Doctoral thesis

Establishment of evaluation systems of antiviral agents for hepatitis C virus and analysis of cyclophilin inhibitors as anti-HCV drugs

（抗 **C** 型肝炎ウイルス剤評価系の構築とそれを用いた抗 **HCV** 薬としてのシクロフィリン阻害剤の阻害メカニズム解析）

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Preface

This thesis is based on the following publications.

Arai M, Tsukiyama-Kohara K, Takagi A, Tobita Y, Inoue K, Kohara M. Resistance to cyclosporine A derives from mutations in Hepatitis C virus nonstructural proteins. Biochem Biophys Res Commun. 2014;

Arai M, Tokunaga Y, Takagi A, Tobita Y, Hirata Y, Ishida Y, Tateno C, Kohara M. Isolation and characterization of highly replicable hepatitis C virus genotype 1a strain HCV-RMT. PLoS One. 2013;8:e82527

Arai M, Suzuki H, Tobita Y, Takagi A, Okamoto K, Ohta A, Sudoh M, Kohara M. Establishment of infectious HCV virion-producing cells with newly designed full-genome replicon RNA. Arch Virol. 2011;156:295-304

Summary

For the development of anti-viral drugs against hepatitis C virus (HCV) infection, the efficient and effective infection systems are required. HCV replicon systems enable in-depth analysis of the life cycle of HCV. However, the previously reported full-genome replicon system is unable to produce authentic virions.

First, I constructed newly designed full-genomic replicon RNA, which is composed of the intact 5′-terminal-half RNA extending to the NS2 region flanked by an extra selection marker gene. Huh-7 cells harboring this full-genomic RNA proliferated well under G418 selection and secreted virion-like particles into the supernatant. These particles, which were round and 50 nm in diameter when analyzed by electron microscopy, had a buoyant density of 1.08 g/mL that shifted to 1.19 g/mL after NP-40 treatment; these figures match the putative densities of intact virions and nucleocapsids without envelope. The particles also showed infectivity in a colony-forming assay. This system may offer another option for investigating the lifecycle of HCV.

Next, multiple genotype 1a clones have been reported, including the very first HCV clone called H77. The replication ability of some of these clones has been confirmed *in vitro* and *in vivo*, although this ability is somehow compromised. I now report a newly isolated GT1a clone, designated HCV-RMT, which has the ability to replicate efficiently in patients, chimeric mice with humanized liver, and cultured cells. An authentic subgenomic replicon cell line was established from

the HCV-RMT sequence with spontaneous introduction of three adaptive mutations, which were later confirmed to be responsible for efficient replication in HuH-7 cells as both subgenomic replicon RNA and viral genome RNA. Following transfection, the HCV-RMT RNA genome with three adaptive mutations was maintained for more than 2 months in HuH-7 cells. One clone selected from the transfected cells had a high copy number, and its supernatant could infect naïve HuH-7 cells. Direct injection of wild-type HCV-RMT RNA into the liver of chimeric mice with humanized liver resulted in vigorous replication, similar to inoculation with the parental patient's serum. A study of virus replication using HCV-RMT derivatives with various combinations of adaptive mutations revealed a clear inversely proportional relationship between *in vitro* and *in vivo* replication abilities. Thus, I suggest that HCV-RMT and its derivatives are important tools for HCV GT1a research and for determining the mechanism of HCV replication *in vitro* and *in vivo*.

Cyclosporine A (CsA) is an immunosuppressive drug that targets cyclophilins, cellular cofactors that regulate the immune system. Replication of HCV is suppressed by CsA, but the molecular basis of this suppression is still not fully understood. To investigate this suppression, I cultured HCV replicon cells (Con1, HCV GT1b, FLR-N cell) in the presence of CsA and obtained nine CsA-resistant FLR-N cell lines. I determined full-length HCV sequences for all nine clones, and chose two (clones #6 and #7) of the nine clones that have high replication activity in the presence of CsA for further analysis. Both clones showed two consensus mutations, one in NS3 (T1280V) and

the other in NS5A (D2292E). Characterization of various mutants indicated that the D2292E mutation conferred resistance to high concentrations of CsA (up to 2 μM). In addition, the missense mutation T1280V contributed to the recovery of colony formation activity. The effects of these mutations are also evident in two established HCV replicon cell lines—HCV-RMT (GT1a) and JFH-1 (GT2a). Moreover, three other missense mutations in NS5A—D2303H, S2362G, and E2414K— enhanced the resistance to CsA conferred by D2292E; these double or all quadruple mutants could resist approximately 8- to 25-fold higher concentrations of CsA than could wild-type Con1. These four mutations, either as single or combinations, also made Con1 strain resistant to two other cyp inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811) or Debio-025. Interestingly, the changes in IC_{50} values that resulted from each of these mutations were the lowest in the Debio-025-treated cells, indicating its highest resistant activity against the adaptive mutation.

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In addition, I express my gratitude to my beloved daughters, Ms. Mifuyu Arai, Chiharu Arai, and wife, Mrs. Kyoko Arai, who always provided moral and material support for me.

Abbreviations

A. A.	: amino acid
bla	: beta lactamase
BSA	: bovine serum albumin
cDNA	: complementary DNA
CsA	: cyclosporine A
cyp	: cyclophilin
ELISA	: enzyme-linked immunosorbent assay
EMCV	: encephalomyocarditis virus
FMDV	: foot-and-mouth disease virus
GT	: genotype
HCV	: hepatitis C virus
IFN	: interferon
IRES	: internal ribosome entry site
IC50	: 50 % inhibitory concentration
JFH-1	: Japan fulminant hepatitis-1

mAb	: monoclonal antibody
neo	: neomycin phosphotransferase II
NS	: non-structural
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
ORF	: open reading frame
RACE	: rapid amplification of cDNA end
RBV	: ribavirin
RNase	: ribonuclease
RT-PCR	: reverse transcription PCR
SCID	: severe combined immune deficient
SVR	: sustained anti-viral response
UTR	: untranslated region

Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [73]. With over 170 million people currently infected [13], HCV is a growing public health burden.

The life cycle of HCV has been difficult to study, because cell culture and small animal models of HCV infection are not available. The recent development of HCV replicon systems has permitted the study of HCV translation and RNA replication in human hepatoma-derived Huh-7 cells *in vitro* [57]. However, these replicon systems cannot produce authentic virions, because they lack the infection steps, and analysis of these infection steps is very important for understanding HCV pathogenesis.

Recently, some groups have successfully established *in vitro* infection systems [56, 66, 85, 91, 92, 94]. The strategies of these systems are basically the same as that of transfection of *in vitro*-generated HCV genome RNA into Huh-7 cell or its derivatives [7]. The non-structural regions used in those studies were from the 2a genotype JFH-1 clone or the 1a genotype H77 clone. The former is known for its exceptionally vigorous amplification and broad permissiveness in culture cells other than Huh-7 [15, 44, 45], while the latter shows only poor replication ability. Another group reported a newly established immortalized hepatocyte susceptible to HCV infection, but only modest improvement was achieved [40]. There are also reports of a system using a full-genome

replicon that has the entire coding region under the control of the EMCV-IRES; however, this system also failed to show infectivity in the G418 selection assay [36, 67], and secretion of particles with the putative nature of HCV virions could not be confirmed [16].

I now report the establishment of infectious virion-producing replicon cells that utilize an ordinary GT1b replicon strain. In order to address the contribution of structural and non-structural gene products to the maturation of HCV particles *in vitro*, I partitioned these regions in the same cistron of the full-genomic sequence, thereby enabling the functions of these structural and non-structural genes to be separately studied. Thus, I termed this construction "divided open reading frame carrying" full-genome replicon, or dORF replicon.

Virus particles secreted from cells containing dORF replicon RNA, as confirmed morphologically using electron microscopy, were shown to be able to infect Huh-7 cells. Replication of dORF replicon RNA was so efficient that infected cells could survive and proliferate under G418 selection to form colonies, as seen in transfection of replicon RNA. In addition, a reporter gene was successfully inserted into the construct, and activity of the reporter gene could be transmitted to naive Huh-7 cells by infection.

I believe that the success of this system is due to the difference in the construction of the replicon, namely, having the intact 5′ half extending to NS2 instead of being divided at the beginning of the core region. Although further investigation is required to elucidate whether the encapsidation

signal of HCV is located through the region that is divided in the full-genome replicon, this is the first report to describe genome-length replicon-containing cells that can produce virus particles that have the putative nature of the HCV virion, in terms of both morphological and biological characteristics.

Next, for *in vitro* research, establishment of an HCV replicon system [6, 57] was an important achievement that allowed research into the function of individual non-structural viral proteins. However, the entire viral life cycle remains enigmatic because no structural proteins are needed in this system. Some reports have been published about full-length replicons with structural proteins in addition to non-structural proteins, although little [36] or no [67] secretion of infectious virions were observed, which may have been partly due to adaptive mutations. Another breakthrough was made with the discovery of a GT2a JFH-1 strain that soon became well known for its vigorous replication as a replicon with no adaptive mutations [15]. JFH-1 can also infect and propagate in cultured cells as a virus, especially in HuH-7 cells or their derivatives [56, 85, 94]. After the discovery of JFH-1, two methods were available for the investigation of how viral proteins other than those of HCV GT2a function during their entire life cycle. The first method was only for structural proteins and involved making a hybrid of the structural region of the clone of interest and the non-structural regions of JFH-1 for efficient replication [26, 66, 91]. The other method utilized the entire viral genome sequence of genotype 1 and made them infectious to HuH-7 derivative cells

by introducing known adaptive mutations [53, 92] or enhancing replication with a casein kinase inhibitor [68]; however, their replication abilities were somehow compromised. In this study, I report the isolation of a new GT1a strain from a patient's serum sample that was highly infectious to human hepatocyte-transplanted chimeric mice, as the viral titer in the blood of the mice was higher than 10^8 copies/ml. I evaluated its replication abilities in four replication systems: subgenomic replicon, virus, *in vitro* infection, and *in vivo* infection. The new HCV clone, which was designated HCV-RMT (GenBank accession number, AB520610), was different from other GT1a clones because it did not require any artificially introduced adaptive mutations for the establishment of replicon cells. With these features, our newly cloned HCV-RMT may be a useful tool for investigating the entire life cycle of genotype 1 HCV.

The genome of the HCV is a single-stranded RNA with positive polarity and is classified in the *Flaviviridae* family. HCVs have been classified into six major genotypic groups (genotypes 1-6) based on genomic RNA sequences; genotype 1 is the most prevalent over most of the world [61]. Treatments with alpha interferon (IFN-α), together with the nucleoside analog ribavirin (RBV), greatly increased the percentage of HCV chronically infected patients able to reach a SVR. Covalent attachment of polyethylene glycol (PEGylated) IFN-α-plus-RBV therapy has a success rate of ~80% in patients with genotype 2 or 3 infections, but only ~50% in patients with genotype 1 infections [48, 78]. The recently approved protease inhibitors boceprevir and telaprevir each

improved the efficacy of IFN-α-plus-RBV therapy [5]. These direct-acting agents (boceprevir, simeprevir, sofosbuvir, faldaprevir and telaprevir etc.) each have the advantage of being highly specific, but each may select for specific resistant mutations, limiting their long-time efficacy. Therefore, antiviral inhibitors targeting host factors crucial for viral replication should be developed to overcome these problems.

Reportedly, several HCV proteins interact with cyclophilins (Cyp) and modulate HCV replication [19, 22, 88]. To date, three Cyp inhibitors—Debio-025, NIM811, and SCY-635—have been deemed safe and effective for patients with HCV in phase I and II studies[21, 32, 52]. Development of Debio-025 has advanced the farthest through phase II studies, and Debio-025 has approved and showed a great deal of promise for decreasing HCV viremia in infected patients. However, emergence of drug-resistant HCV mutants could limit the therapeutic potential of CsA and Cyp inhibitors.

The HCV genome is a positive-sense, single-stranded RNA (about 9.6 kb) that encodes at least 10 viral proteins; these are categorized as structural core proteins (E1, E2) or nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [43, 64]. The nonstructural proteins are involved in HCV RNA replication [64]. NS5A protein comprises three domains linked by two low-complexity sequences (LCS) that are either serine or proline rich; domain I is a highly structured zinc binding domain whose three-dimensional structure shows two dimeric conformations [58, 81]. Domains II

and III have been shown to be unstructured in their native states, but nuclear magnetic resonance and circular dichroism have shown that elements of secondary structure run throughout each of these domains [20, 30, 54]. NS5A is anchored to membranes by an N-terminal amphipathic helix and is an essential component of the viral genome replication complex; it also interacts with other non-structural proteins [77] or cellular factors. NS5A domain II is a substrate for the peptidyl-prolyl cis/trans isomerase activity of Cys A and B [29], and NS5A domain III is reportedly a substrate of CypA [84].

In this study, I used CsA to select for and isolate drug-resistant HCV mutants; I then performed virus genome sequencing to investigate the molecular mechanisms of this drug resistance.

Chapter 1:

Establishment of infectious HCV virion-producing cells with full-genome replicon RNA

1. Background and Aims

The life cycle of HCV has been difficult to study, because cell culture and small animal models of HCV infection are not available. The recent development of HCV replicon systems has permitted the study of HCV translation and RNA replication in human hepatoma-derived Huh-7 cells *in vitro* [57]. However, these replicon systems cannot produce authentic virions, because they lack the infection steps, and analysis of these infection steps is very important for understanding HCV pathogenesis.

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also failed to show infectivity in the G418 selection assay [36, 67], and secretion of particles with the putative nature of HCV virions could not be confirmed [16].

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I believe that the success of this system is due to the difference in the construction of the replicon, namely, having the intact 5' half extending to NS2 instead of being divided at the beginning of the core region. Although further investigation is required to elucidate whether the encapsidation signal of HCV is located through the region that is divided in the full-genome replicon, this is the

first report to describe genome-length replicon-containing cells that can produce virus particles that have the putative nature of the HCV virion, in terms of both morphological and biological characteristics.

2. Materials and Methods

Construction and RNA transcription

To construct dORF replicon RNA, the latter half of the NS2 region of the HCV-R6 strain [82] was replaced in frame with the FMDV 2A protease gene and the neomycin resistance gene, and the EMCV IRES. In addition, the region from NS3 to the beginning of NS5B was replaced with the 1bneo/delS replicon sequence made by the N strain of genotype 1b [28] (kindly provided by Dr. Seeger of Fox Chase Cancer Center). This construct was designated as the “divided open reading frame carrying full-genome” (dORF) replicon. The subgenomic replicon construct was also prepared from the R6 strain and also contained the 1bneo/delS replacement. For the reporter assay, the FMDV 2A protease gene and beta-lactamase gene (bla; Invitrogen, Waltham, CA, USA) were inserted after the remaining NS2 gene to produce the dORF bla replicon construct. Replication-deficient versions of these 3 replicons were also prepared by deleting 27 nucleotides, including the GDD motif of NS5B polymerase.

In vitro transcription of these replicon RNAs was performed using the MEGAscript kit (Ambion, Foster city, MA, USA).

Cell culture and electroporation

Huh-7 cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum. Replicon cells were maintained in the same medium supplemented with 300 μg/mL G418 (Invitrogen). These cells were passaged 3 times a week at a 4:1 splitting ratio. Electroporation of replicon RNA was performed as previously described [57]. The subgenomic replicon (1bneo/delS replicon) cells were treated with 1000 IU of IFN-α for 2 months and cloned by the limited dilution method. Two of these clones were designated as HCV replicon-cured Huh-7 cells F2 and K4. The cell line containing the full-genome replicon of genotype1b, namely the NNC#2 clone [47], was a kind gift from Dr. Shimotohno of Keio University.

Northern blot analysis and quantification of HCV RNA

Total RNA was purified from cells using ISOGEN (Nippon Gene, Tokyo, Japan) for northern blot analysis or ABI prizm6100 (Applied Biosystems) for real-time RT-PCR. Purified RNAs were quantified by absorbance at 260 nm. For northern blot analysis, 30 μg of each total RNA was used with the Northern Max kit (Ambion), according to the manufacturer's instructions. The probe for detection of HCV RNA was a PCR fragment of the NS5B region (nucleotide numbers 7629–7963) that had been biotin-labeled using the BrightStar Psoralen-Biotin kit (Ambion), according to the manufacturer's instructions. Following hybridization of the membranes, the probe

was detected using the BrightStar Biodetect kit (Ambion), according to the manufacturer's instructions, and luminescence was detected by the LAS1000 detection system (Fujifilm, Tokyo, Japan). Quantification of HCV RNA copy number with real-time RT-PCR was performed using an ABI PRISM 7900 system (Applied Biosystems), as described previously [80].

Western blot analysis

Western blot analysis was carried out according to the conventional semi-dry blot method. Cells were lysed with buffer containing 100 mM Tris-HCl (pH 7.4) and 4% sodium dodecyl sulfate. A 10-μg amount of protein from each sample was separated by SDS-PAGE through a 4–20% gradient gel (Invitrogen) and transferred to the membrane according to the gel manufacturer's protocol. The antibodies used in this study were anti-core mouse MAb, anti-E1 MAb, anti-E2 MAb (reported previously; [82]), anti-NS3 anti-sera (reported previously; [82]), anti-NS5B anti-sera (Upstate, New York, NY, USA), and anti-beta-actin MAb (Abcam, Cambridge, UK). Horseradish peroxidase-labeled anti-mouse and anti-rabbit IgG goat antibodies (Santa Cruz Biotechnology, Dallas, TX, USA and DAKO, Glostrup, Denmark respectively) were used as the secondary antibody. The membranes were treated using the ECL-plus kit (Amersham, Amersham, UK), according to the manufacturer's instructions, and luminescence was detected using the LAS1000 system (Fujifilm).

Density gradient analysis and core ELISA

Culture supernatants from replicon cells were loaded onto 10–60% sucrose density gradient tubes with or without 10-fold concentration with Amicon-100 (Millipore, Billerica, MA, USA). The tubes were then ultra-centrifuged at 100,000 *g* for 16 h and fractionated. A final concentration of 0.5% NP-40 was added to the culture supernatants and incubated at 4°C for 30 min. For electron microscopy, the culture supernatant was concentrated and loaded onto a 60% sucrose cushion and ultra-centrifuged at 100,000 *g* for 4 h. The interface between the concentrated medium and the sucrose cushion was collected and separated by the density gradient method described above. A 2-mL fraction from 5 ml to –7 mL from the bottom, with a density of 1.1–1.2 g/mL, was used for observation with electron microscopy after further concentration with the sucrose cushion ultra-centrifugation method described above. The amount of core protein in the fractions was quantified using an Ohso ELISA kit (Ohso, Tokyo, Japan), in accordance with the manufacturer's instructions.

Electron microscopy

The concentrated fraction of core protein was observed by scanning and transmission electron microscopy. For scanning electron microscopy, the sample was allowed to settle on the surface of poly-L-lysine-coated glass coverslips for 30 min, and the attached sample was then fixed

with 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min, washed with 0.1 M phosphate buffer 3 times, and post-fixed with 1% osmium tetroxide in the same buffer for 10 min. After dehydration through a graded series of ethanol, the samples were dried in a freeze dryer (Hitachi ES-2020, Hitachi, Tokyo, Japan) using t-butyl alcohol, coated with osmium tetroxide approximately 2 nm thick using an osmium plasma coater (NL-OPC80; Nippon Laser and Electronics Laboratory, Nagoya, Japan), and then examined using a Hitachi S-4800 field emission scanning electron microscope at an accelerating voltage of 10 kV [79]. For transmission electron microscopy, the sample was allowed to settle on a formvar-coated nickel grid for 10 min, dried in air, incubated with rabbit anti-E2RR6 antibody (prepared as described in the supplementary information), washed with PBS, and then incubated with goat anti-rabbit IgG coupled to 10-nm colloidal gold (British BioCell, Cardiff, UK). After negative staining with 2% uranyl acetate, the sample was examined using a JEM 1200EX transmission electron microscope (JEOL, Akishima, Japan) at an accelerating voltage of 80 kV.

Rabbit anti-E2 RR6 antibody to the HCV-E2 protein was prepared as follows. The E2 gene of HCV type 1b [82] was cloned under the control of the ATI-P7.5 hybrid promoter of vaccinia virus vector pSFB4 and allowed to recombine with the Lister strain of the vaccinia virus to give vector RVV. Rabbits were infected intradermally with 10^8 p.f.u. of RVV and 2 months later were boosted twice with the purified E2 protein. HCV-E2 protein was expressed by RVV and purified by lentil

lectin column chromatography and affinity chromatography using an anti-E2 MAb [82].

Infection

A 2.5-ml aliquot of cleared culture supernatants from replicon cells was added to approximately 70% confluent 25-cm^2 flasks of Huh-7 cells and the same amount of complete DMEM was added 2 h later. Infected cells were passaged into 75-cm^2 flasks the next day and into four 10-cm dishes 2 days later. A 300-μg/mL amount of G418 was added to the medium immediately after the second passage. The 3 types of Huh-7 cells used in this study were the one purchased from J.C.R.B. and the 2 IFN-cured replicon cell lines F2 and K4 described above. The medium was changed every other day. For the blocking experiment, cells were treated with the anti-CD81antibody, as described previously [94]. Cells were fixed with 10% formalin/PBS(-) for 10 min after washing with PBS(-) and staining with 1% crystal violet/PBS(-) for 1 h before washing with water.

Beta-lactamase detection assay

Beta-lactamase activity was detected using the GeneBLAzer *in vivo* kit (Invitrogen), according to the manufacturer's instructions, and observed with fluorescence microscopy (Nikon, Tokyo, Japan) with UV light excitation.

3. Results

dORF replicon RNA can replicate in Huh-7 cells

I began this study with transfection of the dORF replicon RNAs (Figure 3-1A). When 30 μg of each RNA was electroporated into 4×10^6 Huh-7 cells, the dORF and dORF bla RNA-transfected cells formed 20 and 5 colonies, respectively, after 3 weeks of G418 selection. No colonies appeared as a result of transfection of polymerase defective mutants (data not shown). Two of each colony were picked, amplified, and designated as dORF replicon cell #1 and #2, and dORF bla replicon cell #1 and #2. Some of these cells were then used for quantification of HCV RNA and northern blot analysis (Figure 3-1B). Northern blot analysis showed that these clones contained HCV RNAs of the expected size, and that the HCV RNA copy numbers of these clones did not differ substantially from that of the subgenomic replicon, indicating that replication ability had not been hampered by insertion of the structural genes, which is counter to what was expected. Western blot analysis showed that these clones express both structural and non-structural proteins (Figure 3-1C). These results confirmed that transfected dORF HCV RNAs can replicate in Huh-7 cells, just as authentic subgenomic replicon RNAs do.

dORF replicon cells secrete virus particles

In a previous study, HCV subgenomic replicon cells secreted RNase-resistant subgenomic RNA into the culture supernatant [16, 36, 67]. I also detected a similar amount of RNase-resistant HCV RNA in the culture supernatant of our dORF replicon cells, as well as of the subgenomic and full-genome replicon cells. These supernatants showed no significant differences in terms of distribution of HCV RNA in the buoyant density gradient analysis (Figures 3-2A, B, open square). In contrast, there was a clear difference between these supernatants after NP-40 treatment. While almost all the HCV RNA in the supernatant of the subgenomic replicon cells was erased by NP-40 treatment (Figure 3-2A, filled triangle), there remained a peak of HCV RNA at a density of 1.18 g/mL in the supernatant of the dORF replicon cells (Figure 3-2B, filled triangle). These results were confirmed by the same experiment, using concentrated culture supernatant (Figures 3-2C, D). I also reconfirmed the results of previous reports [36, 67], which showed no genomic RNA resistant to NP-40 treatment in the supernatant of full-genome replicon cells (Figure 3-2E). Secreted core proteins in the concentrated supernatant showed a different density gradient distribution compared to genomic RNA (Figure 3-2F, open circle), in that the core proteins were present between densities of 1.1–1.2 g/mL, while HCV RNA was more broadly distributed in the range of 1.06–1.22 g/mL. Thus, HCV RNA and core proteins were not always associated with each other. However, after NP-40 treatment, core proteins were found only in the same fraction as HCV RNA, at 1.19 g/mL (Figure

3-2F, filled triangle). Taken together with the results of the report mentioned above [67] , our replicon cells harboring dORF RNA appeared to secrete both particles with core proteins being assembled as nucleocapsids and those without core proteins and sensitive to NP-40 treatment, such as the particles from subgenomic and full-genome replicon cells. I concluded that the broader distribution of the HCV genome RNA in the density gradient than the core protein was caused by overlapping of the distribution of these 2 particle types, and that the remaining peaks of genome RNA and core protein after NP-40 treatment were of nucleocapsids that had had their envelopes stripped off by NP-40 [41].

According to our hypothesis, the distribution of core proteins in the density gradient represented that of the intact virion and, thus, I tried to observe virions directly by electron microscopy using the fraction in which the core protein was present. I easily identified numerous round-shaped virus particles approximately 50 nm in diameter by scanning electron microscopy (Figure 3-3A). Furthermore, when the immunogold method using anti-E2 RR6 antibody was applied to samples fixed on the mesh, transmission electron microscopy could be used to visualize virus particles labeled with colloidal gold (Figure 3-3B). These findings provide evidence of intact virion production from our dORF replicon cells.

Secreted virus particles can infect naive Huh-7 cells

Next, I examined the infectivity of these virus particles. The culture supernatants of these dORF replicon cells were collected, and 3 kinds of naive Huh-7 cells, one purchased from the J.C.R.B. (Japanese Collection of Research Bioresources) and the other 2 designated as the cured cells F2 and K4 generated by IFN-α treatment of 1bneo/delS replicon cells, were infected with these supernatants. After 2 sequential passages and 3 weeks of G418 selection, as described above, a number of colonies appeared, as shown in Figure 3-4A. The largest number of colonies appeared from the cured cells K4, and slightly fewer colonies appeared from the cured cells F2, while no colonies appeared from the normal Huh-7 cells (data not shown). The same infection experiment carried out with full-genome replicon cells produced no infectivity in the supernatant (data not shown). Under the most efficient conditions, the titer of the supernatant reached as high as 20 cfu (colony forming units) per milliliter when the putative doubling time of these cells was approximately 24 h. Furthermore, the appearance of colonies was abolished by the antibody JS-81 (BD Pharmingen, San Diego, CA, USA), an antibody to a possible co-receptor of HCV, namely CD81 [69] (Figure 3-4B).

Next, I propagated some of these colonies for further analysis. Northern blot analysis showed that these clones carry HCV RNAs of reasonable size (Figure 3-5A), such as subgenomic RNA (7994 bases), dORF RNA (10994 bases), and dORF bla RNA (11840 bases). Western blot

analysis revealed that the cell clones from the dORF replicon cell's supernatant-infected Huh-7 cells express structural proteins (Figure 3-5B), indicating that the colonies were not just the reappearance of subgenomic replicons hidden in the cured cells.

Altogether, our findings indicate that these particles in the supernatant infected the Huh-7 cells through a CD81-associated pathway and that infected cells formed colonies after G418 selection, similar to electroporation of subgenomic RNA.

A reporter gene installed in the dORF replicon RNA can be transmitted through infection

At first, I confirmed that the bla gene in the dORF bla replicon RNA was active in established replicon cell clones and able to process the green fluorescent substrate into blue fluorescent product (Figure 3-6A). Next, I attempted to detect the activity of the bla gene in the cloned infected colonies. Three clones grown from the dORF bla supernatant-infected cells were treated with the GeneBLAzer *in vivo* kit. One clone was positive for blue fluorescence (Figure 3-6B), demonstrating that a reporter gene installed in the dORF replicon could be transmitted to naive Huh-7 cells through secreted virus particles in the culture supernatant.

4. Discussion

There have been several previous reports of full-genome HCV replicons that can replicate well in Huh-7 cells and express sufficient amounts of structural proteins [7, 16, 34, 44, 65]. Pietschmann et al. (2002) observed the secretion of RNase-resistant HCV genome into the supernatant from both full-genome and subgenomic replicon cells and non-specific uptake of these genomes by naive Huh-7 cells. Ikeda et al. (2002) were also unable to detect any infectivity in the supernatant of their full-genome replicon cells. They assumed that the reason for this failure was the inability or incompetence of the Huh-7 cells to release intact virions or to be infected by the virus, although this was later demonstrated not to be the case by a series of reports on infection using the JFH-1 clone [56, 85, 94].

At first, I planned to ameliorate the inadequacies of the full-genome replicon in 2 ways, namely, modification of construction and reduction of genome size. Numerous studies have examined the encapsidation signal in the genomic RNA of positive-sense single-stranded viruses [23, 37, 38]. Frolova et al. showed that the encapsidation signal of the Sindbis virus lies in the nsP1 gene and is 132 nucleotides long [23]. Johansen et al. found that the IRES of the poliovirus had the ability to enhance the efficiency of packaging of the polio subgenomic replicon [38]. I think that these findings indicate that the construction of the genome could affect the efficacy of encapsidation and thus decided to change the site of genome division from the beginning of the core region to the

middle of the NS2 region. Regarding the size of the genome, there have been reports that the insertion of a foreign gene of significant size will result in the deletion of some portion of the chimeric genome in some rounds of replications [59, 60]. I therefore removed the latter half of the NS2 region, because this region appears unnecessary for both replication and packaging in Huh-7 cells, and this deletion was found to have no influence on the efficacy of encapsidation, as there were no apparent differences between the NS2-deleted construct and that with the entire NS2 region (data not shown).

Our established dORF replicon was able to replicate well in Huh-7 cells and to express sufficient amounts of structural proteins, similar to the previously reported full-genome replicon. Though both the dORF replicon cells and the previously reported full-genome replicons secreted RNase-resistant genomes, there was a striking difference between these 2 full-genome replicons when NP-40 treatment was carried out on their supernatants. There was no RNase-resistant genome left in the NP-40-treated supernatant of full-genome replicons, although density gradient analysis of the NP-40-treated supernatant of dORF replicon cells clearly showed the coexistence of the HCV genome and core proteins at a peak of 1.18 g/mL. This peak may represent NP-40-resistant nucleocapsids. The distribution of core proteins in the density gradient analysis of the concentrated supernatant of the dORF replicons did not match that of the HCV genome. A reasonable explanation for this mismatch is that the lighter side of the broad peak of the HCV genome was not

representative of intact virions and is instead an indication of secretion by a pathway used in subgenomic replicon cells, which differs from the natural process. The fact that the peak of the HCV genome of full-genome replicons was located in a narrow range on the lighter side compared to that of the dORF replicons supports this hypothesis. I observed round particles in the concentrated core protein fraction using electron microscopy, and those particles also seemed to contain core proteins. These findings indicate that our dORF replicon cells produced both intact virions and artificial membranous particles, with the former having the morphological and biophysical characteristics of putative virions.

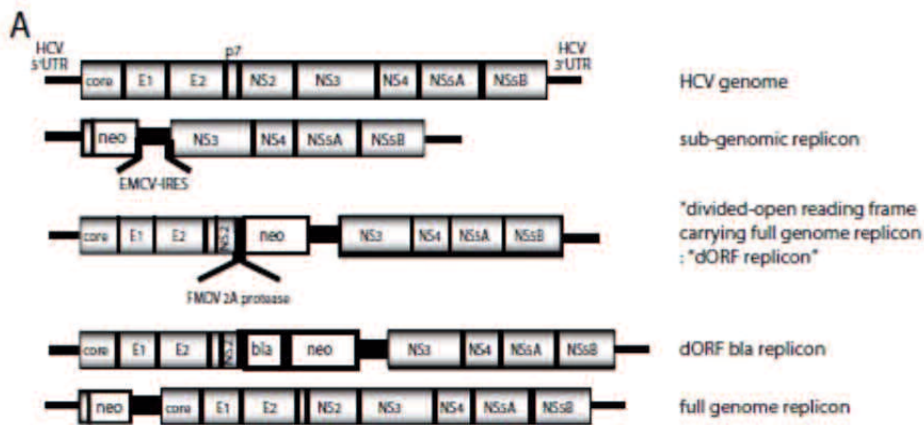
The colony-forming assay clearly demonstrated the ability of the supernatants of our dORF replicon cells to efficiently infect Huh-7 cells. The reason for the difference in efficacy between the 2 cured cells is uncertain, but may involve the ability to support replication or the level of receptor expression. This is an important factor to clarify in order to improve the efficacy of HCV infection *in vitro*. Differences in the efficacy of infection were also noted between clones of the same dORF replicon cells, which may have been due to different mutations beings accumulated in the structural region, although I have not yet confirmed this hypothesis. I also observed colonies appearing from cells treated with supernatant that contained subgenomic replicons, and these colonies most likely represent the so-called "non-specific transduction" of the subgenome. Although this dORF supernatant infection could be blocked by the anti-CD81 antibody reported previously

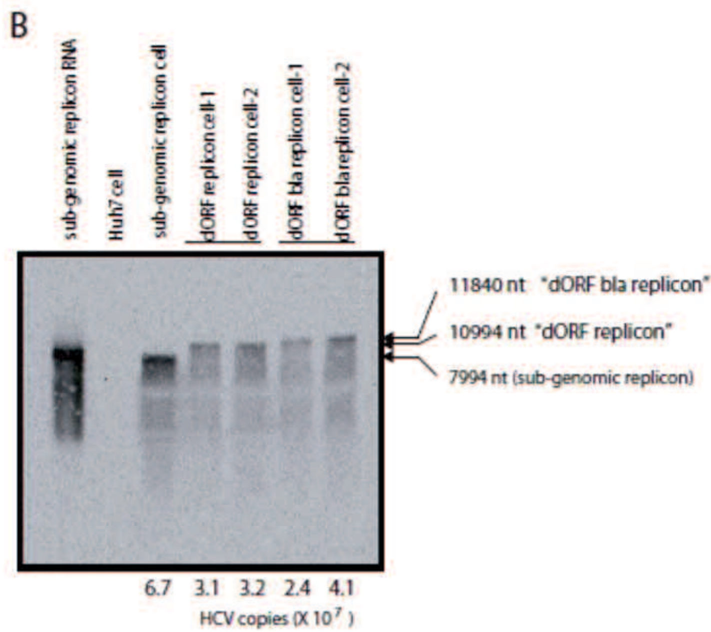
[94], I cannot exclude the possibility that the infection I observed was due to highly efficient "non-specific transduction," as I could not determine whether "non-specific transduction" also could be affected by the anti-CD81 antibody [7], because of the low colony-forming ability of the supernatant of subgenomic replicons.

I also demonstrated that the reporter gene installed in addition to the neomycin resistance gene could be transmitted to the new generation of viruses. This finding raises the possibility of producing sufficient amounts of reporter virus constitutively.

In summary, I established an infectious particle-producing HCV replicon system. This achievement should yield more precise information about the encapsidation signal of HCV, which was kept intact despite the partitioning of the genome. This system also allows analysis of the pathway of HCV infection, including adsorption of virions to cell surface receptors, penetration, uncoating, virus particle assembly, and HCV release. Moreover, the dORF replicon system may be used as a convenient tool to investigate the utility of the newly established siRNA system [46, 86] and evaluation of compounds that are effective against subgenomic replicons.

Although I believe that the reason for our success is our new construct, further examination is necessary to verify our findings.


A
HCV 5'UTR
p7
HCV 3'UTR
core
E1
E2
NS2
NS3
NS4
NS5A
NS5B
HCV genome
neo
sub-genomic replicon
EMCV-IRES
"divided-open reading frame
carrying full genome replicon
: "dORF replicon"
FMDV 2A protease
bla
dORF bla replicon
full genome replicon


B
sub-genomic replicon RNA
Huh7 cell
sub-genomic replicon cell
dORF replicon cell-1
dORF replicon cell-2
dORF bla replicon cell-1
dORF bla replicon cell-2
11840 nt "dORF bla replicon"
10994 nt "dORF replicon"
7994 nt (sub-genomic replicon)
6.7 3.1 3.2 2.4 4.1
HCV copies (X 10 7)

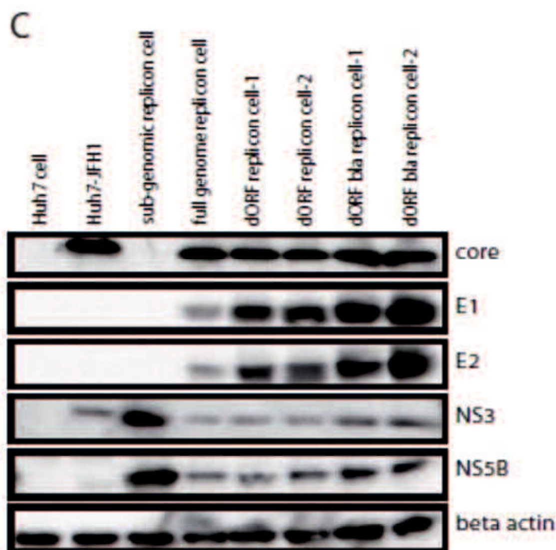

C
Huh7 cell
Huh7-JFH1
sub-genomic replicon cell
full genome replicon cell
dORF replicon cell-1
dORF replicon cell-2
dORF bla replicon cell-1
dORF bla replicon cell-2
core
E1
E2
NS3
NS5B
beta actin

Figure 3-1. Confirmation of "divided open reading frame carrying" (dORF) replicon cells.
(A) Schematic representations of replicon RNAs used in this study. All the replicon constructions were placed just after the T7 promotor. (B) Northern blot analysis. A 10-µg amount of total RNA from each cell sample was loaded. Subgenomic replicon RNA: 108 copies of *in vitro*-generated subgenome RNA. Numbers below lanes are the HCV copy number per microgram of total RNA. Huh-7 cell, subgenomic replicon cell, dORF replicon cell #1, #2, dORF bla replicon cell #1, #2. (C) Western blot analysis. A 10-µg amount of each cell lysate was loaded. Huh-7 cell, Huh-7-JFH-1: Huh-7 cell transfected with JFH-1 viral RNA, subgenomic replicon cell, full-genome replicon cell, dORF replicon cell #1, #2, dORF bla replicon cell #1, #2.

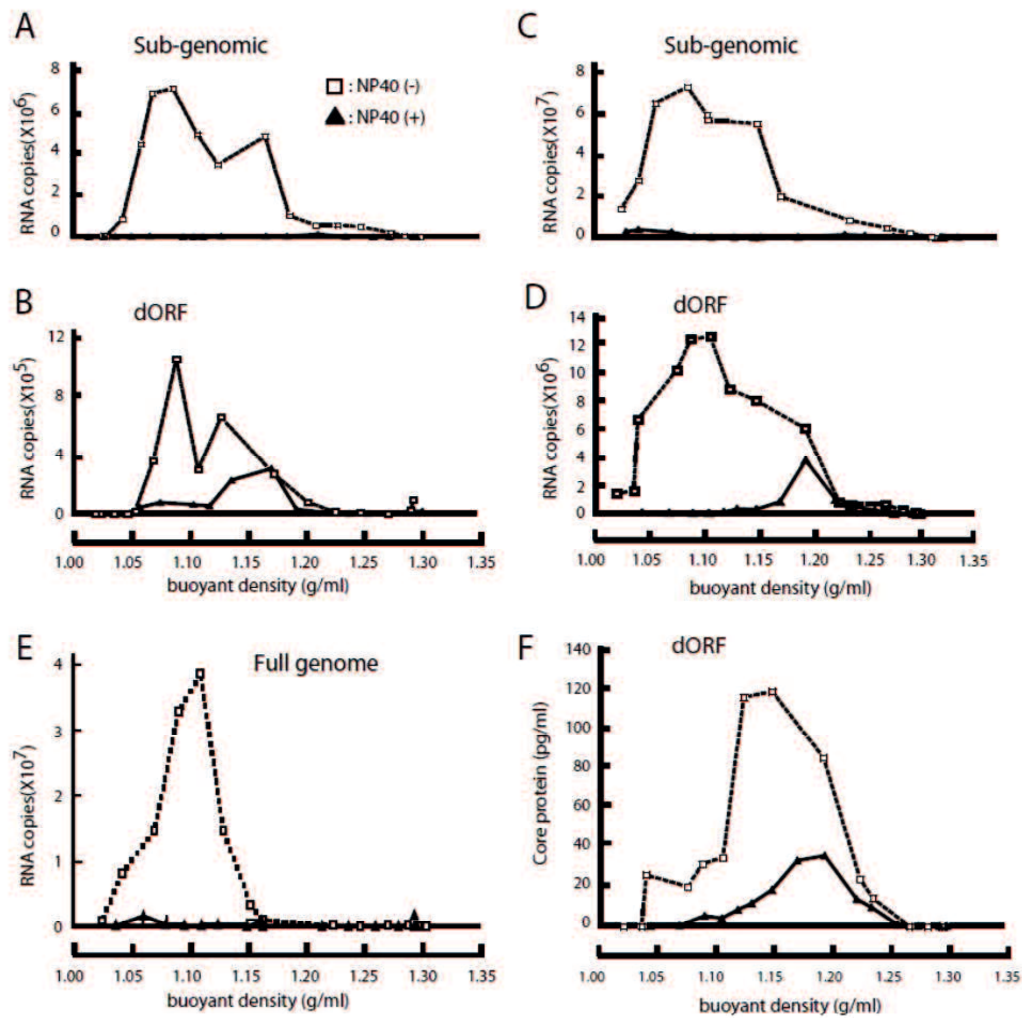


Figure 3-2. HCV Density gradient analysis of supernatants.

Culture supernatants were RNaseA treated and loaded onto a tube of sucrose density gradient directly (open square) or after NP-40 treatment (filled triangle). Quantification of HCV RNA in each fraction of supernatant from the subgenomic replicon (A) and dORF replicon (B). Analysis of concentrated culture supernatant from the subgenomic replicon (C) and dORF replicon (D). Concentrated culture supernatant from the full-genome replicon, NNC#2, was also analyzed (E). Quantification of HCV core protein in each fraction of supernatant from the dORF replicon (F).

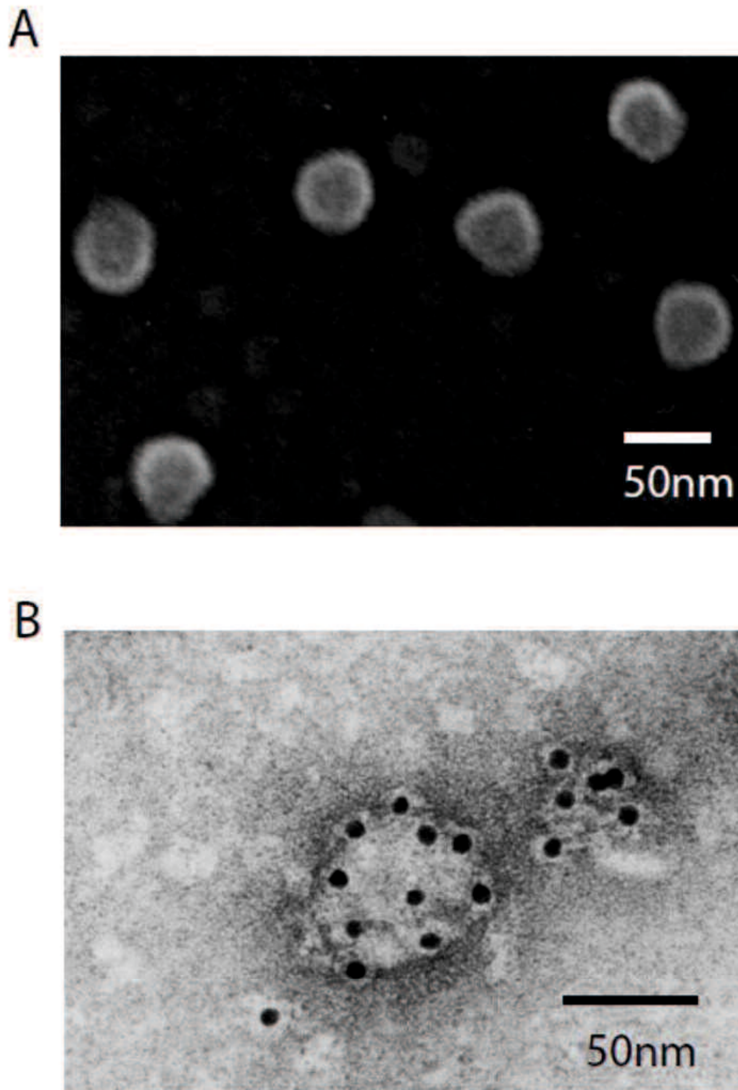
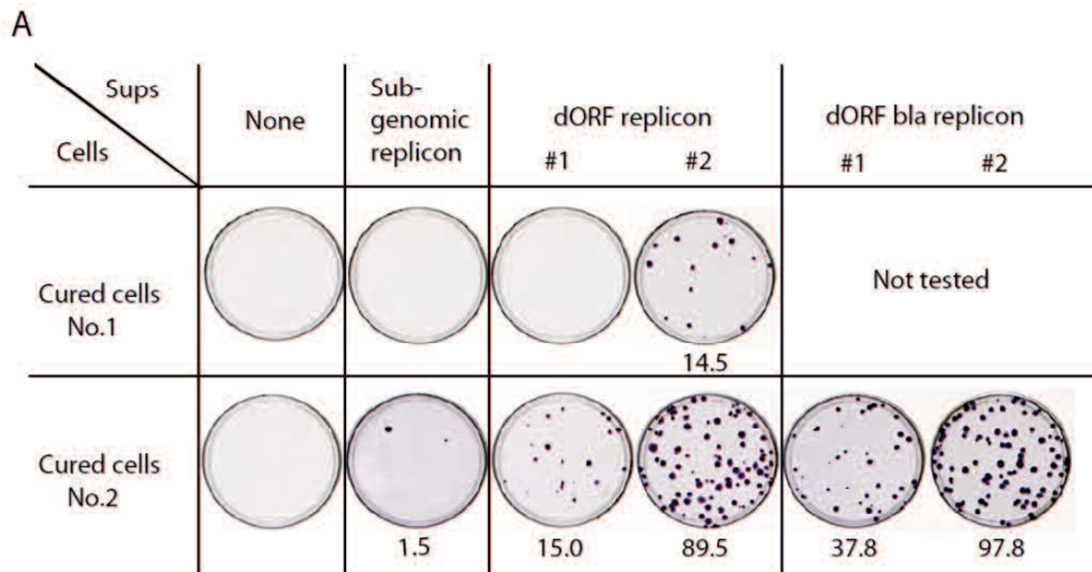


Figure 3-3. Electron microscopy analysis of virus-like particles.

The core-protein-rich fraction collected from the density gradient was further concentrated by ultra-centrifugation and observed by scanning electron microscopy (A). The same fraction attached to formvar-coated grids was incubated with rabbit anti-E2 RR6 antibody, treated with goat anti-rabbit IgG coupled to 10-nm colloidal gold, negatively stained with uranyl acetate, and then examined by transmission electron microscopy (B).



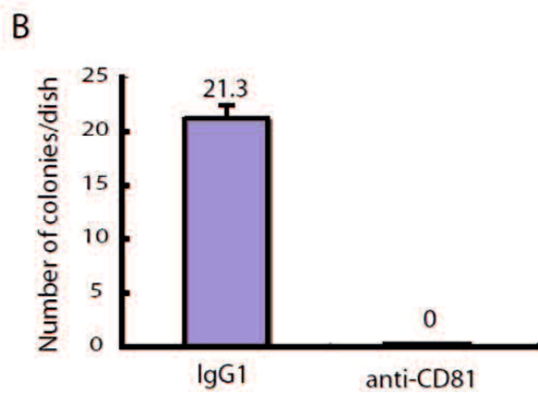
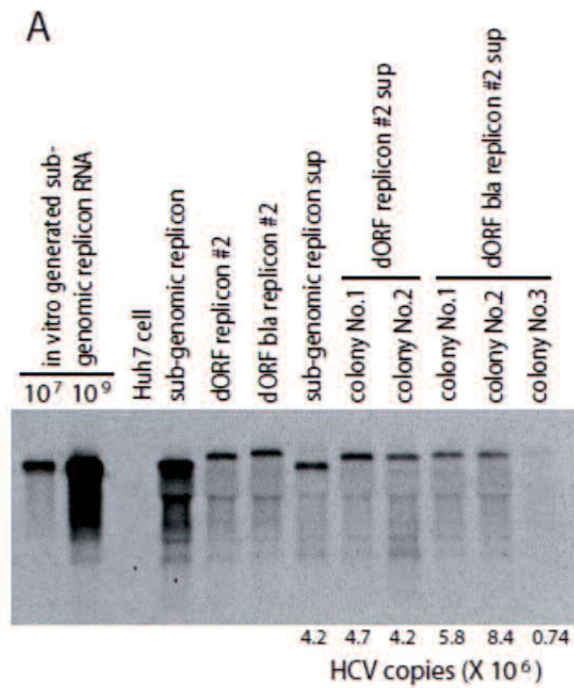


Figure 3-4. Infectivities of supernatants from various replicon cells were examined.
Colonies that appeared from cells infected with the indicated supernatant (A). Numbers indicated below the plates are the average of a total of 4 plates per condition.
Infection was inhibited by anti-CD81 antibody. Cured cell K4 cells were treated with mouse IgG1 as the negative control or anti-CD81 before infection (B).



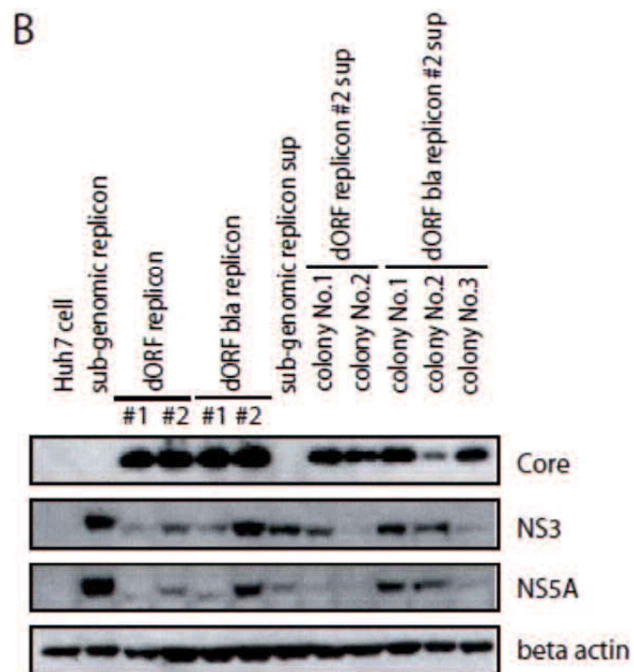


Figure 3-5. Northern blot analysis of colonies formed after infection.

10^7, 10^9: amounts of *in vitro*-generated subgenomic replicon RNA loaded. Numbers below the lanes are the HCV copy number per μg of total RNA (A). Huh-7 cells, subgenomic replicon cells, dORF replicon cell #2, dORF bla replicon cell #2, subgenomic replicon sup: colony from cells transduced with subgenomic replicon supernatant, colony No.1, 2 of dORF replicon #2 sup: colonies from cells infected with dORF replicon #2 supernatant, colony No.1, 2, and 3 of dORF bla replicon #2 sup: colonies from cells infected with dORF bla replicon #2 supernatant. Western blot analysis of colonies formed after infection (B). The order of the lanes is identical to that for the northern blot, except for the dORF and dORF bla replicons, which represent 2 clones in this figure.

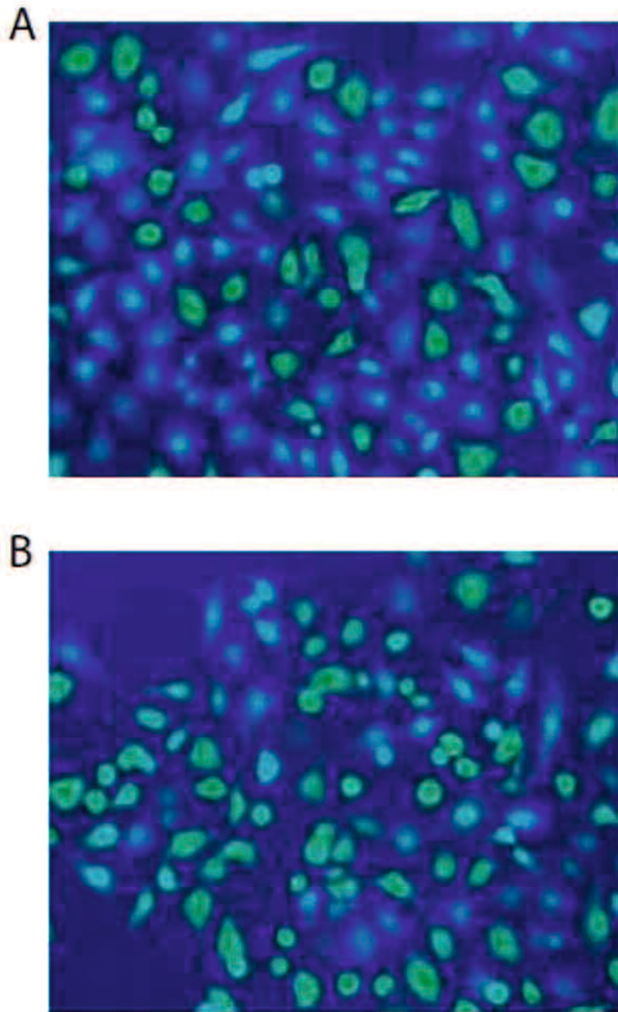


Figure 3-6. Detection of beta-lactamase activity in dORF replicon cells.
Parental dORF bla replicon #2 cell (A) and Colony No. 3 cloned from dORF bla replicon #2 cell supernatant infected cells (B). Blue fluorescence shows high beta-lactamase activity, indicating that the reporter gene functioned normally after infection

Chapter 2:

Isolation and characterization of highly replicable hepatitis C virus genotype 1a strain HCV-RMT

1. Background and Aims

HCV is an enveloped positive-strand RNA virus that belongs to the Flaviviridae family [78]. With over 170 million people currently infected worldwide [13], HCV represents a growing public health burden despite the launch of new antiviral medications that directly inhibit virus replication [55, 83].

Since HCV was first identified in 1989 as the major cause of non-A and non-B hepatitis [13], great progress has been made in understanding the life cycle of HCV. The first propagation system for this disease agent was an *in vivo* chimpanzee model [49, 76, 87]. Although that system is still occasionally used as a pivotal animal model for some drugs, chimeric mice with humanized liver that is generated by transplanting human hepatocytes [39, 62] are more popular now because of the low cost and the absence of ethical concerns associated with the use of chimpanzees. For *in vitro* research, establishment of an HCV replicon system [6, 57] was an important achievement that allowed research into the function of individual non-structural viral proteins. However, the entire viral life cycle remains enigmatic because no structural proteins are needed in this system. Some reports have been published about full-length replicons with structural proteins in addition to non-structural proteins, although little [3] or no [36, 67] secretion of infectious virions was observed, which may have been partly due to adaptive mutations. Another breakthrough was made with the

discovery of a genotype 2a JFH-1 strain that soon became well known for its vigorous replication as a replicon with no adaptive mutations [15]. JFH-1 can also infect and propagate in cultured cells as a virus, especially in HuH-7 cells or their derivatives [56, 85, 94]. After the discovery of JFH-1, two methods were available for the investigation of how viral proteins other than those of HCV genotype 2a function during their entire life cycle. The first method was only for structural proteins and involved making a hybrid of the structural region of the clone of interest and the non-structural regions of JFH-1 for efficient replication [26, 66, 91]. The other method utilized the entire viral genome sequence of genotype 1 and made them infectious to HuH-7 derivative cells by introducing known adaptive mutations [53, 92] or enhancing replication with a casein kinase inhibitor [68]; however, their replication abilities were somehow compromised. In this study, we report the isolation of a new genotype 1a strain from a patient's serum sample that was highly infectious to human hepatocyte-transplanted chimeric mice, as the viral titer in the blood of the mice was higher than 10^8 copies/ml. I evaluated its replication abilities in four replication systems: subgenomic replicon, virus, *in vitro* infection, and *in vivo* infection. The new HCV clone, which was designated HCV-RMT (GenBank accession number, AB520610), was different from other genotype 1a clones because it did not require any artificially introduced adaptive mutations for the establishment of replicon cells. With these features, our newly cloned HCV-RMT may be a useful tool for investigating the entire life cycle of genotype 1 HCV.

2. Materials and Methods

Ethics Statement

This study was carried out in strict accordance with both the *Guidelines for Animal Experimentation* of the Japanese Association for Laboratory Animal Science and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All protocols were approved by the ethics committee of Tokyo Metropolitan Institute of Medical Science.

Cloning and Sequencing

Acute-phase serum from an HCV genotype 1a-infected patient, HCG9 (purchased from International Reagents Corp., Kobe, Japan; discontinued), was supplemented with 0.1 µg/µl yeast tRNA, and total RNA was extracted using ISOGEN-LS (Nippon Gene, Tokyo, Japan) according to the manufacturer's information. Purified RNA (1 µg) was reverse transcribed using LongRange Reverse transcriptase (QIAGEN, Valencia, CA, USA) and a 21-mer oligonucleotide (antisense sequence 9549-9569 of HCV-H77: GenBank accession number AF011751) as the primer. The first PCR amplification was carried out with the generated cDNA and Phusion DNA polymerase (Finnzymes, Vantaa, Finland) using sense primers corresponding to nucleotides 9-28, 2952-2972,

and 5963-5979 (numbers correspond to the HCV-H77 sequence) and antisense primers corresponding to nucleotides 4038-4054, 7042-7057, and 9549-9569. The second nested PCR amplification was carried out with these three products using sense primers corresponding to nucleotides 23-43, 2967-2987, and 5981-6000 and antisense primers corresponding to nucleotides 4018-4033, 7016-7035, and 9534-9554. For the cloning of terminals, total RNA was purified from non-supplemented HCG9 serum. The 5′ terminus was amplified with a 5′ RACE system kit (Invitrogen) using one-fourth of the purified total RNA from 100 μl serum and antisense primers corresponding to nucleotides 255-273 for the first PCR and 241-261 for the second nested PCR. For the 3′ terminus, the poly(A) tail was added to the 3′ terminus of the same amount of RNA with poly(A) polymerase (Takara Bio Inc., Shiga, Japan). Reverse transcription and PCR amplification of this region were carried out using oligo-d(T) as the reverse primer for both reactions and primers corresponding to nucleotides 9385-9408 for PCR.

All fragments were subcloned using a TOPO cloning kit (Invitrogen), and sequences yielding 10 or more clones per fragment were determined with the Big Dye Terminator mix and ABIprism3100 (Applied Biosystems). The consensus sequence was determined by accepting the most frequent nucleotide at each position.

Construction and RNA transcription

To generate full-length viral RNA, the HCV-RMT sequence, which has an endogenous *Xba*I site, was mutated to a silent mutation (T3941C) using a QuikChangeII kit (Stratagene, La Jolla, CA, USA) and cloned into the *Hind*III site of pBR322 with an additional T7 promoter at the beginning and an *Xba*I site at the end. Replicon construction of HCV-RMT was performed by replacing nucleotides 390-3419 of HCV-RMT with the neomycin resistance gene, EMCV-IRES, and an additional start codon at the beginning of the NS3 region. For RNA generation, plasmids were digested with *Xba*I and used as a template for RNA transcription using a RiboMax kit (Promega, Madison, WI, USA).

Cells and Electroporation

HuH-7 cells were cultured in DMEM-GlutaMax-I (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Replicon cells were maintained in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Cells were passaged three times a week at a split of four times. Electroporation of replicon RNA and G418 selection were performed as previously described [6, 57]. The cured replicon cell clone (HuH7-K4) was established as previously described [3]. Briefly, authentic subgenomic replicon cells were treated with 1000 IU IFN-α (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) for 2 months and cloned using the limiting

dilution method.

Quantification of HCV RNA

Total RNA was purified from 1 µl chimeric mouse serum using SepaGene RV-R (Sanko Junyaku, Tokyo, Japan), and total RNA was prepared from cells or liver tissues using the acid guanidium thiocyanate-phenol-chloroform extraction method. Quantification of HCV RNA copy number with real-time RT-PCR was performed using an ABI 7700 system (Applied Biosystems) as described previously [80].

Western blot analysis and immunofluorescence analysis

Western blot analysis was carried out according to the conventional semi-dry blot method. Cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4 containing 1% sodium dodecyl sulfate, 0.5% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 1 mM dithiothreitol). Protein (10 µg) from each sample was separated with SDS-PAGE through a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore). HCV NS3 protein was detected with 5 µg/ml anti-NS3 polyclonal antibody (R212) as described previously [82]. HCV NS5B protein and β-actin were detected with 0.5 µg/ml anti-NS5B polyclonal antibody (ab35586; Abcam) and 0.2 µg/ml anti-β-actin MAb (AC-15; Sigma-Aldrich, respectively.

For immunofluorescence analysis, cells were washed twice with PBS(−) and fixed with 100% methanol (chilled at −80°C) at −20°C for 20 min. Fixed cells were treated with PBS(−) supplemented with 1% BSA and 2.5 mM EDTA overnight at 4°C. Blocking and antibody treatments were also carried out in the same buffer. Stained cells were viewed with a laser scanning confocal microscope LSM510 (Carl Zeiss, Oberkochen, Germany). HCV core proteins were detected with 5 μg/ml α-HCV core MAb (31-2) prepared in our laboratory [65].

***In vitro* infection and α-CD81 blocking**

HuH7-K4 cells were seeded at 6×10^4 cells/well onto a ϕ10-mm coverglass in a 48-well plate 24 h before inoculation with 240 μL culture medium. At 72 h post-inoculation, cells were fixed with 100% methanol (chilled to −80°C) for 20 min at −20°C. HCV core proteins were detected with 5 μg/ml α-HCV core antibody 31-2. Fluorescent-positive foci were counted under fluorescence microscopy, and the focus-forming units (ffu) per milliliter of supernatant were calculated.

For α-CD81 blocking, HuH7-K4 cells were pre-treated with a serial dilution of α-CD81 antibody (JS81, BD Pharmingen) or normal mouse IgG_1 (BD Pharmingen) as an isotype control for 1 h before inoculation.

Drug treatment

#11 cells (5,000 cells/well), which were established using the single cell cloning of HCV-RMTtri-electroporated cells, were seeded in 96-well tissue culture plates and cultivated overnight. Serial dilutions of CsA (Fluka Chemie, Buchs, Switzerland) or IFN-α (Mochida Pharmaceutical Co., Ltd.) were added. After incubation for 72 h, total RNA was extracted from cells, and HCV-RNA was quantified as described above. The experiments were carried out in triplicate.

***In vivo* infection**

Chimeric mice with humanized liver (PhoenixBio, Hiroshima, Japan) were infected with 10 µl patient serum HCG9 by intravenous injection. For analysis of infectivity of the HCV genome clone, mice were directly injected with 30 µg of the generated RNAs into five to six sites in the liver during abdominal surgery. Blood samples were collected once a week and used for quantification of HCV copy number.

3. Results

Cloning of a new HCV genotype 1a strain from the serum of an HCG9-infected mouse

I first infected chimeric mice with humanized liver with patient serum HCG9. HCV in HCG9 serum was classified as genotype 1a with RT-PCR genotyping and showed relatively high replication ability in the patient and comparable or better replication ability in the chimeric mice (Figure 2-1A). In one infected mouse, the HCV copy number in blood reached 1×10^9/ml (data not shown). Using two mice with blood titers of 1×10^8 and 1×10^9 copies/ml, I cloned HCV sequences with the standard PCR amplification method using HCV-H77 as a source of primer sequences. Except for some length variations in the poly-pyrimidine tract region, I found no differences in HCV sequences from mouse blood with titers of 1×10^8 and 1×10^9 copies/ml when considering major consensus nucleotides at all sites (GenBank accession number AB520610). The HCV sequences were identical to the HCV sequence cloned from HCG9 serum itself (data not shown). I designated this sequence as HCV-RMT. Its homology to the HCV-H77 strain was 92.8% for nucleotides and 95.1% for amino acids. The *in vivo* replication ability was confirmed with direct injection of the generated HCV-RMT RNA genome into livers of the chimeric mice. Blood titers were comparable to infection with parental HCG9 serum (Figure 2-1B). JFH-1 infection resulted in a 2-log lower blood titer than HCV-RMT when the same procedure was used.

Establishment of subgenomic replicon cells with the HCV-RMT strain

Next, I generated an authentic subgenomic replicon RNA construct using the HCV-RMT sequence and used it to establish replicon cells. Only two colonies appeared after electroporation with 30 µg of the HCV-RMT replicon RNA and G418 selection. One of these colonies had a reasonable HCV subgenome copy number, and thus, I propagated it and determined the sequence of the subgenome. The determined consensus sequence of the subgenome had three mutations from the wild type: two were located in the NS3 region (E1056V and E1202G), and one was in the NS5A region (A2199T) (Figure 2-1C). I introduced these mutations into the HCV-RMT replicon sequence as a single mutation or combination of mutations and identified the mutations that were responsible for colony formation (Figure 2-1D). The most influential single mutation was E1202G in the NS3 region, although a combination of all three mutations (designated RMTtri) resulted in the best replication ability. Interestingly, western blot analysis and HCV genome quantification revealed that the amount of HCV viral components in cells was independent of the colony-forming ability and seemed to be negatively affected by the most beneficial adaptive mutation (E1202G) (Figure 2-1E).

The HCV-RMT RNA genome with adaptive mutations was maintained in HuH-7 cells

Next, I assessed the *in vitro* replication abilities of HCV-RMT derivatives as a viral genome rather than a replicon. I introduced adaptive mutation(s) into the HCV-RMT sequence

(Figure 2-2A) and electroporated the *in vitro*-generated RNAs into Huh-7.5.1 and HuH7-K4 cells. Electroporated cells were passaged every 2 to 4 days depending on their confluency, and sampling of cells for quantification of the HCV RNA genome was carried out at each passage. The amounts of HCV-RMTtri and JFH-1 were maintained at $\geq 1 \times 10^5$ copies/μg total RNA, in contrast to wild-type HCV-RMT, which was eliminated rapidly (Figure 2-2B). Additionally, different cell preferences were observed with the two strains of HCV: JFH-1 replicated well in Huh-7.5.1 cells compared to HCV-RMTtri, but the opposite was seen in HuH7-K4 cells. Different replication abilities were also observed among derivatives of the HCV-RMT strain and corresponded to the colony-forming ability of the replicon constructs (Figure 2-2C). Immunostaining of HCV core proteins revealed that many cells (19.2%) were stained in HCV-RMTtri RNA electroporated cells compared to small number cells (0.98%) were stained in HCV-RMT with E1202G mutated RNA electroporated cells (Figure 2-2D).

The supernatant of HCV-RMTtri-replicating cells was infectious to naïve HuH7-K4 cells

To assess the infectivity of HCV-RMTtri, I used the limiting dilution method to establish clone number 11 (#11) cells in which HCV-RMTtri was highly replicating. The percent of cells expressing the HCV core protein in #11 cells was 75.3 ± 5.0% as seen with immunostaining, whereas the percent of parental cells expressing the HCV core protein was 6.3 ± 2.2% (Figure 2-3A;

the value was calculated as an average of ten observed areas). The cells maintained 1×10^8 copies/μg total RNA of the HCV-RMTtri RNA genome. I collected the supernatants from #11 cells 2 months after cloning and HuH7-K4 cells carrying JFH-1 2 months after establishment. To evaluate infectivity, I added these supernatants to the medium of naïve HuH7-K4 cells. Cells were stained with anti-HCV core protein antibody 3 days later, and I observed core protein-positive cell foci per 0.78 cm^2 in at least triplicate wells (Figure 2-3B). The calculated ffu of the supernatant was 160 ffu/ml, which was similar to that of H77 with artificially introduced adaptive mutations. This infection was inhibited by anti-CD81 antibody in a similar concentration-dependent manner as *in vitro* infection of JFH-1 (Figure 2-3C). #11 cells were also useful for evaluating anti-HCV agents such as CsA and IFN-α (Figure 2-3D) when 5,000 cells/well (96 well plate) of #11 cells were treated with inhibitors for 72 h beginning 1 day after passaging.

***In vivo* replication abilities of HCV-RMT derivatives were inversely proportional to their *in vitro* abilities.**

I assessed the *in vivo* replication abilities of HCV-RMT derivatives carrying combinations of the three adaptive mutations using chimeric mice with humanized liver. *In vitro*-generated HCV genomic RNAs were injected directly into the livers of the chimeric mice during abdominal surgery. Mice were monitored for amounts of genomic RNA in the blood once a week for 6 weeks, and virus

titers in the livers were quantified after sacrifice of the mice. As shown in Figure 2-4A, in contrast to the vigorous *in vitro* replication ability, the clone that was most active *in vitro*, HCV-RMTtri, showed no evidence of replication *in vivo*, whereas the wild type showed replication that was comparable to the parental virus in the patient's serum, HCG9. In addition, the double mutant (E1056V, A2199T), which showed little replication *in vitro*, showed a similar replication ability as the wild-type clone. The most positively influential adaptive mutation (E1202G) seemed to hamper its *in vivo* replication ability. Quantification of HCV genomic RNA in liver (Figure 2-4B, C) showed a conserved serum/liver ratio among HCV-RMT derivatives. Thus, the blood titers directly reflected the titers in liver, although the ratio was considerably different than that of JFH-1. Table 1 shows the replication abilities of derivatives and JFH-1 both *in vitro* (HuH7-K4 cells) and *in vivo* (chimeric mice), clearly showing the inversely proportional relationship between them, including the replication ability of JFH-1, which corresponds to data in a previous report [31].

4. Discussion

In this report, we investigated many types of HCV replication systems using our newly cloned HCV-RMT.

The first type is replication in cultured cells as an authentic replicon construction. This system only depends on the ability to replicate in cells. HCV-RMT was the first genotype 1a clone that could be established in authentic replicon cells without artificially introduced adaptive mutations that are required by H77 [6, 90], although the three spontaneously occurring mutations (E1056V, E1202G, and A2199T) are not novel [57, 90]. Among the mutants with single mutations or a combination of these three adaptive mutations, the amounts of HCV genome and viral proteins did not reflect the colony-forming abilities (Figure 2-1D, E). The A2199T mutation, which least affects the colony-forming ability (no stable replicon cell line was established with this single mutation). However, combination of mutations including A2199T, triple (E1056V, E1202G and A2199T) and double (E1056V and A2199T), allowed HCV subgenomic replicon cells to produce high amounts of HCV proteins. This observation illustrates the complex nature of HCV subgenomic replicon-establishing factors, especially NS5A-related factors [2, 18]. This hypothesis requires further investigation.

The second type is replication of the virus itself in cultured cells. This system also only

depends on the ability to replicate in cells as long as the presence of structural protein regions does not cause any differences. Electroporation of HCV genomes resulted in constant replication when the combination of active derivatives of HCV-RMT and HuH7-K4 cells was used; replication lasted for more than 2 months (data not shown). The order of the replication ability of mutants in cultured cells as a virus appeared to be nearly consistent with the colony-forming ability of replicons of each sequence, although some constructs with "weak" adaptive mutation(s) showed no difference from the wild type (Figure 2-2C). Thus, these two types of replication may be basically the same despite the different constructs. HCV-RMT derivatives replicated better than JFH-1 in HuH7-K4 cells. In contrast, replication was much less efficient in Huh-7.5.1 cells (Figure 2-2B), which are well known to support replication of JFH-1 [6]. These materials appear to be good tools for investigating the mechanism of HCV replication in cultured cells.

The third type is *in vitro* infection using established HCV-infected cultured cells as the source of inoculum. Because this system has more steps than the first two, the outcome is more difficult to understand. Infection systems using strains other than JFH-1 seem to be rare because the magnitude of their replication is somehow compromised [51, 68, 92], in contrast to several studies examining the JFH-1 strain or its chimeric constructs with other genotypes [26, 56, 66, 85, 91, 94]. For observation of the infection process, selection of efficiently replicating cell clones from HCV-RMT RNA-electroporated cells was required. That clone, designated HuH7-K4-#11 cells, had

approximately 1×10^8 copies/μg total RNA of the HCV-RMT genome, and more than 80% of cells were core protein positive (Figure 2-3A). I were able to infect naïve HuH7-K4 cells with its supernatant (Figure 2-3), and the infectivity reached approximately 160 ffu/ml, which was comparable to that of the artificially mutated H77 strain. Thus, our HCV-RMT strain was unique among all genotype 1a clones. I could only detect infectivity using HCV-RMTtri, likely because the abundance of HCV-positive cells was high compared to other mutants (data not shown).

Many reports investigating the cellular and/or viral factors required for *in vitro* infection of HCV have been published [9, 12, 33, 35, 42, 50, 63, 69, 75, 93]. Almost all of these reports used a combination of JFH-1 and Huh-7.5 or Huh-7.5.1 cells. Our system using genotype 1a HCV-RMT and HuH7-K4 cells could complement these studies, considering the fact that the replication abilities of HCV-RMT and JFH-1 were quite different in the two derivatives of HuH-7 cells (Figure 2-2). Among the host factors reported to influence the virus life cycle *in vitro* and *in vivo*, CD81 was the first reported receptor to be involved in the *in vitro* infection process [26, 42, 50, 69, 93], and CD81 was also necessary in our HCV-RMT infection system because infection was blocked by anti-CD81 mAb (Figure 2-3).

Pietschmann et al. reported that the production of virus particles is impaired by replication-enhancing mutations [68]. Because I could not detect any infectivity in any supernatants from HCV-RMT derivative-electroporated cells at early times, I could not determine whether this

idea applies to these derivatives. Our observation of *in vitro* infectivity only with HCV-RMTtri may be due to the balance of replication ability and budding ability. Our system may be sufficiently efficient to quantify the infectivity titer.

Other cellular factors such as lipid droplets that interact with core proteins [9, 63, 75] and apolipoproteins [12, 33, 35] have been reported previously, although their contribution to our new infection system requires further studies.

The last type of HCV replication system I investigated was *in vivo* infection. The chimpanzee model was the first animal model established for HCV infection and was frequently used in important studies despite its high cost and ethical problems. Studies using chimpanzees have revealed that *in vitro*-adapted HCV mutants require back mutation(s) at specific site(s) for efficient replication *in vivo* [11]. In our studies using chimeric mice with humanized liver, I also did not observe amplification of HCV-RMTtri, which was the most active *in vitro* mutant. In addition, I observed a clear inversely proportional relationship between the *in vitro* and *in vivo* replication abilities of each mutant (Table 1), suggesting that the same factor(s) may work in both *in vitro* replication enhancement and *in vivo* replication inhibition. Although I have not confirmed whether back mutations or other new complimentary mutations were present, the characteristics of these three mutations were clarified by analysis of these four types of replication: replicon, virus, *in vitro* infection, and *in vivo* infection. E1202G, one of the two NS3-adaptive mutations, was the most

important mutation for *in vitro* replication, but it also severely hampered *in vivo* amplification. This mutation appears to impact the colony-forming ability comparable to the triple mutation, although virus replication was relatively lower than with the triple mutation. E1056V, another NS3 mutation, had a mild impact on the colony-forming ability, but it did not hamper the efficient replication of wild-type RMT *in vivo* in combination with A2199T, which seemed to have little influence on HCV replication alone except for increasing the amounts of virus genome and viral proteins in replicon cells. These two "weak" adaptive mutations provide the E1202G single mutant the ability to efficiently replicate *in vitro*. These effects may be dependent on the colony-forming ability of E1056V, the genome- and protein-increasing ability of A2199T, or both. At the same time, the weak *in vivo* replication ability of the E1202G single mutant, the replication of which was detected in only two of three mice injected and which showed a relatively low titer, was destroyed by addition of these two mutations, although the combination of these mutants had little effect on the replication ability of the wild type. These results suggest that the putative mechanism that renders *in vitro*-active clones deficient *in vivo* is not caused by a single factor such as the phosphorylation status of the NS5A protein, but a balance of many factors controlling mechanisms that are directly related to HCV replication both *in vitro* and *in vivo*.

I evaluated the amounts of HCV genome both in blood and liver, and the blood:liver ratios of replicable mutants varied little. This observation seems to be inconsistent with the hypothesis that

the *in vivo* ability of *in vitro* active mutants is compromised because of adaptive mutations that make the HCV genome more replicable but impair its virion-producing ability. Whether this occurs in certain conditions or is universal must be elucidated.

Recently, Li et al. reported the efficient replication and infection of Huh-7.5 cells of a genotype 1a clone named TN with artificially introduced adaptive mutations [53]. Similar reports have been published regarding an infectious genotype 1 HCV genome in Huh-7.5 cells or their derivatives by introducing adaptive mutations or using replication enhancing reagent [68, 92]. Although these appear to be more infectious than our HCV-RMT strain, I believe that our system is valuable because of the cells I used. HuH7-K4 cells are not a derivative of Huh-7.5 cells and are apparently distinct from them in terms of the ability to support HCV replication (Figure 2-2).

Our newly cloned HCV-RMT strain is unique because of its vigorous replication ability in chimeric mice, compared to the first HCV strain, H77, or the JFH-1 strain that is well known for its efficient replication *in vitro*. I believe that the different levels of *in vitro* replication abilities of these HCV-RMT mutants with an inversely proportional relationship to *in vivo* replication are valuable tools for investigating the factors required for HCV replication both *in vitro* and *in vivo*.

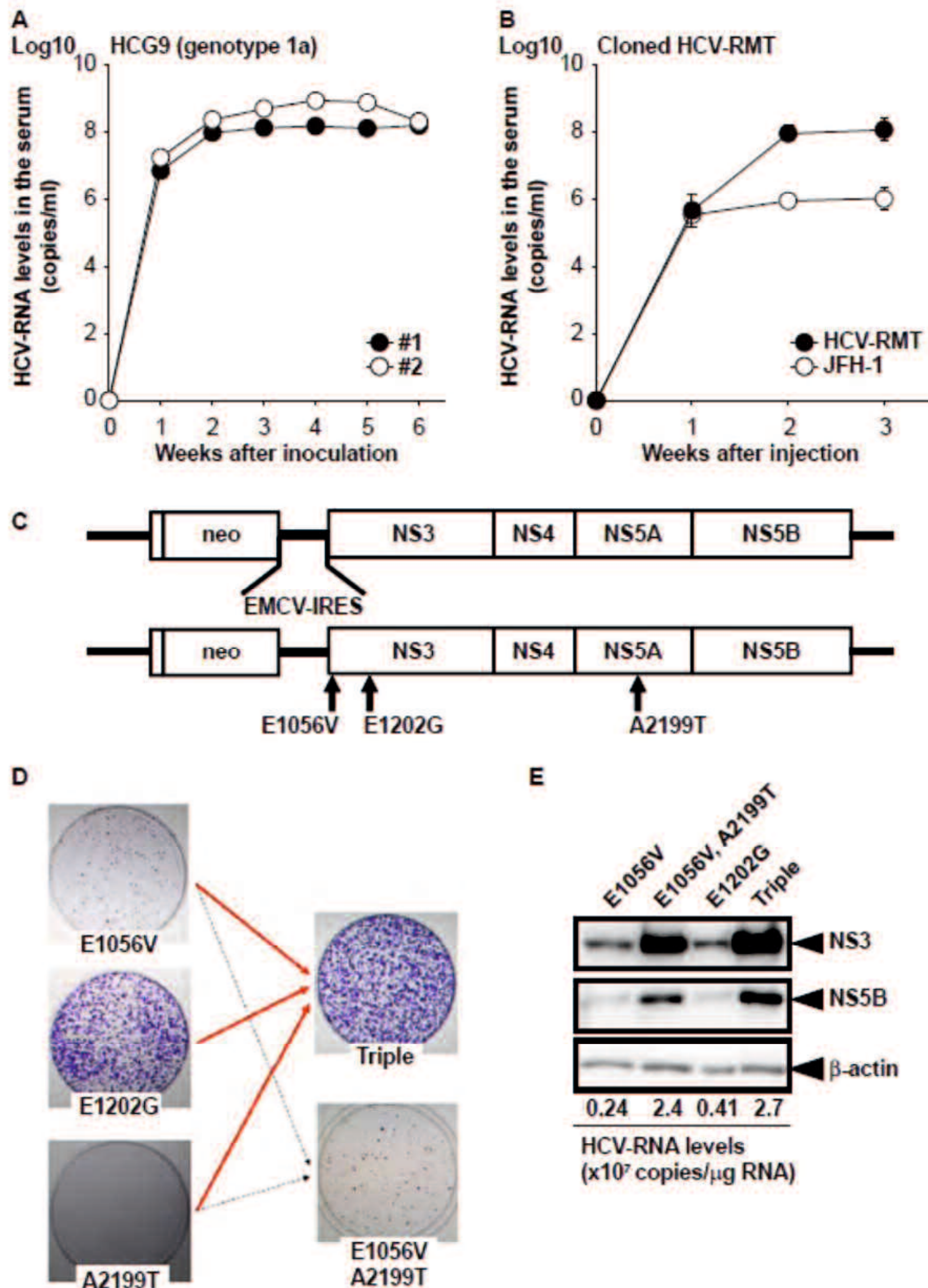


Figure 2-1. Basic characteristics of the HCV-RMT clone.

Change in HCV copy number in chimeric mice. (A) Two mice were intravenously infected with 10 μl patient serum HCG9. (B) Three mice per group were directly injected with 30 μg HCV RNAs of the HCV-RMT strain or the JFH-1 strain into the liver. Data are indicated as the mean ± S.D. (C) Schematic representation of construction of the replicon and the sites of adaptive mutations. (D) Colony formation assay of replicon clones with adaptive mutations. Each RNA (1 μg) was

electroporated into HuH7-K4 cells. (E) Western blot analysis of replicon cells. Each culture of replicon RNA-electroporated cells was maintained and passaged with G418 selection for 2 weeks. Cell lysates (10 μg) were loaded onto an SDS-PAGE gel.

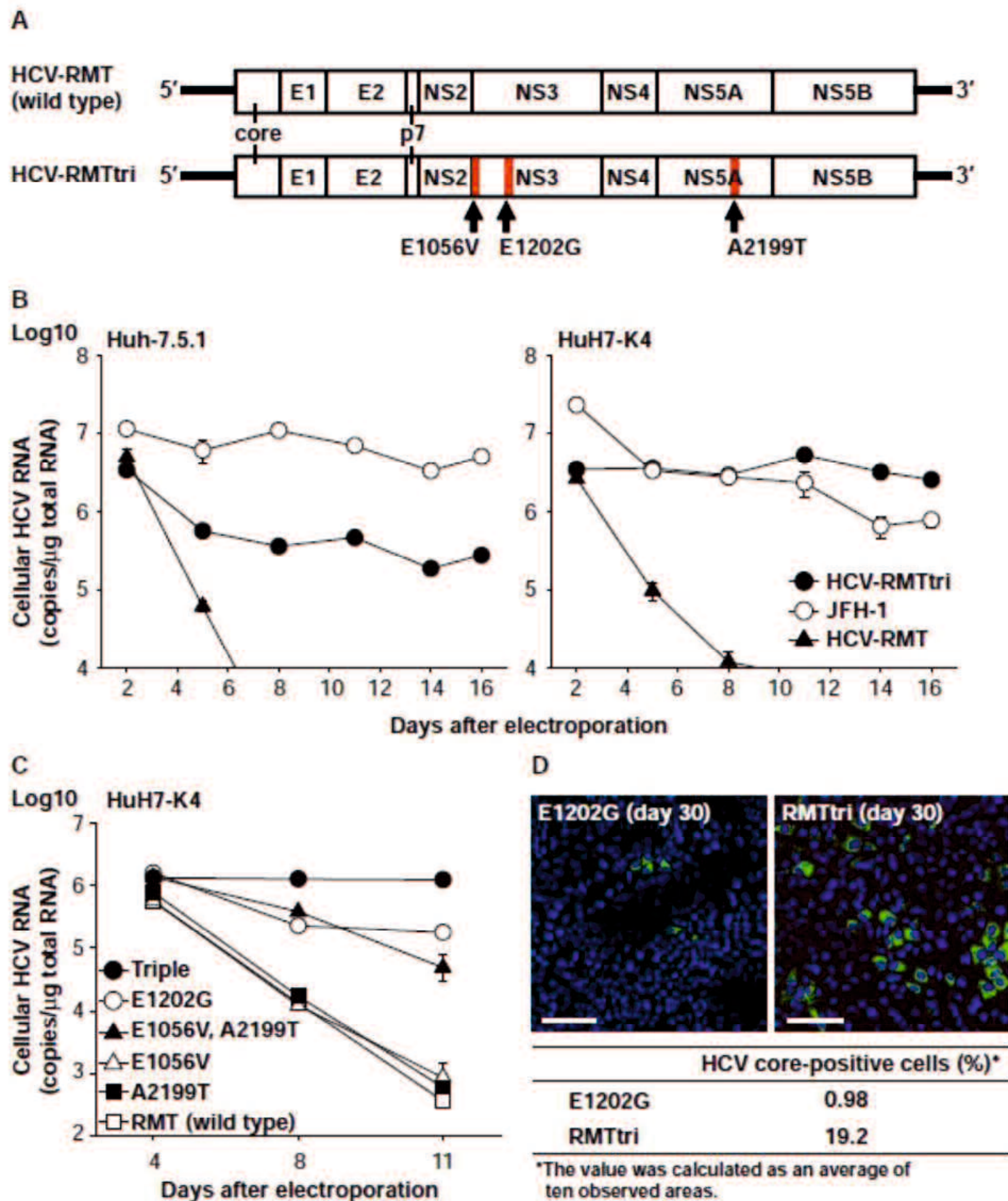


Figure 2-2. *In vitro* replication ability of HCV-RMT derivative genomes.

(A) Schematic representation of construction of the HCV genome and the sites of adaptive mutations (red bars). (B) Electroporation of the generated HCV-RNA genomes of wild-type HCV-RMT (closed triangles), HCV-RMT with triple mutations (HCV-RMTtri; closed circles), and the JFH-1 strain (open circles) into Huh-7.5.1 or HuH7-K4 cells. The experiments were carried out in duplicate. (C) Comparison of the *in vitro* replication ability of each HCV-RMT derivative in HuH7-K4 cells. The experiments were carried out in duplicate. Wild type: open squares, E1202G: open circles, E1056V: open triangles, A2199T: closed squares, E1056V and A2199T: closed triangles, triple mutations: closed circles. (D) Immunostaining for the HCV core protein in HCV-RNA-electroporated cells.

Scale bar = 100 μm. The percent of HCV core protein-positive cells (%) was calculated as an average of ten observed areas.

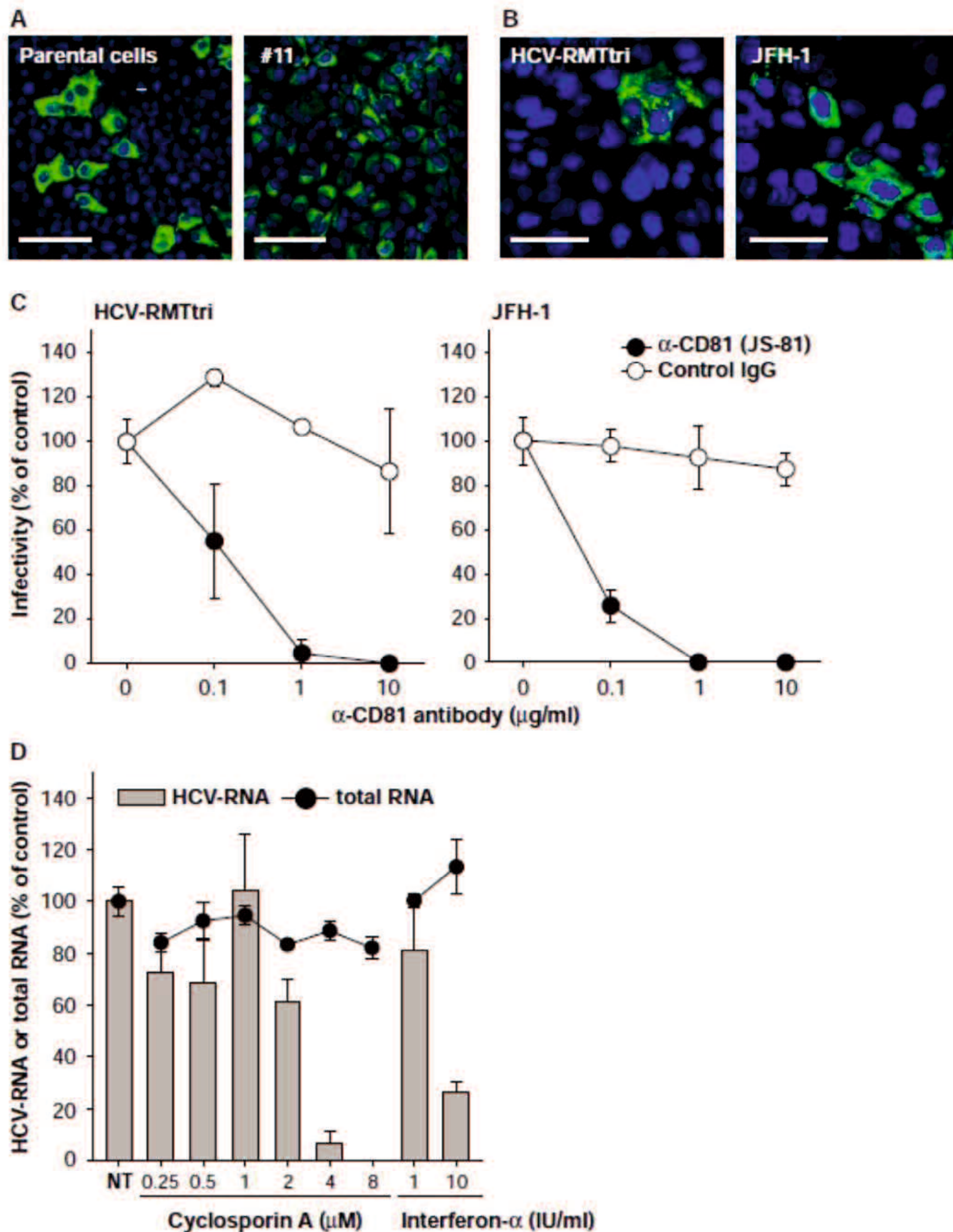


Figure 2-3. Establishment of HCV-RMTtri highly replicating #11 cell and infectivity of its supernatant on naïve HuH7-K4 cells.

(A) Immunostaining for the HCV core protein in HCV-RMTtri-electroporated parental cells and the cell clone (#11) obtained by limiting dilution cloning. Scale bar = 100 μm. (B) Immunostaining for the HCV core protein in naïve HuH7-K4 cells infected with supernatants of HCV-RMTtri- or

JFH-1-replicating cells. Scale bar = 50 μm. (C) Infection with the HCV-RMTtri supernatant was inhibited with anti-CD81 antibody in a similar manner as JFH-1. Control IgG (normal mouse IgG1): open circles, anti-CD81 mAb (JS-81): closed circles. Data are indicated as the mean ± S.D. (D) Replication of HCV-RMTtri in HuH7-K4 cells was inhibited by HCV replication inhibitors such as CsA and IFN-α. Drugs were added to #11 cells in 96-well plates 1 day after passaging, and cells were harvested after 72 h of treatment. NT: no treatment.

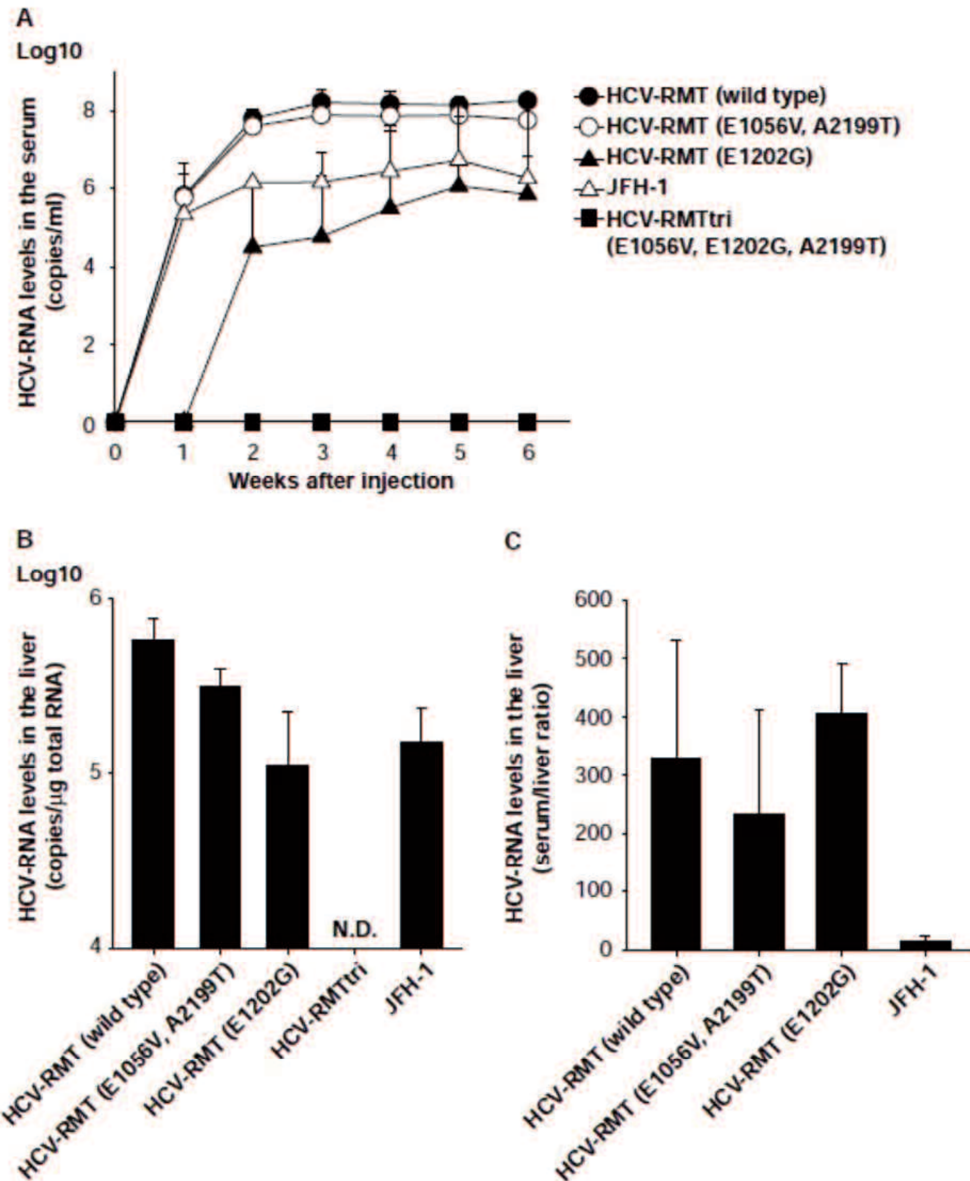


Figure 2-4. ***In vivo*** **replication ability of HCV-RMT derivatives.**

(A) Change in HCV copy numbers in the serum of chimeric mice in which the HCV genome was directly injected into the livers. HCV-RMT (wild type): closed circles, HCV-RMT (E1056V and A2199T): open circles, HCV-RMT (E1202G): closed triangles, HCV-RMTtri: closed squares, JFH-1: open triangles. Data are indicated as the mean ± S.D. (B) HCV copy number in the livers. N.D.: not detected. (C) Serum/liver ratio of HCV copy number.

Table 2-1. Relationship between the *in vitro* and *in vivo* replication ability of HCV-RMT derivatives.

Clones	*in vitro*	*in vivo*
HCV-RMT (wild type)	-	+++
HCV-RMT (E1056V, A2199T)	-	+++
HCV-RMT (E1202G)	+	+
HCV-RMTtri	+++	-
JFH-1	++	+

For the *in vitro* column, +++: maximum replication ability, ++: approximately 1 log lower than the maximum, +: approximately 2 logs lower than the maximum, -: no difference compared to the wild-type strain. For the *in vivo* column, +++: maximum replication ability, ++: approximately 1 log lower than the maximum, +: approximately 2 logs lower than the maximum, -: no replication.

Chapter 3:

Analysis of resistance of Hepatitis C virus mutants to cyclophilin inhibitors as anti-HCV agents

1. Background and Aims

HCVs have been classified into six major genotypic groups (genotypes 1-6); genotype 1 is the most prevalent over most of the world [61]. Treatments with IFN-α, together with the nucleoside analog ribavirin (RBV), greatly increased the percentage of HCV chronically infected patients able to reach a SVR [10].

Covalent attachment of polyethylene glycol (PEGylated) IFN-α-plus-RBV therapy has a success rate of ~80% in patients with genotype 2 or 3 infections, but only ~50% in patients with genotype 1 infections [48, 80]. The recently approved protease inhibitors boceprevir and telaprevir each improved the efficacy of IFN-α-plus-RBV therapy [5]. These direct-acting agents (boceprevir, simeprevir, sofosbuvir, faldaprevir and telaprevir etc.) each have the advantage of being highly specific, but each may select for specific resistant mutations, limiting their long-time efficacy [74]. Therefore, antiviral inhibitors targeting host factors crucial for viral replication should be developed to overcome these problems.

Reportedly, several HCV proteins interact with cyclophilins (Cyp) and modulate HCV replication [19, 22, 88]. To date, three Cyp inhibitors—Debio-025, NIM811, and SCY-635—have been deemed safe and effective for patients with HCV in phase I and II studies [21, 32, 52]. Development of Debio-025 has advanced the farthest through phase II studies, and Debio-025 has approved and showed a great deal of promise for decreasing HCV viremia in infected patients.

However, emergence of drug-resistant HCV mutants could limit the therapeutic potential of CsA and Cyp inhibitors.

The HCV genome is a positive-sense, single-stranded RNA (about 9.6 kb) that encodes at least 10 viral proteins; these are categorized as structural core proteins (E1, E2) or nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [43, 64]. The nonstructural proteins are involved in HCV RNA replication [64]. NS5A protein comprises three domains linked by two low-complexity sequences (LCS) that are either serine or proline rich; domain I is a highly structured zinc binding domain whose three-dimensional structure shows two dimeric conformations [58, 81]. Domains II and III have been shown to be unstructured in their native states, but nuclear magnetic resonance and circular dichroism have shown that elements of secondary structure run throughout each of these domains [20, 30, 54]. NS5A is anchored to membranes by an N-terminal amphipathic helix and is an essential component of the viral genome replication complex; it also interacts with other non-structural proteins [77] or cellular factors. NS5A domain II is a substrate for the peptidyl-prolyl cis/trans isomerase activity of Cyp A and B [29], and NS5A domain III is reportedly a substrate of CypA [84].

In this study, I used CsA to select for and isolate drug-resistant HCV mutants; I then performed virus genome sequencing to investigate the molecular mechanisms of this drug resistance.

2. Materials and Methods

Cells, Electroporation and Ethics statement

HuH-7 cells were cultured in DMEM-GlutaMax-I (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Replicon cells were maintained in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Cells were passaged three times a week, and at each passage, each culture was split into four subcultures. Electroporation of replicon RNA and G418 selection were performed as previously described [89]. All experimental protocol was approved by the regional research institute.

Establishment of Cyclosporine A resistant replicon clones

FLR3-1 cells derived from Con1 (AJ238799)-based, luciferase-harboring HCV subgenomic replicon cell were treated with both 2 μM of CsA and 0.5 mg/ml of G418 for 24 days. Surviving cells were further treated with 3 μM CsA for 2 days, 4μM for 4 another days, and finally 6 μM for the last 10 days. Using limiting dilution cloning, I established nine clonal cell lines. Using real-time RT-PCR (ABI 7700 system, Applied Biosystems) as described previously [80], I systematically measured HCV RNA copy number in each of these nine clonal lines.

Determination of Consensus Sequence of Resistant Clones

LongRange Reverse transcriptase (QIAGEN) and an oligonucleotide primer (antisense sequence 9549-9569 of HCV-Con1) were used to reverse transcribe purified RNA (1 μg). The resulting cDNA, Phusion DNA polymerase (Finnzymes, Vantaa, Finland), and primers recognizing each non-coding region were used for PCR amplification of the entire non-structural protein coding region of the subgenomic replicon. The TA cloning kit (Invitrogen) was used to introduce each fragment into a separate plasmid; I picked up eight clones from each resistant cell line and their nucleotide sequences were determined.

Construction and RNA transcription

The pFK I389neo/NS3-3'/5.1 and pFK I389luc/NS3-3'/5.1 plasmids (ReBlikon, Baden-Württemberg, Germany) were used to generate HCV constructs with regions of the subgenomic replicon with mutations (Figure 1-2A). The QuikChangeII kit (Stratagene) was used to introduce specific mutations into the HCV sequences. To generate RNA, plasmids were digested with XbaI and used as a template for RNA transcription; RiboMax (Promega) was used for each transcription reaction.

Drug treatment

For the drug resistance assays, established CsA-resistant replicon clones were seeded onto 24-well tissue culture plates (10,000 cells/well) and cultivated overnight. Then cells were treated with various concentrations of CsA (0~8μM) for 4 days. Surviving cells were stained with crystal violet.

For HCV replication inhibition assays, replicon cells were seeded in 96-well tissue culture plates (5,000 cells/well) and cultivated overnight. Serial dilutions of CsA (Fluka Chemie) or NIM811 (Novartis) and Debio-025 (Debiopharma) were then added to sets of wells. After incubation for 72 h, ABI prizm 6100 (Applied Biosystems) was used to extract total RNA from cells, and HCV-RNA was measured as described above. Each assay was carried out in triplicate. For another HCV replication inhibition assay, mutant replicon RNA derived from pFK I389luc/NS3-3'/5.1 plasmid were introduced into HuH7 cells via electroporation, and the transformed/transfected cells were seeded to 96-well tissue culture plates. Drugs were added 24 h after electroporation. Luciferase activities were evaluated 4 h or 72 h after electroporation, which corresponded to 20 h before drug treatment or 48 h after drug treatment, respectively; the Blight-Glo kit (Invitrogen) and Envision (Perkin Elmer, Waltham, MA, USA) were used to take all measurements, and values at 72 h were normalized relative to the values from 4 h.

3. Results

Establishment of CsA-resistant clones

To establish CsA-resistant clones, I treated HCV FLR-N replicon cells with CsA (Figure 1-1A) and obtained nine resistant clonal cell lines. I measured the amount of HCV RNA in each resistant clonal line and chose for further study the three lines that consistently had the largest amount of HCV RNA (Figure 1-1B). I then determined the entire HCV sequence from 16 sub clones; I isolated two groups of eight sub clones (one group each from clones #6 and #7), because I could not establish clone #2; each sub clone was isolated by treating a CsA-resistant clone (#6 or #7) with 6 μM CsA [1]. Although there were several mutations in the NS3~NS5B protein-coding regions, common mutations were isoleucine (I) to valine (V) at A. A. 1280 (T1280V) and aspartic acid (D) to glutamic acid (E) at A. A. 2292 (D2292E). At 1280, original Con1 has threonine (T) and was mutated into (I) in Con1 replicon cells.

Identification of mutations responsible for CsA resistance

To define the mutations responsible for CsA resistance, I constructed various chimeric clones that each contained specific mutation that arose from CsA selection (Figure 1-2A). I could thereby evaluate each mutation with regard to its effect on CsA resistance. I found that mutations in

two proteins—NS5A and NS4A —significantly enhanced the resistance against CsA treatment (Figure 1-2B). I also cultured replicon cells with these mutants in the presence of CsA (up to 2 μM); I found that cells with a D2292E mutation could survive, but cells with wild-type NS5A or T1280V mutation could not (Figure 1-3A). The effect of T1280V mutation on colony formation was further evaluated (Figure 1-3B). Introduction of the T1280V mutation in cis to the D2292E mutation rescued the colony-formation defect of the D2292E mutant replicon cells; specifically, the T1280V-D2292E double-mutant replicon cells had the same colony-forming ability as the parental replicon cells.

Evaluation of mutations for CsA resistance in other HCV genotypes

I evaluated whether the mutations that conferred CsA resistance to the HCV Con1 strain (genotype 1b) also conferred CsA resistance to the RMT (genotype 1a; AB520610) and JFH-1 (genotype 2a; AB047639) strains (Figure 1-4A, B and Table 1-2). D2292E conferred CsA resistance to the HCV strains RMT and JFH-1, but T1280V did not (Table 1-2), as observed with HCV Con1 strain (Figure 1-2E). The amino acid sequences surrounding mutations other than D2292E showed some differences among three genotypes (1a, 1b, and 2a) (Figure 1-4B). D2292E mutants of these three genotypes showed resistance to CsA (Figure 1-2E, Table 1-2) but the fold increase of resistance in genotype 1a and 2a was lower than that of genotype 1b (Table 1-2 and 1-3). Therefore, there

might be some residue(s) other than D2292E to influence the resistance to CsA.

Efficacy of mutations in NS5A for conferring CsA resistance

Although D2292E clearly conferred CsA resistance to HCV, other mutations in NS5A may also have had an effect because constructs with all four of the original NS5A mutations found in clone #6 mutations were more resistant to CsA than were constructs with only the D2292E mutation (Figure 1-2B, E). I constructed HCV-luciferase replicons, each with one or more of four mutations (D2292E, D2303H, S2362G, and E2414K). HuH-7 cells were transiently transfected with RNA of each construct; I then treated the transfected cells with CsA (Table 1-3). Of the four single mutants, all but S2362G conferred some CsA resistance to HCV-luciferase replicons; notably, combinations of mutations had additive effects and conferred greater CsA resistance than any single mutation. The HCV replicon with all four mutations showed the strongest CsA resistance.

Evaluation of CsA-resistant mutants for resistance to cyp inhibitors

I further evaluated each of the NS5A mutants for their ability to confer resistance to each of two other cyp inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811, Table 1-4) and Debio-025 (Table 1-5). Of the four single mutants, D2292E conferred the highest resistance, and the combination of all four mutations conferred the overall highest resistance to NIM811 and to

Debio-025. When I compared CsA, NIM811, and Debio-025, the mutation-mediated increases in

IC50 values were lowest with the Debio-025 treatment (Table 1-3, 1-4 and 1-5).

4. Discussion

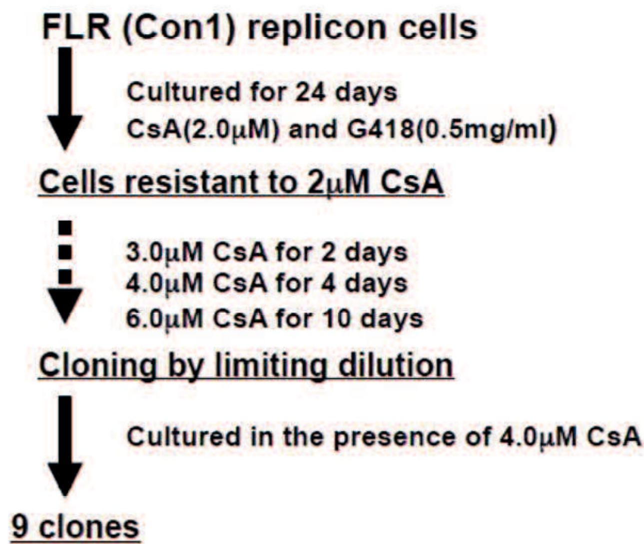
Here, I investigated two of nine HCV subgenomic replicon cell clones (CsA-resistant HCV mutants) isolated following long-term dual treatment with CsA and G418. Comparing the HCV sequences of these two clones (#6 and #7), only two of many mutant sites were shared between the mutant HCV sequences. Specifically, both clones #6 and #7 had a D2292E missense mutation in NS5A and a T1280V missense mutation in NS3. D2292E is known to confer CsA resistance to some HCV genotypes [14, 24, 25, 70], and as a single mutation, it conferred CsA resistance to three separate HCV strains in our hands. In contrast, T1280V in NS3 was not previously identified as a CsA-resistance mutant, and in our hands, it had no impact on CsA resistance as a single mutation (Figure 1-2E, 1-3A).

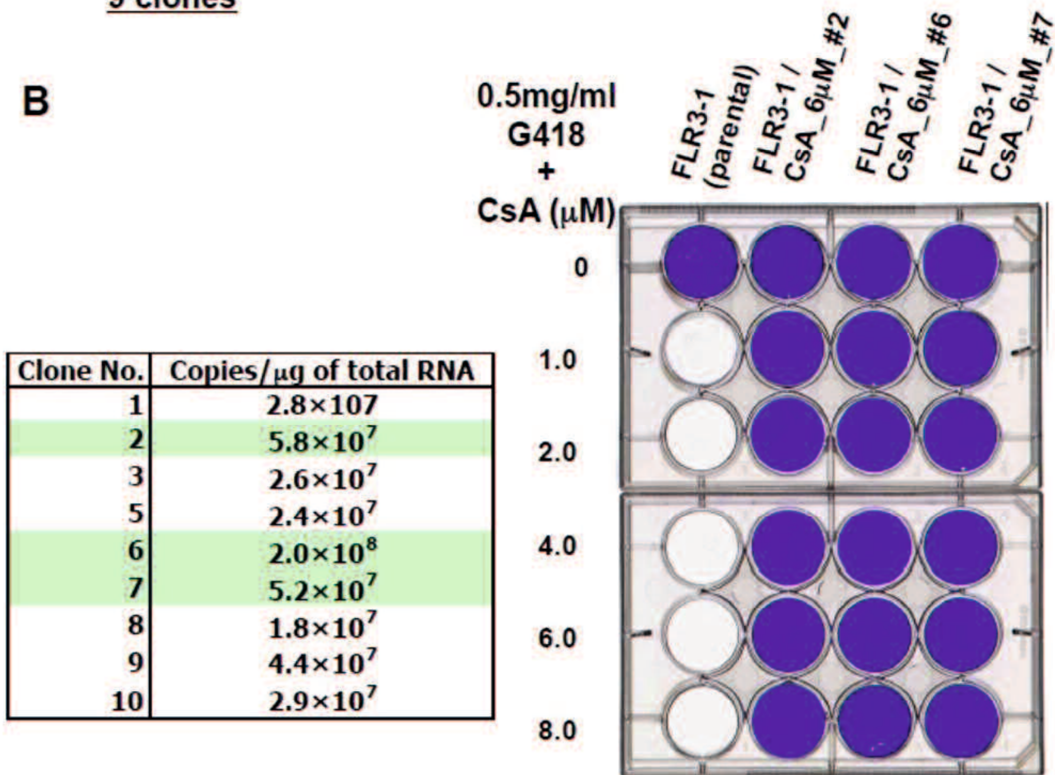
D2292E was the most significant resistance mutation in this study (Figure 1-4C). This mutation is also significant in the regulation of HCV genome replication [72] , and close to the CypA binding region [27]. With several genotypes (1a, 1b, 2a, 3, 4, and 6), D2292E is frequently observed after DEBIO-025 selection [1, 24]. Other different mutations in NS5A and NS5B were identified in other studies of CsA resistance [19]; therefore, various mutations could influence HCV resistance to CsA. In addition to the D2292E mutation, the T1280V mutation in NS3 was present in both clones #6 and #7. Despite its presence in both clones, it did not confer CsA resistance as a single mutant, nor did it enhance the effects of the NS5A CsA-resistant mutants (Figure 1-2E). Instead, it partially

rescued the colony-forming defect caused by D2292E (Figure 1-3B). I used three assays—colony formation assay without CsA treatment (Figure 1-3B), cell survival assay of established replicon cells with CsA and G418 dual-treatment (Figure 1-3A), and HCV replication inhibition assay without G418 treatment (Figure 1-2E, Table 1-1 and 1-2)—to evaluate the HCV replication competence of each of these two mutations (D2292E, T1280V). It is difficult to fully explain all of the results, and comparison of the two CsA-resistant clones (clone #6 and #7) leaves some questions unanswered. These clones were similar to each other when considering survival during CsA and G418 dual-treatment (Figure 1-1B), but they show differences in their resistance in HCV subgenome replication assay (Figure 1-2B, E). Apparently, each mutation in clone #7, except for D2292E, had no effect on the results of the HCV subgenome replication inhibition assay with CsA. These findings might suggest that these mutations were important to G418 resistance, but not to the resistance of HCV to CsA treatment. In contrast, each of three other mutations in NS5A (D2303H, S2362G, and E2414K) that were found in clone #6 were required for the maximum level of drug resistance conferred by a mutant NS5A in this study. To our knowledge, D2303H is a novel CsA resistant mutation, and as a single mutation, it conferred CsA resistance comparable to D2292E. D2303H, like D2292E, was located in carboxy-terminal of domain II of NS5A, which is reportedly a CypA binding site [22]. S2362G and E2414K were mutations in domain III of NS5A, and these mutations may have influenced the peptidyl-prolyl isomerase enzymatic catalytic activity of CypA [84]. The

V1681A mutation in NS4A identified in clone #6 greatly enhanced the CsA resistance of a HCV construct that had NS3 and NS5A replaced with Cs6#6 sequences (Figure 1-2B-D). Though I have not assessed V1681A as single mutant, analyzing its mechanism of CsA resistance and its cooperation with other mutations in NS3 and NS5A must be worthwhile because V1681A greatly enhanced the CsA resistance of some constructs. In all, I evaluated three cyp inhibitors—CsA, NIM811, and Debio-025. Among them, Debio-025 showed the strongest inhibition (IC50 values to any mutants) and was tolerated by CsA-resistant mutations (IC50 index change values, Figure 1-2 and Table 1-3, 1-4 and 1-5). It was interesting that the resistant mutants differed so greatly in their tolerance of these three inhibitors because all three inhibitors have the same mode of action. Garcia-Rivera et al. concluded that CsA resistance of HCV mutants were solely derived from dependence of the NS5A proteins on cyps [24]. Our results might indicate that other factors are important to CsA resistance, in addition to residual cyp activity. Drugs that are intended to treat chronic HCV infection and that target important non-structural HCV proteins—the serine protease NS3/4A, the large phosphoprotein NS5A , or the RNA-dependent RNA polymerase NS5B—have reached the clinical trial stage of drug development [8, 17, 34]. Two oral HCV protease inhibitors were approved by the FDA, and some of the drugs could achieve a SVR [71]. However, to develop treatments that eradicate individual chronic HCV infections, additional studies on the emergence of drug-resistant HCV mutants and on the molecular interactions at HCV replication complexes are

necessary. Our new findings provided insights into the way by which HCV acquires resistance to cyp inhibitors, and these insights will facilitate the development of this type of anti-HCV drug for clinical use.



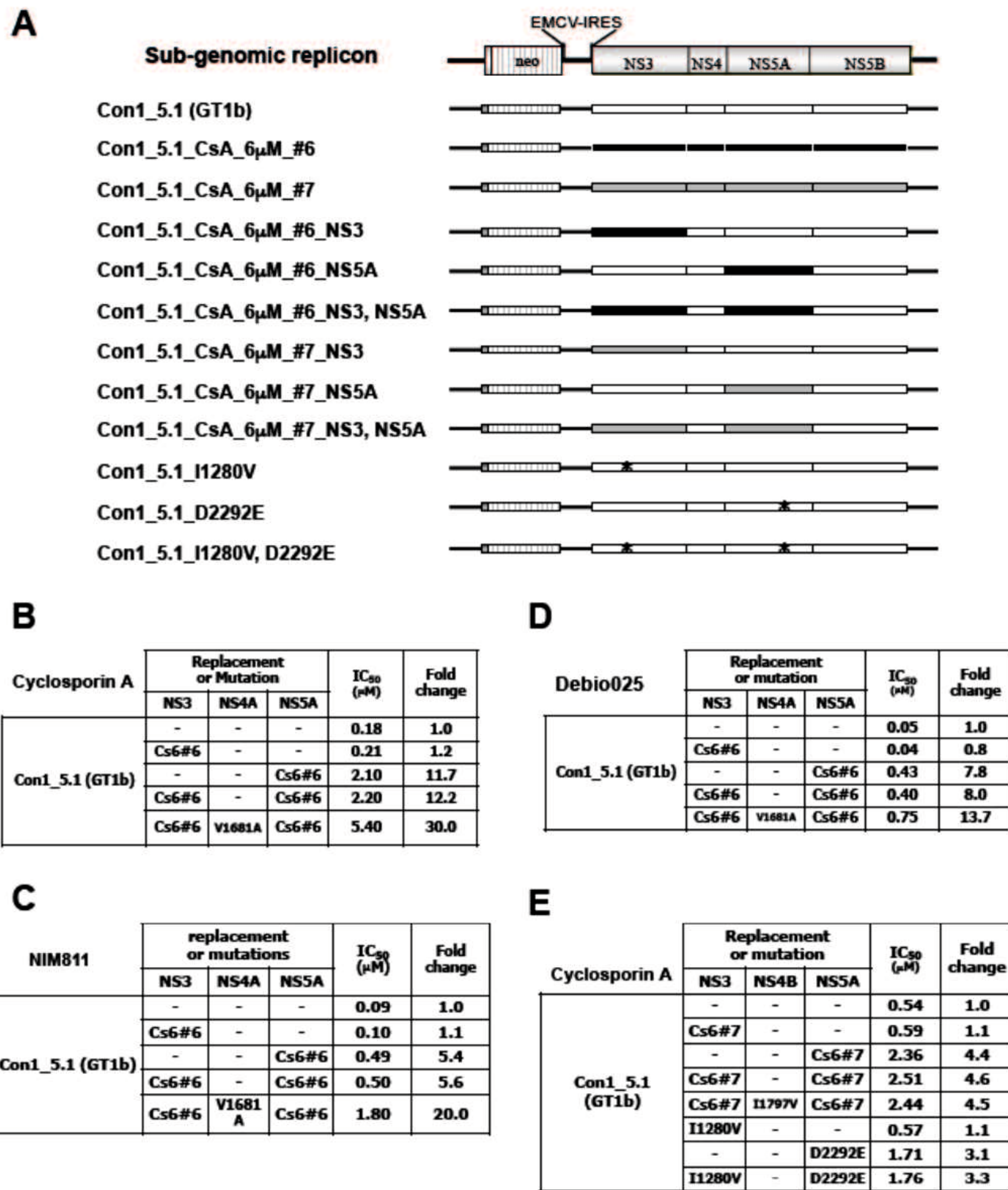


Clone No.	Copies/μg of total RNA
1	2.8×107
2	5.8×10^{7}
3	2.6×10^{7}
5	2.4×10^{7}
6	2.0×10^{8}
7	5.2×10^{7}
8	1.8×10^{7}
9	4.4×10^{7}
10	2.9×10^{7}

Figure 1-1. Basic characteristics of the nine Cyclosporine A-resistant clones.

(A) Flow chart outlining the selection of CsA-resistant HCV replicon clones.

(B) Real-time PCR was used to determine the copy number of each CsA-resistant clone. The three clones with the highest HCV genome copy number are highlighted in green (Left). Colony formation assay of mutant #2, 6 and 7 (Right).



B

Cyclosporin A	Replacement or Mutation			IC$_{50}$ (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.18	1.0
	Cs6#6	-	-	0.21	1.2
	-	-	Cs6#6	2.10	11.7
	Cs6#6	-	Cs6#6	2.20	12.2
	Cs6#6	V1681A	Cs6#6	5.40	30.0

C

NIM811	replacement or mutations			IC$_{50}$ (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.09	1.0
	Cs6#6	-	-	0.10	1.1
	-	-	Cs6#6	0.49	5.4
	Cs6#6	-	Cs6#6	0.50	5.6
	Cs6#6	V1681 A	Cs6#6	1.80	20.0

D

Debio025	Replacement or mutation			IC$_{50}$ (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.05	1.0
	Cs6#6	-	-	0.04	0.8
	-	-	Cs6#6	0.43	7.8
	Cs6#6	-	Cs6#6	0.40	8.0
	Cs6#6	V1681A	Cs6#6	0.75	13.7

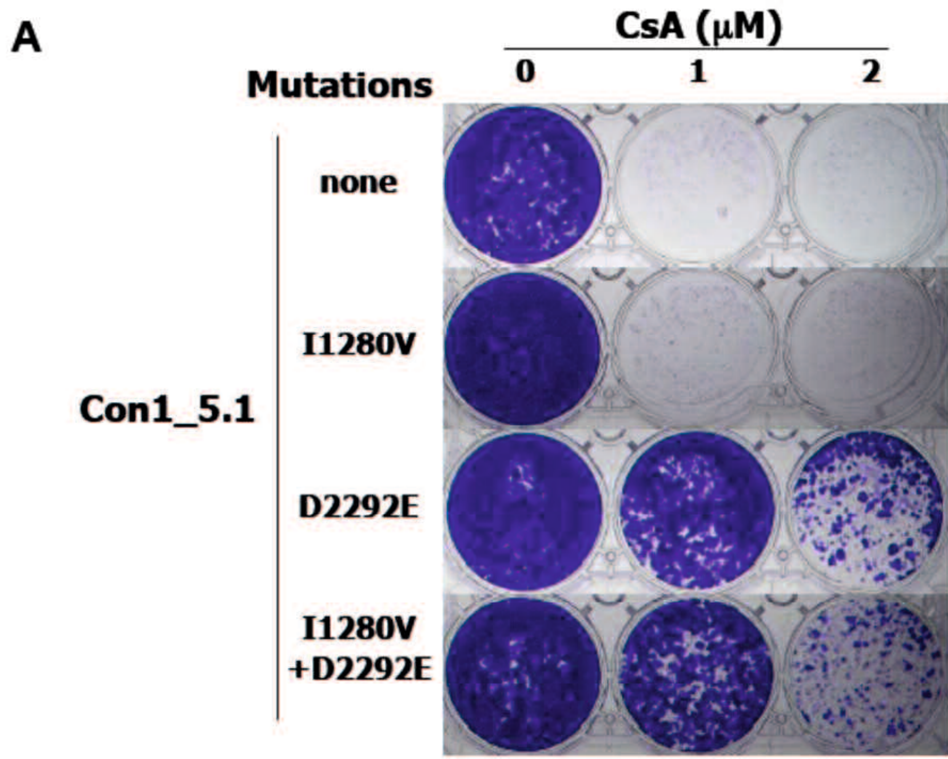
E

Cyclosporin A	Replacement or mutation			IC$_{50}$ (μM)	Fold change
	NS3	NS4B	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.54	1.0
	Cs6#7	-	-	0.59	1.1
	-	-	Cs6#7	2.36	4.4
	Cs6#7	-	Cs6#7	2.51	4.6
	Cs6#7	I1797V	Cs6#7	2.44	4.5
	I1280V	-	-	0.57	1.1
	-	-	D2292E	1.71	3.1
	I1280V	-	D2292E	1.76	3.3

Figure 1-2. Characterizations of established CsA resistant clones

(A) Schematic representations of 12 Con1 replicon-derived constructs.

(B, C, D) Evaluation of Cs6#6 constructs with regard to resistance to CsA or to each of two CsA derivatives (NIM811 and Debio-025). Real-time PCR was used to measure HCV subgenome copy number in cells, and IC50s were then determined from the copy number values. For each construct, the fold change represents the ratio of IC50 values from the construct and the parental Con1 replicon (IC50Construct:IC50Parental). (E) Resistance to CsA of three Cs6#7 derivative constructs that represent the T1280V and D2292E mutations as each single mutation or as a double mutation.



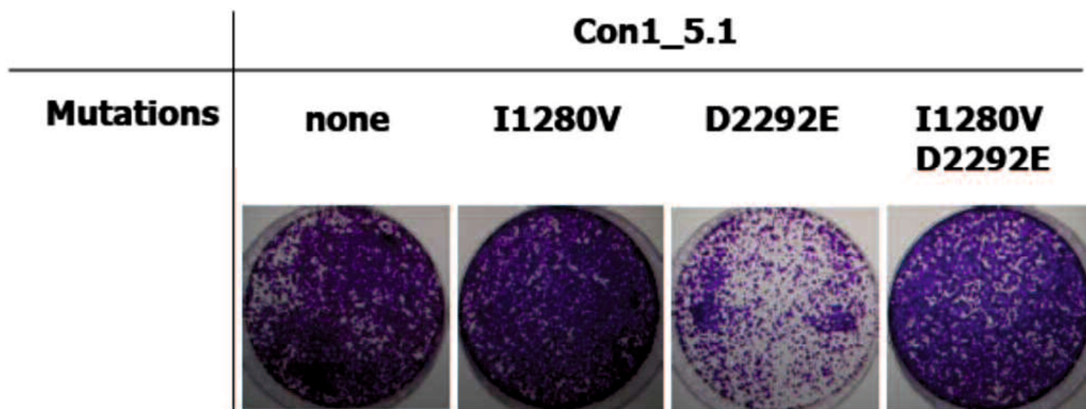


Figure 1-3. (A) Resistance to CsA of T1280V and D2292E mutants. While under G418 selection, established replicon cells were treated with CsA at the indicated doses. (B) Standard methods described in Materials and methods were used to determine the colony-forming abilities of T1280V and D2292E mutants.

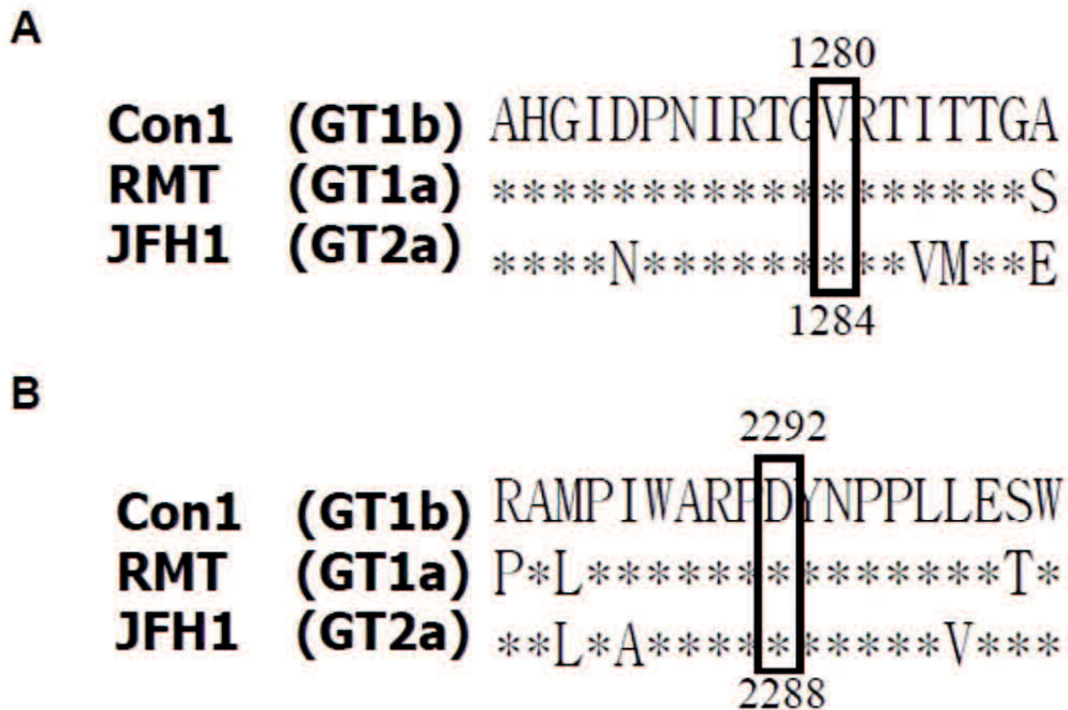


Figure 1-4.

Amino acid sequences of HCV-RMT-tri (GT1a) and HCV-JFH-1 (GT2a) around (A) T1280V and (B) D2292E. (C) Location of the CsA resistant mutations in NS5A. Amino acid sequences around the positions of four CsA resistant mutations.

Table 1-1. The list of each mutated amino acid sequences in 16 clones throughout whole non-structural region.

		NS3						4A	4B	NS5A											5B
A.A. No.		1062	1275	1280	1560	1609	1612	1681	1797	2109	2179	2197	2231	2269	2292	2303	2320	2362	2387	2414	2992
Con1_5.1		V	D	I	S	K	I	V	I	D	S	P	L	S	D	D	K	S	S	E	M
CsA_6μM_#6	1	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	D	D	K	**G**	S	**K**	M
	2	V	D	**V**	S	**E**	I	V	I	**N**	S	P	L	S	D	D	K	S	S	E	M
	3	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	4	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	5	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	6	V	D	**V**	**G**	K	**T**	**A**	I	**E**	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	7	V	D	**V**	**G**	K	**T**	**A**	I	**E**	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	8	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
CsA_6μM_#7	1	**I**	**G**	**V**	S	**E**	I	V	I	**N**	S	P	**P**	**P**	**E**	D	K	S	P	**G**	**T**
	2	V	D	**V**	S	**E**	I	V	I	**N**	S	P	**P**	**P**	**E**	D	K	S	P	**G**	**T**
	3	**I**	**G**	**V**	S	K	I	V	**V**	**N**	**P**	**L**	L	S	**E**	D	**T**	S	S	E	M
	4	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	S	**E**	D	**M**	S	S	E	M
	5	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	S	**E**	D	**T**	S	S	E	M
	6	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	S	**E**	D	**T**	S	S	E	**T**
	7	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	**P**	**E**	D	K	S	P	**G**	**T**
	8	V	D	**V**	S	**E**	I	V	I	**N**	S	P	**P**	**P**	**E**	D	K	S	P	**G**	**T**

The two gray-highlighted lines were selected as the representative sequences of CsA_6μM_#6 and #7 and used to generate the derivative constructs.

Table 1-2. Evaluation of amino acid mutations in NS5A that conferred CysA resistance.

	Mutations in NS5A				IC50 (uM)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.11	1.0
	○				0.88	7.9
		○			0.52	4.7
			○		0.12	1.0
				○	0.30	2.7
		○	○	○	1.0	9.4
	○	○			1.8	16.6
	○		○		0.95	8.5
	○			○	1.5	13.1
	○	○	○	○	2.80	25.7

Table 1-3. Evaluation of amino acid mutations in NS5A that conferred NIM811 resistance.

	Mutations in NS5A				IC50 (uM)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.054	1.0
	○				0.324	6.0
		○			0.184	3.4
			○		0.056	1.0
				○	0.125	2.3
		○	○	○	0.455	8.4
	○	○			0.635	11.8
	○		○		0.403	7.5
	○			○	0.599	11.1
	○	○	○	○	0.923	17.1

Table 1-4. Evaluation of amino acid mutations in NS5A that conferred Debio-025 resistance.

	Mutations in NS5A				IC50 (uM)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.024	1.0
	○				0.095	4.0
		○			0.074	3.1
			○		0.028	1.2
				○	0.024	1.8
		○	○	○	0.139	5.8
	○	○			0.198	8.3
	○		○		0.139	5.8
	○			○	0.185	7.8
	○	○	○	○	0.263	11.0

Table 1-5. Evaluation of resistance to CsA of mutants that have single mutations or combinations of multiple mutations.

	Mutations		IC50 (uM)	Fold change
	NS3	NS5A		
RMT-tri (GT1a)	-	-	0.79	1.0
	-	D2292E	2.1	2.7
	T1280I	-	0.96	1.2
	T1280I	D2292E	2.46	3.1
	T1280V	-	0.91	1.2
	T1280V	D2292E	2.54	3.2
JFH-1 (GT2a)	-	-	0.49	1.0
	-	D2292E	1.3	2.7
	T1284I	-	0.51	1.0
	T1284I	D2292E	1.38	2.8
	T1284V	-	0.69	1.4
	T1284V	D2292E	1.2	2.4

Threonine at site 1280 (RMT-tri) or 1284 (JFH-1) were mutated to isoleucine (adaptive mutation of Con1 replicon) or valine (major mutation of CsA resistant clones). Aspartic acid at 2292 was mutated to glutamic acid.

Conclusion

In this study, I attempted to establish HCV infectious clone and evaluate the mechanism to gain resistance to CsA.

First, I constructed newly designed full-genomic HCV replicon RNA, which is composed of the intact 5′-terminal-half RNA extending to the NS2 region flanked by an extra selection marker gene. HuH-7 cells harboring this full-genomic RNA proliferated well under G418 selection and secreted virion-like particles into the supernatant. These particles, which were round and 50 nm in diameter when analyzed by electron microscopy, had a buoyant density of 1.08 g/mL that shifted to 1.19 g/mL after NP-40 treatment; these figures match the putative densities of intact virions and nucleocapsids without envelope. The particles also showed infectivity in a colony-forming assay. This system may offer another option for investigating the lifecycle of HCV.

Next, I isolated a genotype 1a clone, designated HCV-RMT, which has the ability to replicate efficiently in patients, chimeric mice with humanized liver, and cultured cells. An authentic subgenomic replicon cell line was established from the HCV-RMT sequence with spontaneous introduction of three adaptive mutations, which were later confirmed to be responsible for efficient replication in HuH-7 cells as both subgenomic replicon RNA and viral genome RNA. Following transfection, the HCV-RMT RNA genome with three adaptive mutations was maintained for more than 2 months in HuH-7 cells. One clone selected from the transfected cells had a high copy number,

and its supernatant could infect naïve HuH-7 cells. Direct injection of wild-type HCV-RMT RNA into the liver of chimeric mice with humanized liver resulted in vigorous replication, similar to inoculation with the parental patient's serum. A study of virus replication using HCV-RMT derivatives with various combinations of adaptive mutations revealed a clear inversely proportional relationship between *in vitro* and *in vivo* replication abilities. Therefore, HCV-RMT and its derivatives are important tools for HCV genotype 1a research and for determining the mechanism of HCV replication *in vitro* and *in vivo*.

Finally, CsA is an immunosuppressive drug that targets cyclophilins, cellular cofactors that regulate the immune system. Replication of HCV is suppressed by CsA, but the molecular basis of this suppression is still not fully understood. To investigate this suppression, I cultured HCV replicon cells (Con1, HCV genotype 1b, FLR-N cell) in the presence of CsA and obtained nine CsA-resistant FLR-N cell lines. I determined full-length HCV sequences for all nine clones, and chose two (clones #6 and #7) of the nine clones that have high replication activity in the presence of CsA for further analysis. Both clones showed two consensus mutations, one in NS3 (T1280V) and the other in NS5A (D2292E). Characterization of various mutants indicated that the D2292E mutation conferred resistance to high concentrations of CsA (up to 2 μM). In addition, the missense mutation T1280V contributed to the recovery of colony formation activity. The effects of these mutations are also evident in two established HCV replicon cell lines—HCV-RMT [4], genotype 1a) and JFH-1

(genotype 2a). Moreover, three other missense mutations in NS5A—D2303H, S2362G, and E2414K— enhanced the resistance to CsA conferred by D2292E; these double or all quadruple mutants could resist approximately 8- to 25-fold higher concentrations of CsA than could wild-type Con1. These four mutations, either as single or combinations, also made Con1 strain resistant to two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811) or Debio-025. Interestingly, the changes in IC_{50} values that resulted from each of these mutations were the lowest in the Debio-025-treated cells, indicating its highest resistant activity against the adaptive mutation.

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