into each cell line and cells were cultured with G418 for 3 weeks before staining with crystal violet as described in the Experimental Procedures.
Fig. 3. **Detection of replicon RNA in cloned HepG2 (A) and IMY-N9 (B).**

Total RNA (6 µg for HepG2 and 4 µg for IMY-N9) from cloned cells in each cell line was analyzed by Northern blotting with the DNA probes of the neo’ - EMCV IRES and β-actin genes. *In vitro* synthesis of $10^8$ and $10^7$ copies of transcribed + strand RNAs were loaded as controls as indicated. Arrow heads indicate target positions of replicon RNA and β-actin.

Fig. 4. **Detection of neo’ gene integration in the genomic DNA of cloned cells.**

Genomic PCR was performed with cellular DNA from pSGR-JFH1 RNA transfected HepG2 cell clones (upper) and IMY-N9 cell clones (lower) using neo’ and β-globin gene-specific primers. The neo’ gene DNA was mixed with DNA extracted from Huh7 and used as a positive control (lane P) and genomic DNA from un-transfected cells as a negative control (lane H, HepG2 and Lane I, IMY-N9). Fragment target sizes are indicated by arrow heads or arrows on the right side.

Fig. 5. **Detection of HCV NS5A antigens in cloned cells of HepG2 (A) and IMY-N9 (B) by Western blot analysis.**

Cell lysates were prepared from pSGR-JFH1 RNA transfected Huh7 cell clones (lanes 4-1 and C6) as positive controls, or un-transfected parental HepG2 and IMY-N9 (lane N) as negative controls. Anti-NS5A polyclonal
antibodies were used to detect HCV antigens. The target size of NS5A proteins is indicated by arrow heads.

Fig. 6. **Subcellular localization of HCV antigens determined by immuno-fluorescence.**

Replicon RNA un-transfected or cloned HepG2 and IMY-N9 were cultured on cover slips, fixed in acetone-methanol, and incubated with anti-NS5A polyclonal antibodies. Representative clones of HepG2 and IMY-N9 are indicated.
TABLE I. Mutations of JFH-1 replicon in HepG2 cell

<table>
<thead>
<tr>
<th>Clone</th>
<th>Nucleotide(^{a})</th>
<th>Amino acid(^{b})</th>
<th>Affected region</th>
<th>Replicon titer(^{c})</th>
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<tr>
<td>1</td>
<td>6826 C→A</td>
<td>2714 Q→K</td>
<td>NS5B</td>
<td>2.55 x 10(^7)</td>
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<tr>
<td>5</td>
<td>7125 T→A</td>
<td>none</td>
<td></td>
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<tr>
<td>8</td>
<td>6580 T→A</td>
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<tr>
<td></td>
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<td>NS5B</td>
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<td>1718 R→G</td>
<td>NS4B</td>
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<td>6601 C→A</td>
<td>2639 P→T</td>
<td>NS5B</td>
<td></td>
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<td>6110 A→G</td>
<td>2475 Y→C</td>
<td>NS5B</td>
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</table>

\(^{a}\) position of mutated nucleotide within the subgenomic replicon

\(^{b}\) position of mutated amino acid within the complete ORF of full-length JFH1

\(^{c}\) represented as copies / µg RNA
<table>
<thead>
<tr>
<th>Clone</th>
<th>Nucleotide$^a$</th>
<th>Amino acid$^b$</th>
<th>Affected region</th>
<th>Replicon titer$^c$</th>
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<tr>
<td>3</td>
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<td>P→A</td>
<td>NS4B</td>
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<td>M→I</td>
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<td>6</td>
<td>C→A</td>
<td>D→E</td>
<td>NS3</td>
<td>2.04 x 10$^7$</td>
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<tr>
<td>7</td>
<td>A→G</td>
<td>T→A</td>
<td>NS5A</td>
<td>8.12 x 10$^7$</td>
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<tr>
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<td>G→C</td>
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<tr>
<td>8</td>
<td>T→G</td>
<td>V→G</td>
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<td>11</td>
<td>T→A</td>
<td>L→Q</td>
<td>NS3</td>
<td>2.54 x 10$^7$</td>
</tr>
</tbody>
</table>

$^a$ position of mutated nucleotide within the subgenomic replicon

$^b$ position of mutated amino acid within the complete ORF of full-length JFH1

$^c$ represented as copies / µg RNA
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 6
Genotype 2a hepatitis C Virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells
Tomoko Date, Takanobu Kato, Michiko Miyamoto, Zijiang Zhao, Kotaro Yasui, Masashi Mizokami and Takaji Wakita

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