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Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders

Ikuo Shoji *, Lin Deng and Hak Hotta

Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan

Edited by:
Yasuko Yokota, National Institute of Infectious Diseases, Japan

Reviewed by:
Koji Ishii, National Institute of Infectious Diseases, Japan
Kohji Moriishi, University of Yamanashi, Japan

****Correspondence:***
Ikuo Shoji, Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan.
e-mail: ishoji@med.kobe-u.ac.jp

Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including metabolic disorders. Chronic HCV infection is often associated with type 2 diabetes. However, the precise mechanism underlying this association is still unclear. Glucose is transported into hepatocytes via glucose transporter 2 (GLUT2). Hepatocytes play a crucial role in maintaining plasma glucose homeostasis via the gluconeogenic and glycolytic pathways. We have been investigating the molecular mechanism of HCV-related type 2 diabetes using HCV RNA replicon cells and HCV J6/JFH1 system. We found that HCV replication down-regulates cell surface expression of GLUT2 at the transcriptional level. We also found that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells. HCV infection transcriptionally up-regulated the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. Gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with the FoxO1-dependent increased gluconeogenesis. This paper will discuss the current model of HCV-induced glucose metabolic disorders.

Keywords: HCV, diabetes, gluconeogenesis, GLUT2, FoxO1, JNK, NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein of about 3,000 amino acids, which is cleaved by cellular signalases and viral proteases to generate at least 10 viral proteins, such as core, envelope 1 (E1) and E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Choo et al., 1991; Lemon et al., 2007).

Hepatitis C virus is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 170 million people worldwide are chronically infected with HCV (Poynard et al., 2003). Persistent HCV infection causes not only liver diseases but also extrahepatic manifestations. It is well established that HCV perturbs the glucose metabolism, leading to insulin resistance and type 2 diabetes in predisposed individuals. Several epidemiological, clinical, and experimental data suggested that HCV infection serves as an additional risk factor for the development of diabetes (Mason et al., 1999; Negro and Alaei, 2009; Negro, 2011). HCV-related glucose metabolic changes and insulin resistance and diabetes have significant clinical consequences, such as accelerated fibrogenesis, increased incidence of hepatocellular carcinoma, and reduced virological response to interferon (IFN)-α-based therapy (Negro, 2011). Therefore, it is very important to clarify the molecular mechanism of HCV-related diabetes. However, the precise mechanisms are poorly understood.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway. Transgenic mice expressing HCV core gene exhibit insulin resistance (Shintani et al., 2004; Koike, 2007). In this transgenic mice model, both tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 and IRS-2 are decreased. These decreases are recovered when the proteasome activator PA28γ is deleted, suggesting that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway (Miyamoto et al., 2007). Several other reports also showed a link of the HCV core protein with insulin resistance (Kawaguchi et al., 2004; Pazienza et al., 2007).

Hepatocytes play a crucial role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. Gluconeogenesis is mainly regulated at the transcriptional level of the glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, whereas glycolysis is mainly regulated by glucokinase (GK). Gluconeogenesis and glycolysis are coordinated so that one pathway is highly active within a cell while the other is relatively inactive. It is well known that increased hepatic glucose production via gluconeogenesis is a major feature of type 2 diabetes (Clore et al., 2000).

To identify a novel mechanism of HCV-related diabetes, we have been investigating the effects of HCV on glucose production in hepatocytes using HCV RNA replicon cells (Lohmann et al., 1999) and HCV J6/JFH1 cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Bungyoku et al., 2009). We previously reported that HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporter 2 (GLUT2; Kasai et al., 2009). Furthermore, we recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (**Figure 1**). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (**Figure 1**). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (**Figure 1**).

HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4α (HNF-4α; Hirota et al.,

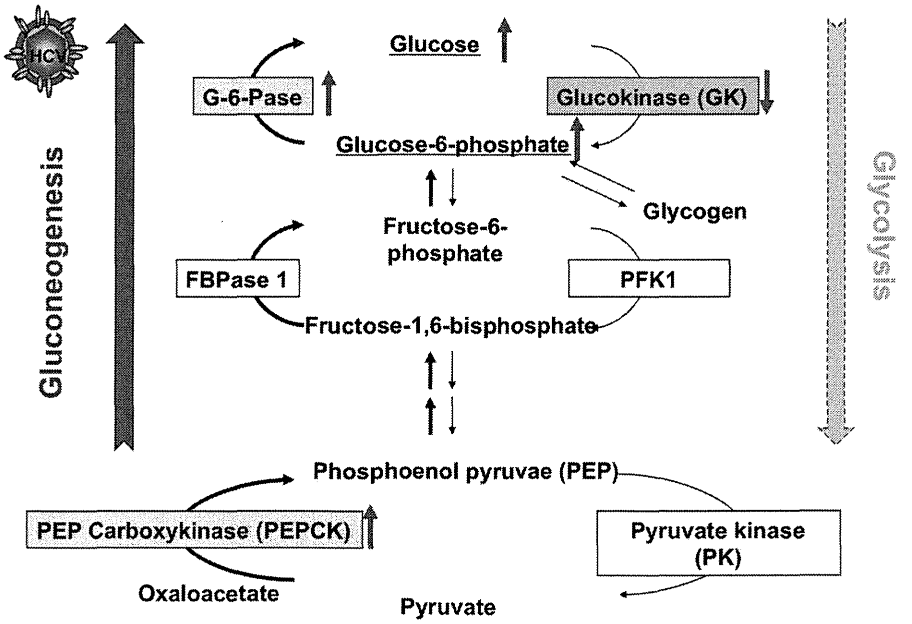

FIGURE 1 | Regulation of Gluconeogenesis and Glycolysis in the HCV-infected cells. HCV infection promotes gluconeogenesis via transcriptional up-regulation of the genes for PEPCK and G6Pase, the rate-limiting enzymes for hepatic gluconeogenesis, and transcriptional down-regulation of the gene for GK, the rate-limiting enzyme for hepatic glycolysis.

2008), Krüppel-like factor 15 (KLF15; Takashima et al., 2010), and cyclic AMP (cAMP) response element binding protein (CREB; Rozance et al., 2008). While we were analyzing these factors in both HCV replicon cells and HCV J6/JFH1-infected cells, we found the involvement of the FoxO1 in the transcriptional activation of G6Pase and PEPCK (Deng et al., 2011). It is known that the FoxO1 enhances gluconeogenesis through the transcriptional activation of various genes, including G6Pase and PEPCK (Gross et al., 2008). The function of FoxO1 is regulated by post-translational modifications, including phosphorylation, ubiquitylation, and acetylation (Tzivion et al., 2011). The phosphorylated form of FoxO1 is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (**Figure 2**). Phosphorylation status of FoxO1 at Ser319 is critical for FoxO1 nuclear exclusion (Zhao et al., 2004). Although the total amounts of FoxO1 protein were unchanged, FoxO1 phosphorylation at Ser319 was markedly suppressed in HCV-infected cells compared to that in the mock-infected cells. It is known that the FoxO1 is phosphorylated by the protein kinase Akt and is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Tzivion et al., 2011). The majority of FoxO1 was accumulated in the nuclear fraction in HCV-infected cells, whereas in control cells FoxO1 was distributed in both the nuclear and cytoplasmic fractions. Akt phosphorylation was enhanced in HCV-infected cells, although the protein levels of total Akt protein were comparable, which is consistent with the report by Burdette et al. (2010). Our findings suggest an interesting scenario in which the HCV-mediated suppression in FoxO1 phosphorylation is caused by an unknown mechanism independent of Akt activity.

HCV-INDUCED JNK ACTIVATION IS INVOLVED IN THE SUPPRESSION OF FoxO1 PHOSPHORYLATION

It is known that the stress-sensitive serine/threonine kinase JNK regulates FoxO at multiple levels (van der Horst and Burgering, 2007; Karpac and Jasper, 2009). We demonstrated that HCV infection induces phosphorylation and activation of JNK in a time-dependent manner, which is similar to that observed for the suppression of FoxO1 phosphorylation. As a result, c-Jun, a key substrate for JNK, got phosphorylated and activated in HCV-infected cells. The JNK inhibitor SP600125 clearly prevented the phosphorylation of c-Jun, and concomitantly recovered the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV activates the JNK/c-Jun signaling pathway, resulting in the nuclear accumulation of FoxO1 by reducing its phosphorylation status. The detailed mechanisms of HCV-induced suppression of FoxO1 phosphorylation via the JNK/c-Jun signaling pathway remain to be explored. There are at least two possibilities. The JNK/c-Jun signaling pathway (1) suppresses a protein kinase, or (2) activates a protein phosphatase to reduce phosphorylation of FoxO1.

HCV-INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION IS INVOLVED IN INCREASED GLUCOSE PRODUCTION THROUGH JNK ACTIVATION

Hepatitis C virus infection increases mitochondrial reactive oxygen species (ROS) production (Deng et al., 2008). *N*-acetyl cysteine (NAC; a general antioxidant) clearly prevented the phosphorylation of JNK, and concomitantly canceled the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that

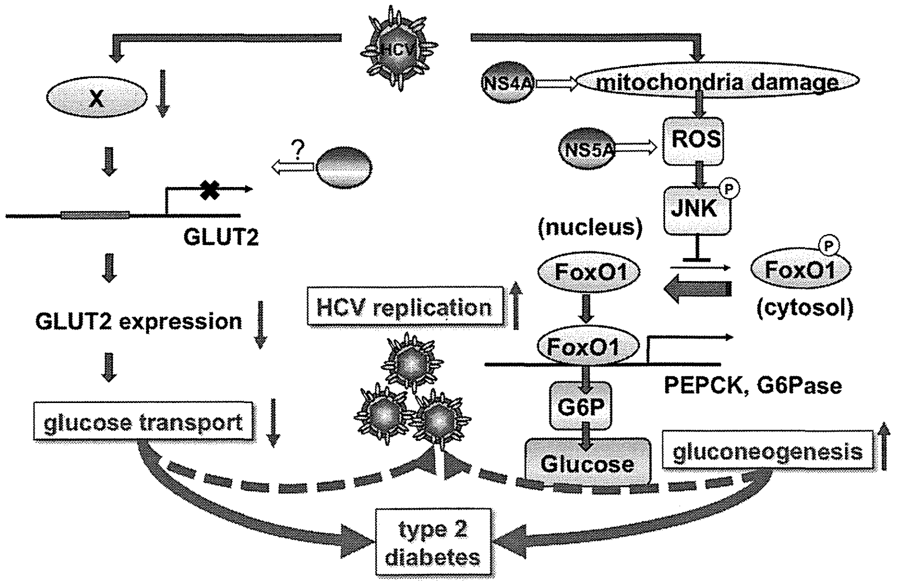

FIGURE 2 | A proposed mechanism of HCV-induced glucose metabolic disorders. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. HCV infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage. HCV NS5A protein is involved in ROS production. HCV-induced ROS production causes JNK activation, resulting in the decreased phosphorylation and nuclear accumulation of FoxO1. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis. High glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

HCV-induced ROS production is involved in the JNK activation. There was no significant difference in HCV RNA replication or infectious virus release between SP600125- or NAC-treated HCV-infected cells and non-treated HCV-infected cells. These results suggest that ROS-mediated JNK activation plays a key role in the suppression of FoxO1 phosphorylation, nuclear accumulation of FoxO1, and enhancement of glucose production in HCV-infected cells (Deng et al., 2011).

HCV NS5A IS INVOLVED IN THE ENHANCEMENT OF GLUCOSE PRODUCTION

Then we sought to determine which HCV protein(s) is involved in the enhancement of glucose production. Transient expression of NS5A protein in Huh-7.5 cells significantly promoted the gene expression levels of G6Pase and PEPCK determined by real time quantitative RT-PCR. Promoter assay revealed that the level of PEPCK promoter activity was significantly higher in NS5A-expressing cells than in the control cells. Our results suggest that NS5A activate both the PEPCK promoter and the G6Pase promoter, leading to an increase in glucose production (Deng et al., 2011). The study by Banerjee et al. (2010) suggests that the HCV core protein modulates FoxO1 and FoxA2 activation and affects insulin-induced metabolic gene regulation in human hepatocytes. Our results, however, suggest that the HCV core protein is not significantly involved in the increased gluconeogenesis (Deng et al., 2011). The difference between these two studies needs to be explored.

There were previous reports suggesting that ROS production is induced in NS5A-expressing cells (Dionisio et al., 2009) or in hepatocytes of NS5A transgenic mice (Wang et al., 2009). We therefore sought to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. NS5A-expressing cells displayed a much stronger signal of ROS than in control cells. NS5A-expressing cells promoted phosphorylation level at Ser63 of c-Jun and suppressed FoxO1 phosphorylation at Ser319, suggesting that NS5A mediates JNK/c-Jun activation and FoxO1 phosphorylation suppression. These results suggest that NS5A play a role in the HCV-induced enhancement of hepatic gluconeogenesis through JNK/c-Jun activation and FoxO1 phosphorylation suppression.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, we propose a model of HCV-induced glucose metabolic disorders as shown in **Figure 2**. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. As GLUT2 is a facilitative GLUT, it ensures large bidirectional fluxes of glucose in and out the cell due to its low affinity and high capacity (Leturque et al., 2009). Down-regulated cell surface expression of GLUT2 results in disruption of bidirectional transport of glucose in hepatocytes. Even in the fasting state, down-regulation of GLUT2 may result in low glucose uptake of hepatocytes, causing hyperglycemia. In the fed state, glucose secretion from hepatocytes may be suppressed due to low level cell surface expression of GLUT2, as GLUT2 is a bidirectional transporter.

Hepatitis C virus infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage (Nomura-Takigawa et al., 2006). HCV NS5A protein is involved in ROS production (Dionisio et al., 2009; Wang et al., 2009; Deng et al., 2011). HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1 by an unidentified mechanism. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis (Deng et al., 2011).

These two pathways, HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis, may contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis may result in high concentration of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentration in the hepatocytes inhibits HCV replication (Nakashima et al., 2011). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

Our understanding of HCV-induced glucose metabolic disorders will require much more work to fully unfold this pathway. Further investigation including the mechanism of HCV-induced GLUT2 downregualtion, JNK-mediated decreased phosphorylation of FoxO1, and the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle and host cells are currently under way.

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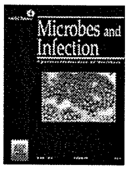

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Original article

Generation of a recombinant reporter hepatitis C virus useful for the analyses of virus entry, intra-cellular replication and virion production

Kazuya Kamada [a], Ikuo Shoji [a], Lin Deng [a], Chie Aoki [a,b], Suratno Lulut Ratnoglik [a,b], Takaji Wakita [c], Hak Hotta [a,b,*]

[a] *Division of Microbiology, Kobe University, Graduate School of Medicine, 7-5-1 Kusunoki-cho, Cyuo-ku, Kobe, Hyogo 650-0017, Japan*
[b] *JST/JICA SATREPS, Japan*
[c] *Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan*

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Abstract

The lack of a culture system that efficiently produces progeny virus has hampered hepatitis C virus (HCV) research. Recently, the discovery of a novel HCV isolate JFH1 and its chimeric derivative J6/JFH1 has led to the development of an efficient virus productive culture system. To construct an easy monitoring system for the viral life cycle of HCV, we generated bicistronic luciferase reporter virus genomes based on the JFH1 and J6/JFH1 isolates, respectively. Transfection of the J6/JFH1-based reporter genome to Huh7.5 cells produced significantly greater levels of progeny virus than transfection of the JFH1 genome. Furthermore, the expression of dominant-negative Vps4, a key molecule of the endosomal sorting complex required for transport machinery, inhibited the virus production of JFH1, but not that of J6/JFH1. These results may account for the different abilities to produce progeny virus between JFH1 and J6/JFH1. Using the J6/JFH1/Luc system, we showed that the two polyanions heparin and polyvinyl sulfate decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner. We also analyzed the function of microRNA on HCV replication and found that miR-34b could affect the replication of HCV. The reporter virus generated in this study will be useful for investigating the nature of the HCV life cycle and for identification of HCV inhibitors.

Keywords: HCV; Reporter virus; Virus production; ESCRT; microRNA

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus and has a positive-stranded RNA genome of about 9.6 kb [1,2]. HCV persistently infects hepatocytes, and the persistent infection can lead to liver cirrhosis and hepatocellular carcinoma. Considering that approximately 170 million people are infected with HCV worldwide [3], HCV is a major public health problem throughout the world. A combination therapy of pegylated interferon-α and ribavirin has been established as the standard of care for treating HCV infection [3,4]. Nonetheless, approximately 50% of individuals with chronic HCV infection are still unable to resolve infection [4,5]. For this reason, more effective therapies are greatly needed against the disease caused by HCV infection [6].

The HCV genome encodes a 3000 amino acid polyprotein which is cleaved by host and viral proteases to yield the mature structural proteins, composed of core and glycoprotein E1 and E2, and the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [1–3]. Translation of the HCV open reading frames is mediated via the 5′ untranslated region and a part of the core coding region carrying the internal ribosome entry site (IRES) [1,7].

In 1999, Bartenschlager and his colleagues produced the HCV replicon system, a tissue culture system that recapitulated the RNA replication of HCV in a human hepatoma cell line [8]. In the initial subgenomic replicon system, genes

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* Corresponding author. Division of Microbiology, Kobe University, Graduate School of Medicine, 7-5-1 Kusunoki-cho, Cyuo-ku, Kobe, Hyogo 650-0017, Japan. Tel.: +81 78 382 5500; fax: +81 78 382 5519.
E-mail address: hotta@kobe-u.ac.jp (H. Hotta).

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unessential for RNA replication that contained the core, E1, E2, p7 and NS2 of the HCV genome were replaced with a genetic cassette carrying an antibiotics resistance gene and IRES from encephalomyocarditis virus (EMCV). The development of a subgenomic replicon system became a driving force for the studies on the mechanism of HCV replication, and these studies revealed numerous biological features of HCV replication. However, the resulting systems were unable to produce progeny virus. Therefore, the nature of the HCV, i.e., the virus production and virus entry, remained unclear for a long while.

Wakita and his colleagues isolated a full-length HCV genome from the sera of a patient with fulminant hepatitis [9]. The HCV strain, designated JFH1, belongs to genotype 2a. The transfection of the Huh7 hepatoma cell line with the JFH1 genome yields a progeny virus called HCVcc that is infectious both *in vivo* and *in vitro*. The HCVcc system allowed us to perform virological studies to investigate the nature of HCV [9,10]. However, the analyses using HCVcc have not been suitable for carrying out high-throughput screening due to the labor-intensive quantitative reverse transcription-PCR methods used in screening and the difficulties presented by the low signal-to-noise ratios.

In this study, to develop a robust tool for use in the screening of HCV replication, we have constructed a genome-length luciferase reporter HCV derived from the JFH1 and J6/JFH1 strains, and used it to analyze the intra-cellular RNA replication and extra-cellular progeny virus production. We demonstrated here that our recombinant reporter HCV system was useful for studying viral genome replication, virus entry, and virion production of HCV.

2. Materials and methods

2.1. Plasmids

The plasmid pFGR-JFH1/Luc, which encodes bicistronic constructs of HCV IRES-driven firefly luciferase reporter genes and the EMCV IRES-driven full-genomic JFH1 genome, was constructed by insertion of the JFH1 full genome of pJFH1 [9] into pSGR-JFH1 [11]. The plasmid pFL-J6/JFH1, which contains a chimeric full-genome composed of the 5′NCR to NS2 region derived from J6 and NS3 to the 3′NCR region from JFH1 [10], was kindly supplied by C.M. Rice of the Center for the Study of Hepatitis C, Rockefeller University. To yield the bicistronic luciferase reporter construct composed of full-length J6/JFH1, the JFH1 full genome of pFGR-JFH1/Luc was replaced with the J6/JFH1 full genome of pFL-J6/JFH1 by digestion with BstZ17I, and the resultant plasmid was designated as pFGR-J6/JFH1/Luc. As a negative control for the HCV replication, a non-synonymous mutation at NS5B (GDD to GND), which disrupts NS5B polymerase activity, was introduced into the pFGR-J6/JFH1/Luc NS5B region by site-directed mutagenesis, and the resultant plasmid was designated pFGR-J6/JFH1/Luc (GND).

2.2. Cell culture and indirect immunofluorescence

All experiments described in this study were performed by using Huh7.5 human hepatoma cells, a highly HCV-susceptible subclone of Huh7 cells. The cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 0.01% streptomycin, and were subcultured twice weekly. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis as previously reported [12]. The primary antibody used was derived from an HCV-infected patient's serum. The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (MBL, Nagoya, Japan).

2.3. In vitro transcription and electroporation

Plasmid DNA was linearized with XbaI, extracted with phenol and chloroform, precipitated with ethanol, and dissolved in RNase-free water. The purified DNA was used for *in vitro* RNA transcription using a T7 Megascript kit (Ambion, Austin, TX) following the manufacturer's protocols. The concentration was determined by measurement of the optical density at 260 nm, and the RNA integrity was checked by agarose gel electrophoresis. The *in vitro*-transcribed RNA (10 μg) was transfected into Huh7.5 cells by means of electroporation (975 μF, 270 V) using a Gene Pulser (Bio-Rad, Hercules, CA). The cells were then cultured in complete medium. The culture fluid of transfected cells was harvested and cleared by passing through 0.45-μm-pore-size filters and stored at −80 °C until use.

2.4. Luciferase assay

The *firefly* luciferase activity was measured by a luciferase assay system (Promega, Madison, WS). The cells were harvested, washed twice with dication-free phosphate buffered saline (PBS), and lysed in a passive lysis buffer supplied by the manufacturer. A 20-μl sample of the lysate was subjected to a luciferase assay. The luminescence was measured at 10 s after an initial 2 s delay according to the manufacturer's instructions, using a Lumat LB9501 luminometer (Berthold, Freiburg, Germany). The assays were performed in duplicate at least three times, and the mean and standard error were computed.

2.5. Vectors of ESCRT family proteins and DNA transfection

The cDNA of the endosomal sorting complex required for transport (ESCRT) family proteins was amplified from Huh7.5 cells by RT-PCR and cloned into pcDNA3.1-FLAG [13], an expression vector containing a CMV promoter and FLAG tag sequence in pcDNA3.1 (Invitrogen, Carlsbad, CA). For the expression of each ESCRT family protein, Huh7.5 cells were transfected with each ESCRT expression vector by using TransIT LT1 transfection reagents (Takara, Kyoto, Japan). The expression levels of the three ESCRT family proteins in transfected Huh7.5 cells were monitored by immunoblotting using the anti-FLAG antibody (Sigma–Aldrich, St. Louis, MO).

2.6. Quantification of HCV core protein

HCV core protein in the cells or cell-culture supernatants was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics). To determine the intra-cellular amounts of core, cell lysates were prepared as described by Schaller et al. [14].

2.7. Blocking of virus attachment and entry with anti-CD81 antibody

Blocking of virus attachment and entry with anti-CD81 antibody was performed essentially as described previously [9]. Huh7.5 cells (6×10^4 cells/well of a 24-well plate) were pre-treated with anti-CD81 antibody (clone JS-81; BD Biosciences) or an isotype-matched control antibody (purified mouse IgG1, isotype control; BD Biosciences) as indicated for 1 h. Cells were then infected with the reporter viruses for 6 h. The viruses were removed, and then the culture medium was replaced with complete DMEM. On day 2 post-infection, the cells were lysed with a passive lysis buffer as mentioned above. The efficiency of infection was monitored by measuring the luciferase activity of the cell lysate.

2.8. Transfection of microRNA inhibitor

Huh7.5 cells were electroporated with luciferase reporter HCV RNA as mentioned above, and then the cells were seeded in a well of a 24-well plate. To analyze the effect of inhibition of microRNA (miR), both a specific miRNA inhibitor (Anti-miR™ miRNA) and a non-targeting negative control (Anti-miR™miRNA Inhibitors—Negative Control) were purchased from Ambion, Inc. 50 pmol of a specific miRNA inhibitor or negative control were transfected into luciferase reporter RNA-electroporated Huh7.5 cells by using a siPORT™ NeoFX™ Transfection Agent (Ambion) according to the manufacturer's instructions. At 48 h post-transfection, the cells were harvested, and viral replication was determined by luciferase assay of the cell lysate.

2.9. Polyions

The polyanions heparin (mol. wt. 3000), dextran sulfate (mol. wt. 50,000) and polyvinyl sulfate (mol. wt. 150,000), and the polycations polybrene (mol. wt. 3000), DEAE-dextran (mol. wt. 100,000), and poly-L-lysine (mol. wt. 500,000) (all purchased from Sigma) were dissolved in PBS.

3. Results

3.1. Construction and characterization of luciferase reporter HCV

To construct a reporter HCV that can permit easy monitoring of both virus production and intra-cellular viral growth kinetics, we constructed the bicistronic HCV constructs by inserting a luciferase reporter gene into the 5′ end of the coding sequence of the JFH1 or J6/JFH1 full-genome plasmids clone as shown in Fig. 1A. In the transcript derived from bicistronic reporter HCV clone, the HCV and EMCV IRESs are responsible for the translation of the luciferase protein and all HCV proteins, respectively. A reporter construct with NS5B GDD to GND mutation, which disrupts viral polymerase function, was also constructed by site-directed mutagenesis, and served as a negative control for viral genome replication. To examine the replication level of reporter HCVs, we prepared the RNAs from each construct by *in vitro* transcription, and then transfected them into Huh7.5 cells by an electroporation technique. The viral replication was quantified up to 10 days post-transfection by using an HCV core-specific ELISA and luciferase reporter assay. As shown in Fig. 1B, the transfection of RNAs of both the JFH1/Luc and J6/JFH1/Luc reporter clones induced intra-cellular HCV core protein expression, which peaked on day 2 post-transfection. Both JFH1/Luc and J6/JFH1/Luc showed similar kinetics, and the high level core protein expression continued until day 10 post-transfection. As expected, the GND mutant exhibited 100-fold lower intra-cellular core protein expression on day 2 post-transfection. The level of core expression by the GND mutant continued to decline thereafter, and fell below the detection limit on day 10 post-transfection. As shown in Fig. 1C, both JFH1/Luc and J6/JFH1/Luc induced similar levels of luciferase activity in Huh7.5 cells at 4 h after electroporation. This result indicated that both RNAs were electroporated with similar efficiency because RNA replication had not started at that time and all the luciferase was translated from the input RNA. At 4 days post-electroporation, the luciferase activities of both JFH1/Luc and J6/JFH1/Luc were 10-fold greater than those measured at 4 h after electroporation. Subsequently, JFH1/Luc and J6/JFH1/Luc showed almost the same kinetics of luciferase activity until 10 days post-transfection. At 3 days post-electroporation, both JFH1/Luc and J6/JFH1/Luc electroporated cells were stained with HCV-positive patient sera, and the rate of intra-cellular replication was then visualized using immunofluorescent microscopy as previously reported [12]. As a result, the HCV-positive rates were 17% and 19% for JFH1/Luc and J6/JFH1/Luc, respectively (Fig. 1D). These results indicated that the luciferase activity of reporter HCV-transfected cells reflected the intra-cellular viral replication, and also suggested that both JFH1 and J6/JFH1 had similar intra-cellular replication ability in Huh7.5 cells.

3.2. Production of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells

Next, we assessed the potential of the reporter HCV to produce infectious progeny virions. Huh7.5 cells were electroporated with the reporter RNAs, and the culture supernatant was collected at various time points. To analyze the release of progeny virions from the reporter RNA-electroporated cells, the amounts of core protein in culture supernatants were

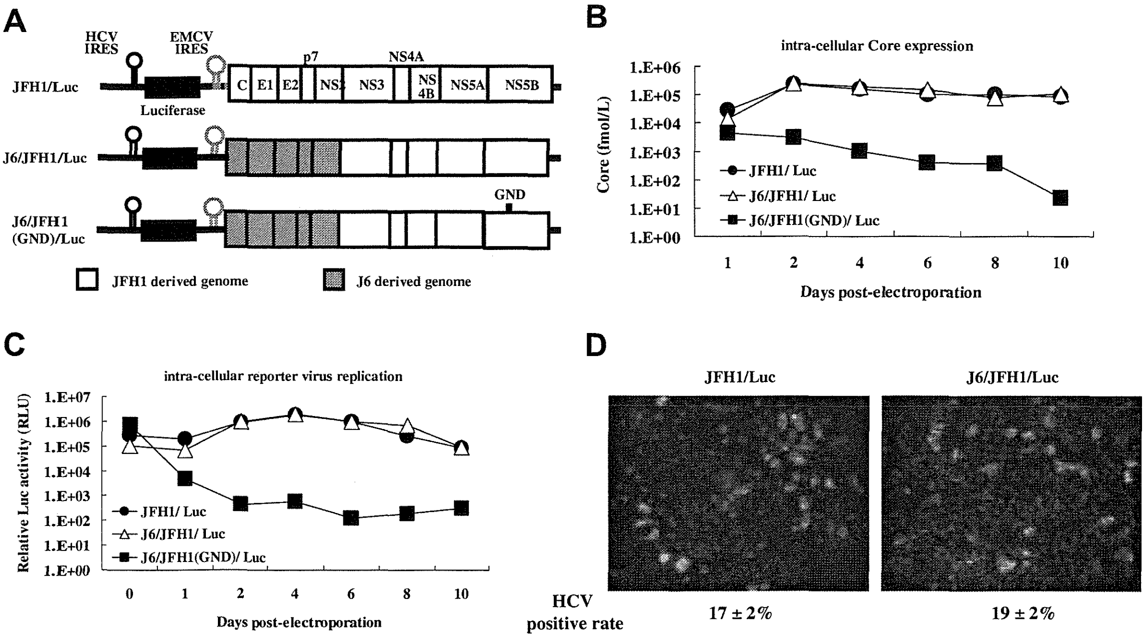

Fig. 1. Schematics of luciferase reporter HCV in this study. (A) Organization of luciferase reporter HCV. The luciferase gene is depicted as a black box. The JFH1-derived open reading frame and J6-derived open reading frame are depicted as a gray box and white box, respectively. As a negative control, a GND mutation was introduced to NS5B RdRp. (B, C) Virus replication kinetics in Huh7.5 cells of luciferase reporter HCV. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and the cells were assayed for core protein ELISA (B) and luciferase activity (C) at intervals as indicated. The assays were repeated at least three times, and the mean values are presented. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis at 3 days post-electroporation (D). Cells were incubated with an HCV-infected patient's serum followed by FITC-labeled goat anti-human IgG (green). In parallel, the cells were stained with Hoechst 33342 to visualize the nuclei (blue). The HCV-positive rate was calculated by counting the number of HCV-positive cells among the total cells, and the data represent the means and SE of three independent experiments.

analyzed by ELISA. As shown in Fig. 2A, electroporation of both reporter viral RNAs with Huh7.5 cells released the HCV core protein into the culture supernatants. The levels of core protein released from both reporter HCV RNAs peaked at 6 days post-electroporation. The amount of core protein of the J6/JFH1/Luc supernatants was 2–4 fold greater than that of JFH1/Luc among all the time points tested. In parallel, to analyze the infectivity of progeny virions produced from reporter RNA-electroporated cells, these supernatants were used as inocula for naïve Huh7.5 cells. The cells inoculated with these supernatants were harvested at 48 h post-inoculation, and the luciferase activity of the cell lysate was analyzed (Fig. 2B). These supernatants infected naïve Huh7.5 cells, and transduced luciferase activity in the cells. The infectious virus of both reporter HCVs was initially detected on day 2 and peaked on day 4 post-electroporation. However, the infectivity was decreased after day 6 post-electroporation. Furthermore, the infectivity of J6/JFH1/Luc supernatants was significantly higher than that of JFH1/Luc (approximately 10-fold). To compare the luciferase activity and the virus titer, we

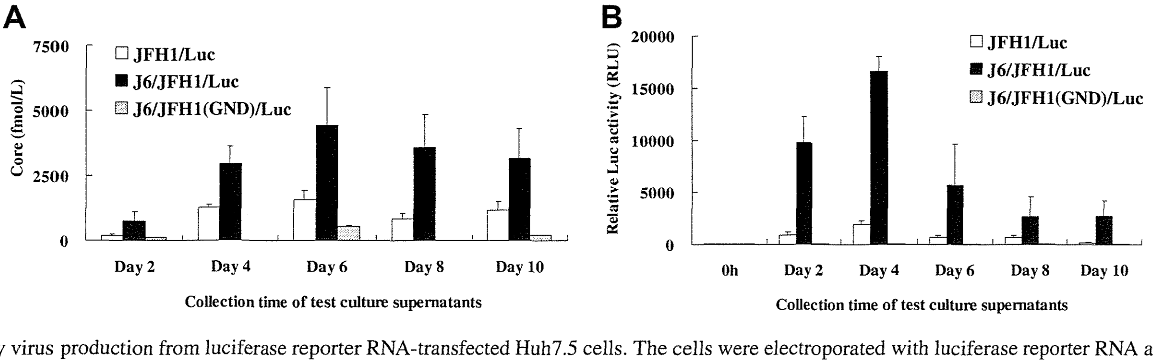

Fig. 2. Progeny virus production from luciferase reporter RNA-transfected Huh7.5 cells. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and culture supernatants of the cells were collected at the indicated time points. The amount of progeny virus in the supernatant was measured by the HCV core protein ELISA. (A) In parallel, the supernatants were added to naïve Huh7.5 cells. At 48 h post-addition, the cells were lysed, and assayed for luciferase activity to assess the infectivity of progeny virus from reporter HCV RNA. (B) The assays were repeated at least three times, and the mean values are presented.

performed standard virion titration by immunofluorescent antibody staining. The result showed that the virus titer of J6/JFH/Luc supernatant, collected at day 4 post-RNA transfection, was 5×10^3 fluorescent-focus forming units (ffu) per ml. In contrast, the titer of JFH/Luc supernatant was below the detection limit ($<1 \times 10^2$ ffu/ml). Interestingly, the peaks of the core release and the infectivity were slightly different, i.e., the peak of the core release of J6/JFH1 was on day 6, and that of the infectivity was on day 4 post-electroporation. Collectively, these data revealed that J6/JFH1 had a greater ability to release progeny virions than JFH1, though the levels of intracellular replication were comparable between J6/JFH1/and JFH1.

3.3. *Characterization of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells*

Next, we examined whether the J6/JFH1/Luc-derived supernatants had the features of a virus and thus could be used as a surrogate for HCV. The supernatants collected from each culture of reporter RNA-electroporated cells were irradiated with ultra-violet (UV) for 5 min, and the supernatants were then inoculated into naïve Huh7.5 cells. As shown in Fig. 3A, the infectivity of the reporter virus was completely abrogated by UV-irradiation. The results indicated that the luciferase activity transduced by the supernatants was derived from the genome of the reporter virus, not from incorporation of the luciferase protein into the virion. The entry of the HCV virion was mediated by binding between the cellular surface protein CD81 and the HCV envelope protein E2 [15]. Therefore, the naïve Huh7.5 cells were pre-treated with a recombinant monoclonal antibody against CD81. After 1 h pre-treatment, the J6/JFH1/Luc supernatant was inoculated into the cells, and the luciferase activity of cells was analyzed at 48 h post-inoculation (Fig. 3B). Normal mouse IgG showed no effect on the infectivity of the J6/JFH/Luc supernatant. In contrast, the infectivity of the J6/JFH/Luc supernatant was decreased by pre-treatment with anti-CD81 antibody in a dose-dependent manner. The results suggested that the supernatant from luciferase reporter J6/JFH1/Luc-transfected cells contained a virus with characteristics similar to HCV, and that this reporter virus could be utilized to investigate all the steps of virus replication, including the intra-cellular viral replication, the virus production and the virus entry as a surrogate model of HCV.

3.4. *Analysis of a potential role for ESCRT family proteins in HCV virus production*

Prior to the recent establishment of the JFH1-based cell-culture system, there was no system for producing the HCV virus, and thus many aspects of the virus production of HCV still remain poorly understood. Generally, the production of the enveloped virus requires a multi-step process that includes the proper transport of viral proteins and organization of viral proteins on the cellular membrane, and these steps are coordinated by a variety of cellular factors [16,17]. From numerous intensive studies, it has been revealed that the process of budding of many enveloped viruses utilizes the ESCRT machinery, which is responsible for the formation of luminal vesicles of endosomal multivesicular bodies (MVB) [16,18–20]. The ESCRT machinery consists of a number of cellular proteins that make up three functional sub-complexes – ESCRT-I, ESCRT-II and ESCRT-III – and other related factors; i.e., Vps4 and AIP/Alix are also participated in the function of ESCRT machinery [20]. A series of analyses about ESCRT networks has revealed the consensus amino acid motifs of viral proteins; the P(T/S)AP motif was observed to interact with Tsg101, and the YPxL motif was seen in the case of AIP/Ailx [19]. We searched for these motifs in the J6 and JFH1 genomes, and found one AIP/Alix interacting the YPxL motif in the NS5B region (aa. 2604 to 2607; YPDL). Therefore, the relation between ESCRT and HCV was examined by analyzing the virus production using a luciferase reporter HCV system. First, we constructed the expression plasmids of the ESCRT-I protein Tsg101, and the ESCRT-associated proteins Nedd4L and AIP/Alix. The ESCRT expression plasmids were transfected into the J6/JFH1/Luc or JFH1/Luc RNA-transfected Huh7.5 cells. After 48 h of transfection, the culture supernatants were collected and inoculated into the culture of the naïve Huh7.5 cells. The effects of over-expression of ESCRT proteins on intra-cellular virus

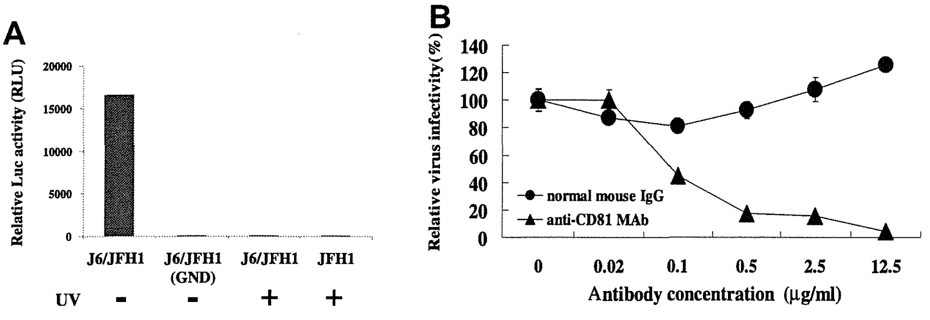

Fig. 3. Anti-CD81 antibody blocks luciferase reporter HCV infection. The reporter viruses containing supernatants were prepared as described in Materials and methods. (A) The JFH1/Luc and J6/JFH1/Luc supernatants were irradiated with UV at 5 min, and then added to naïve Huh7.5 cells. The infectivity was analyzed by luciferase assay. (B) Huh7.5 cells were pre-treated with anti-CD81 monoclonal antibody or control mouse IgG at 1 h before infection. Cells were then infected with J6/JFH1/Luc reporter viruses for 6 h. At 48 h post-infection, the cells were lysed and assayed for luciferase activity. Activities are expressed as the relative activity compared to that of the null antibody-treated sample. The assays were repeated at least three times, and the mean values are presented.

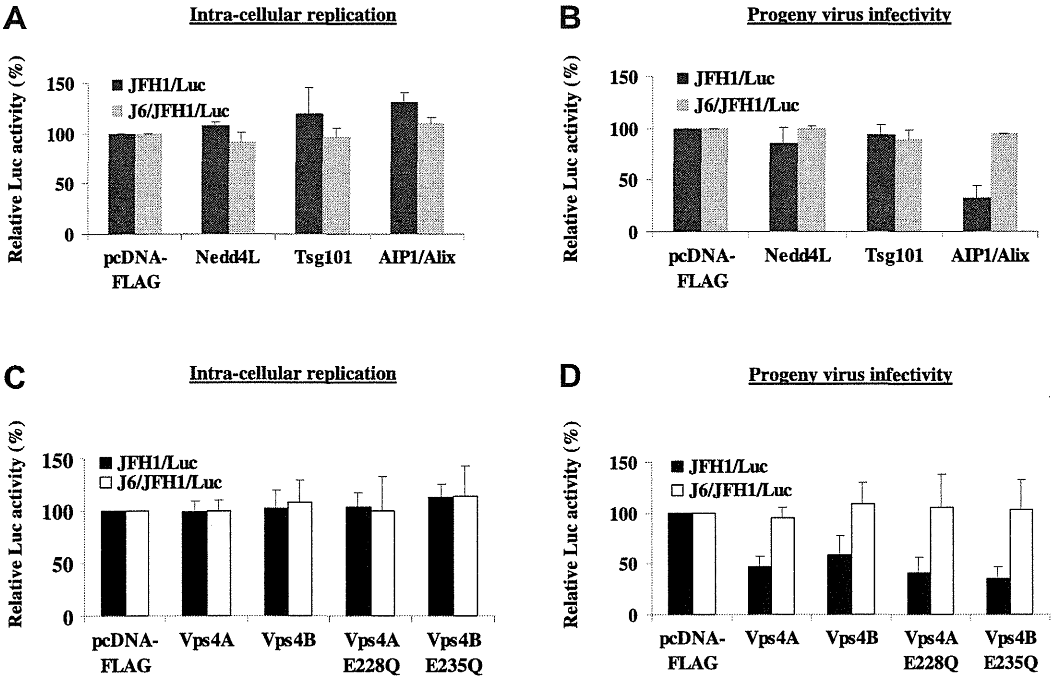

Fig. 4. Effect of ESCRT family protein expression on intra-cellular replication and progeny virus production in Huh7.5 cells. Huh7.5 cells were electroporated with JFH1/Luc and J6/JFH1/Luc RNA, respectively. The RNA-electroporated cells were then transfected with ESCRT protein expression plasmids. At 96 h after transfection, the culture supernatants were collected, and the cells were harvested. (A, C) Cell lysates were assayed for luciferase activity to assess intra-cellular virus replication. (B, D) Collected supernatants were added to naïve Huh7.5 cells and incubated for 48 h, and then the luciferase activity of the cells was analyzed to assess progeny virus infectivity. The data relative to that of luciferase activity in the absence of ESCRT protein (pcDNA-FLAG) is indicated. The assays were repeated at least three times, and the mean and standard error are presented.

replication and virus production were analyzed by monitoring the luciferase activity of reporter RNA-transfected cells (Fig. 4A), and the luciferase activity expressed by supernatant virus (Fig. 4B). As shown in Fig. 4A, the overexpression of Nedd4L, Tsg101, and AIP/Alix had no effect on the intra-cellular replication of either reporter HCV. As shown in Fig. 4B, the virus production from J6/JFH1/Luc also was not affected by these ESCRT protein expressions. In contrast, the expression of AIP/Alix decreased the virus production from JFH1/Luc by 50%. This result implied that the ESCRT machinery might have played some role in the difference in the efficacy of virus production observed between JFH1 and J6/JFH1. AAA-ATPase Vps4, which is present in humans in two isoforms (Vps4A and Vps4B), is a key modulator protein for the final step of ESCRT machinery. To analyze the role of ESCRT in HCV virus production, we constructed expression vectors for Vps4A and 4B, as well as expression vectors for a dominant-negative Vps4A(E228Q) and Vps4B(E235Q) [19]. As shown in Fig. 4C, the intra-cellular replications of JFH1 and J6/JFH1 were not influenced by the wild-type or dominant-negative Vps4 expression. In contrast, the levels of virus production of JFH1/Luc were reduced up to 50% by the expression of both dominant-negative Vps4 mutants (Fig. 4D). Interestingly, neither dominant-negative Vps4 influenced the virus production of J6/JFH1/Luc. These results implied that JFH1 might utilize the ESCRT machinery for release of infectious virus particles.

3.5. Effect of polyions on the infectivity of the J6/JFH1/Luc reporter virus

Next, we tested the usefulness of the J6/JFH1/luc reporter system for virus entry analysis. The binding of the viral and cellular receptors is coordinated with the ionic conditions, indicating that compounds that affect the ionic charge of the receptor surface might be potent inhibitors of virus infection [21,22]. Polyions with a positive or negative charge are frequently used for virus entry analyses, and exhibit inhibitory activity on virus infection [21,22]. Therefore, we investigated the effect of different polyions on the infectivity of the J6/JFH1/Luc virus in order to clarify the influence of electrostatic interactions in virus binding to cell membranes. As candidate compounds, we used both polymers having a positive charge (polybrene (size of 3000 Da), DEAE-dextran (100,000 Da), and poly-L-lysine (500,000 Da)) and those having a negative charge (heparin (15,000 Da), dextran sulfate (50,000 Da), and polyvinyl sulfate (150,000 Da)). These polymers were added to the Huh7.5 cells at 1 h before inoculation of the J6/JFH1/Luc virus into the cells. After 48 h of inoculation, the cells were harvested and the luciferase activity was analyzed (Fig. 5A and B). As shown in Fig. 5A, two polyanions, heparin and polyvinyl sulfate, decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner, whereas one polyanion, dextran sulfate, enhanced the infectivity up to 2-fold. In the case of polycations, the addition of polybrene enhanced virus

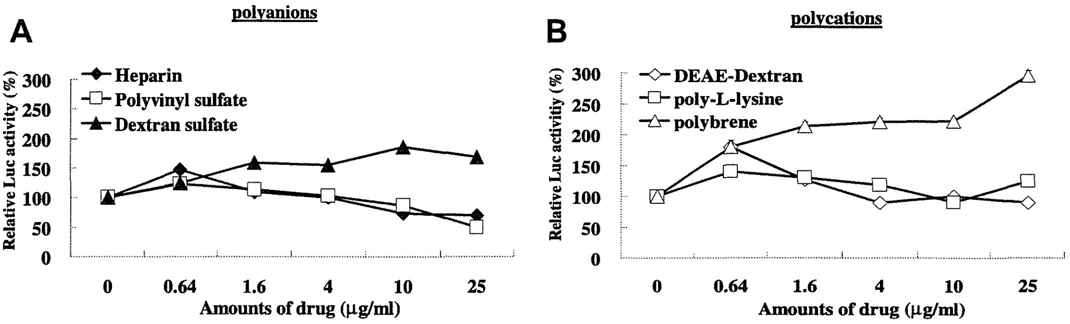

Fig. 5. Effect of multiple polyions on J6/JFH1/Luc virus infection of Huh7.5 cells. Huh7.5 cells were infected with J6/JFH1/Luc virus in the presence of each of the polyions for 6 h, and a luciferase assay was performed 48 h later. The data was expressed as the relative activity compared to the luciferase activity in the absence of polyions. The assays were repeated at least three times, and the mean values are presented.

infection up to 3-fold in a dose-dependent manner, although poly-L-lysine and DEAE-dextran showed no effect on the infectivity of the J6/JFH1/Luc virus (Fig. 5B). The effect shown by compounds belonging to positive and negative polyions suggested that the electric charge is not sufficient by itself to explain the inhibitory or enhancing activity of these drugs on the HCV virus entry. These results indicated that the J6/JFH1/Luc virus was useful to easily monitor HCV virus entry.

3.6. Screening of microRNA inhibition on intra-cellular HCV replication

To confirm the usefulness of the J6/JFH1/Luc reporter system in the analysis targeting intra-cellular replication of HCV, we analyzed the possible involvement of micro RNAs (miRNAs) in HCV infection. miRNAs are evolutionarily conserved, small, non-coding RNA molecules that regulate gene expression at the level of translation [23,24]. Recently, it has been reported that some miRNAs influence the replication of HCV in the cells [25–27]. For example, the expression of miR-122 in the cells might be essential for HCV replication [25]. In addition, the number of miRNAs has been increasing due to numerous strenuous analyses in recent years. Therefore, we compared the full sequences of the viral genome among 4 different HCV strains (H77C, Con1, J6, JFH1) with the sequences of 630 human miRNAs using the miRNA database program (RegRNA: http://regrna.mbc.nctu.edu.tw/index.php), and then identified 54 miRNAs that matched with at least one HCV strain. 10 of the 54 miRNAs matched with all four HCV strains. Hence, we focused on analysis of the function of the 10 miRNAs on HCV replication and prepared commercially available miRNA inhibitors (Anti-miR™ miRNA inhibitor, Ambion) that were chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous target miRNA molecules. The J6/JFH1/Luc RNA-electroporated cells were transfected with each of the 10 specific miRNA inhibitors and the luciferase activities were analyzed at 48 h post-transfection of the inhibitors. None of the miRNA inhibitors significantly affected the cell viability (data not shown). As shown in Fig. 6A, the inhibition of miR-122 reduced the level of intra-cellular virus replication by up to 50% as previously reported [25]. A similar reduction of viral replication was also observed by treatment with the miR-34b inhibitor. The treatment with an anti-miR negative control that is a random sequence anti-miR molecules that has been extensively tested in human cell lines and validated to not produce identifiable effects on known miRNA functions showed no significant effect on HCV replication. None of the other inhibitors showed any significantly greater effect on the

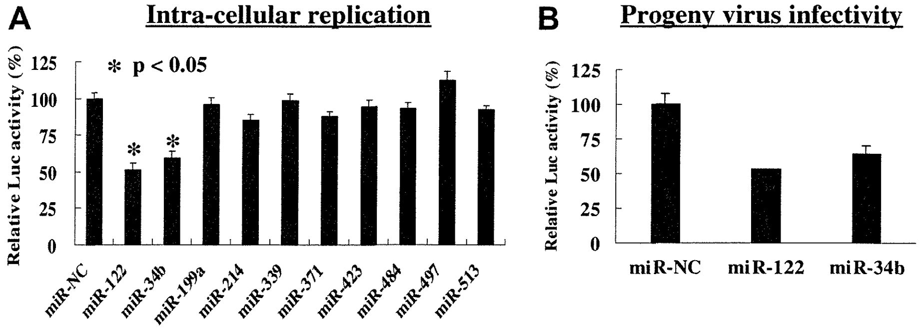

Fig. 6. Effect of miRNA inhibitor on intra-cellular replication of J6/JFH1/Luc RNA in Huh7.5 cells. Target cells were electroporated with J6/JFH1/Luc RNA, and then transfected with a miRNA-specific or a non-target negative control miRNA inhibitor. At 48 h post-transfection, cells were harvested and analyzed for luciferase activity (A). In parallel, the culture supernatants were collected at 48 h post-transfection to assess the effect of miRNA inhibitors on the virus production. The supernatants were added to naïve Huh7.5 cells, and the progeny virus infectivities were then analyzed by luciferase assay. (B) The data relative to the luciferase activity obtained from a non-target negative control miRNA inhibitor are indicated. The assays were repeated at least three times, and the mean and standard error are presented. Statistical significance relative to the negative control miRNA samples as calculated by *t*-test is shown ($^*p < 0.05$).

virus replication than the anti-miR negative control. The miR-34b and miR-122 inhibitors decreased the virus production to the levels 64% and 53% of the control, respectively (Fig. 6B). Since the extent of the reduction in virus production was comparable with that of intra-cellular HCV RNA levels (Fig. 6A), it was likely that these miRNA inhibitors affected the intra-cellular viral replication rather than interfering with the particle formation and the release of the virion. These results suggest that the function of miR-34b could affect the replication of HCV, and also suggested that the J6/JFH1/Luc system was useful to analyze the intra-cellular replication of HCV.

4. Discussion

In this report, we generated two bicistronic luciferase reporter HCV clones from JFH1 and J6/JFH1, and established a unifying system that can monitor intra-cellular viral replication, virion production, and virus entry. Using two constructs, we initially compared the potential of intra-cellular viral replication and virus production. After transfection of reporter RNAs, the level of the intra-cellular core protein and the luciferase activity in RNA-transfected cells showed similar kinetics for JFH1/Luc and J6/JFH1/Luc (Fig. 1B and C). In contrast, both the efficacy of core protein production into the culture supernatant and the infectivity of supernatant virus from J6/JFH1/Luc were significantly higher than that of JFH1/Luc (Fig. 2A and B). These results indicated two possibilities that JFH1 and J6/JFH1 utilize different machinery for progeny virus packaging and budding, or that they utilize the same machinery for the virus production but to a different degree. To evaluate the difference in the virus production between JFH1 and J6/JFH1, we analyzed the role of ESCRT machinery in virus production (Fig. 4A–D). Dominant-negative Vps4 expression inhibited JFH1/Luc virus production, but did not influence J6/JFH1/Luc virus production. In the course of preparing this manuscript, Corless et al. reported that HCV requires late components of the ESCRT pathway for release of infectious virus particles [28]. They showed that a dominant-negative Vps4 expression inhibited the production of virus-like particles derived from JFH1 in a dose-dependent manner. The findings reported by Corless et al. and the findings of our present study emphasize that the ESCRT machinery plays an essential role in JFH1 virus production.

To examine the virus entry, we analyzed the effect of anti-CD81 antibody and polyions on reporter virus infectivity (Figs. 3B and 5A and B). The pre-treatment with anti-CD81 antibody decreased the infectivity of the reporter J6/JFH virus in a dose-dependent manner. The result suggested that the reporter J6/JFH1 virus, similar to HCVcc, utilized the CD81 as a major entry receptor, and that our reporter virus could be used as a surrogate model of HCV entry analysis. As a result of polyions analysis, one of the polycations (dextran sulfate) and one of the polyanions (polybrene) increased the reporter virus infectivity, and the remainder of the polyions inhibited the virus infectivity. These results indicate the possibility that not only the electrostatic condition of polyions but also their molecular weight may be a determinant of the receptor binding of HCV. Considering that several membrane molecules have been identified as candidate cellular receptors for HCV entry [15,29,30], the polyions could interact with a different molecule(s) to influence virus production. As for heparin, it was reported that cell surface heparan sulfate proteoglycans play an important role in mediating HCV envelope–target cell interaction [31]. Basu et al. [32] also reported that heparin treatment completely blocked HIV/HCV E1-E2 pseudotype infection. In their analysis, however, the inhibitory effect of heparin against cell culture-grown HCV H77 was somewhat lower than that of HIV/HCV E1-E2 pseudotypes. In our present study, the level of inhibitory effect of heparin on J6/JFH1 reporter virus infection was not so prominent. Collectively, these data suggest a possibility that cell surface heparan sulfate proteoglycans contribute to the infection of both HIV/HCV E1–E2 pseudotype and cell culture-grown HCV with a different degree. Therefore, to develop a polyion-based anti-HCV drug, a more detailed assessment of the interaction between each candidate receptor and polyion is necessary.

Using microRNA inhibitors, the decrease of miR-34b expression suppressed intra-cellular HCV replication (Fig. 6A). miR-34b belongs to the evolutionary conserved microRNA family of miR-34s [33], known for their role in the p53 tumor suppressor network [34]. miR-34s have been shown to be controlled in a tissue-specific manner by p53. Both wild-type and mutant-type p53 protein expressions in serum and cytoplasm of liver tissue were more pronounced in patients with hepatocellular carcinoma associated with HCV infection [35]. Wild-type p53 binds to a transcriptional regulatory element of miR-34s, thereby up-regulating miR-34 expression [34]. However, it is not understood whether the mutant-type p53 increases miR-34b expression. Furthermore, HCV replication in chronic hepatitis is higher than that of hepatocellular carcinoma [36]. Therefore, more detailed research is needed to reveal the significance of miR-34b expression in HCV replication and hepatocellular carcinoma.

As mentioned above, we have generated a recombinant luciferase reporter HCV, and have shown that the reporter HCV could be used for the quantitative analyses of intra-cellular replication, virus entry, and virion production. In general, the intra-cellular HCV replication has been analyzed by the quantitative real-time RT-PCR method that could detect a small amount of viral RNA because of the greatly high sensitivity. However, the real-time RT-PCR method involves multi-step procedures of the RNA extraction, the reverse transcription and the PCR reaction, which require skillfulness to perform. The high sensitivity and the multiple-steps of the real-time RT-PCR system sometimes cause an experimental error(s) when conducted by less-experienced individuals. On the other hand, our HCV luciferase reporter system is simpler and easier to perform compared to the real-time PCR system. The significant advantage of the reporter HCV is that it can analyze a large number of samples at a time in a time- and cost-saving manner. Also, it can be used to evaluate all the events of viral life cycle. By using it, we have started the screening of anti-HCV substances from the natural resource chemical libraries and found a number of potential candidates for the analysis. Thus, this system can be applicable for robust screening analyses of chemical compounds to discover a potential therapeutic target of HCV.

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Production of Infectious Hepatitis C Virus by Using RNA Polymerase I-Mediated Transcription$^{\triangledown}$

Takahiro Masaki,[1]† Ryosuke Suzuki,[1]† Mohsan Saeed,[1,4] Ken-ichi Mori,[2] Mami Matsuda,[1]
Hideki Aizaki,[1] Koji Ishii,[1] Noboru Maki,[2] Tatsuo Miyamura,[1] Yoshiharu Matsuura,[3]
Takaji Wakita,[1] and Tetsuro Suzuki[1]*

*Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan[1];
Advanced Life Science Institute, Wako, Saitama 351-0112, Japan[2]; Department of Molecular Virology,
Research Institute for Microbial Diseases, Osaka University, Suita-shi, Osaka 565-0871, Japan[3]; and
Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan[4]*

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In this study, we used an RNA polymerase I (Pol I) transcription system for development of a reverse genetics protocol to produce hepatitis C virus (HCV), which is an uncapped positive-strand RNA virus. Transfection with a plasmid harboring HCV JFH-1 full-length cDNA flanked by a Pol I promoter and Pol I terminator yielded an unspliced RNA with no additional sequences at either end, resulting in efficient RNA replication within the cytoplasm and subsequent production of infectious virions. Using this technology, we developed a simple replicon *trans*-packaging system, in which transient transfection of two plasmids enables examination of viral genome replication and virion assembly as two separate steps. In addition, we established a stable cell line that constitutively produces HCV with a low mutation frequency of the viral genome. The effects of inhibitors of N-linked glycosylation on HCV production were evaluated using this cell line, and the results suggest that certain step(s), such as virion assembly, intracellular trafficking, and secretion, are potentially up- and downregulated according to modifications of HCV envelope protein glycans. This Pol I-based HCV expression system will be beneficial for a high-throughput antiviral screening and vaccine discovery programs.

Over 170 million people worldwide have been infected with hepatitis C virus (HCV) (22, 33, 37), and persistence of HCV infection is one of the leading causes of liver diseases, such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (16, 25, 38). The HCV genome is an uncapped 9.6-kb positive-strand RNA sequence consisting of a 5′ untranslated region (UTR), an open reading frame encoding at least 10 viral proteins (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a 3′UTR (46). The structural proteins (Core, E1, and E2) reside in the N-terminal region.

The best available treatment for HCV infection, which is pegylated alpha interferon (IFN-α) combined with ribavirin, is effective in only about half of patients and is often difficult to tolerate (25). To date, a prophylactic or therapeutic vaccine is not available. There is an urgent need to develop more effective and better tolerated therapies for HCV infection. Recently, a robust system for HCV production and infection in cultured cells has been developed. The discovery that some HCV isolates can replicate in cell cultures and release infectious particles has allowed the complete viral life cycle to be studied (23, 49, 53). The most robust system for HCV production involves transfection of Huh-7 cells with genomic HCV RNA of the JFH-1 strain by electroporation. However, using this RNA transfection system, the amount of secreted infectious viruses often fluctuate and mutations emerge in HCV genome with multiple passages for an extended period of time (54), which limits its usefulness for antiviral screening and vaccine development.

DNA-based expression systems for HCV replication and virion production have also been examined (5, 15, 21). With DNA-based expression systems, transcriptional expression of functional full-length HCV RNA is controlled by an RNA polymerase II (Pol II) promoter and a self-cleaving ribozyme(s). DNA expression systems using RNA polymerase I (Pol I) have been utilized in reverse genetics approaches to replicate negative-strand RNA viruses, including influenza virus (12, 29), Uukuniemi virus (11), Crimean-Congo hemorrhagic fever virus (10), and Ebola virus (13). Pol I is a cellular enzyme that is abundantly expressed in growing cells and transcribes rRNA lacking both a 5′ cap and a 3′ poly(A) tail. Thus, viral RNA synthesized in cells transfected with Pol I-driven plasmids containing viral genomic cDNA has no additional sequences at the 5′- or 3′ end even in the absence of a ribozyme sequence (28). The advantages of DNA-based expression systems are that DNA expression plasmids are easier to manipulate and generate stable cell lines that constitutively express the viral genome.

We developed here a new HCV expression system based on transfection of an expression plasmid containing a JFH-1 cDNA clone flanked by Pol I promoter and terminator sequences to generate infectious HCV particles from transfected cells. The technology presented here has strong potential to be the basis for *trans*-encapsidation system by transient transfection of two plasmids and for the establishment of an efficient and reliable screening system for potential antivirals.

MATERIALS AND METHODS

DNA construction. To generate HCV-expressing plasmids containing full-length JFH1 cDNA embedded between Pol I promoter and terminator se-

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* Corresponding author. Present address: Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan. Phone: 81-53-435-2336. Fax: 81-53-435-2337. E-mail: tesuzuki@hama-med.ac.jp.

† T.M. and R.S. contributed equally to this study.

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quences, part of the 5′UTR region and part of the NS5B to the 3′UTR region of full-length JFH-1 cDNA were amplified by PCR using primers containing BsmBI sites. Each amplification product was then cloned into a pGEM-T Easy vector (Promega, Madison, WI) and verified by DNA sequencing. Both fragments were excised by digestion with NotI and BsmBI, after which they were cloned into the BsmBI site of the pHH21 vector (a gift from Yoshihiro Kawaoka, School of Veterinary Medicine, University of Wisconsin-Madison [29]), which contains a human Pol I promoter and a mouse Pol I terminator. The resultant plasmid was digested by AgeI and EcoRV and ligated to JFH-1 cDNA digested by AgeI and EcoRV to produce pHHJFH1. pHHJFH1/GND having a point mutation at the GDD motif in NS5B to abolish RNA-dependent RNA polymerase activity and pHHJFH1/R783A/R785A carrying double Arg-to-Ala substitutions in the cytoplasmic loop of p7 were constructed by oligonucleotide-directed mutagenesis. To generate pHHJFH1/ΔE carrying in-frame deletions of parts of the E1 and E2 regions (amino acids [aa] 256 to 567), pHHJFH1 was digested with NcoI and AscI, followed by Klenow enzyme treatment and self-ligation. To generate pHH/SGR-Luc carrying the bicistronic subgenomic HCV reporter replicon and its replication-defective mutant, pHH/SGR-Luc/GND, AgeI-SpeI fragments of pHHJFH1 and pHHJFH1/GND were replaced with an AgeI-SpeI fragment of pSGR-JFH1/Luc (20). In order to construct pCAG/C-NS2 and pCAG/C-p7, PCR-amplified cDNA for C-NS2 and C-p7 regions of the JFH-1 strain were inserted into the EcoRI sites of pCAGGS (30). In order to construct stable cell lines, a DNA fragment containing a Zeocin resistance gene excised from pSV2/Zeo2 (Invitrogen, Carlsbad, CA) was inserted into pHH21 (pHHZeo). Full-length JFH-1 cDNA was then inserted into the BsmBI sites of pHHZeo. The resultant construct was designated pHHJFH1/Zeo.

Cells and compounds. The human hepatoma cell line, Huh-7, and its derivative cell line, Huh7.5.1 (a gift from Francis V. Chisari, The Scripps Research Institute), were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. *N*-Nonyl-deoxynojirimycin (*N*N-DNJ) and kifunensine (KIF) were purchased from Toronto Research Chemicals (Ontario, Canada), castanospermine (CST) and 1,4-dideoxy-1,4-imino-D-mannitol hydrochloride (DIM) were from Sigma-Aldrich (St. Louis, MO), 1-deoxymannojirimycin (DMJ) and swainsonine (SWN) were from Alexis Corp. (Lausen, Switzerland), and *N*-butyl-deoxynojirimycin (*N*B-DNJ) was purchased from Wako Chemicals (Osaka, Japan). BILN 2061 was a gift from Boehringer Ingelheim (Canada), Ltd. These compounds were dissolved in dimethyl sulfoxide and used for the experiments. IFN-α was purchased from Dainippon-Sumitomo (Osaka, Japan).

DNA transfection and selection of stable cell lines. DNA transfection was performed by using FuGENE 6 transfection reagent (Roche, Mannheim, German) in accordance with the manufacturer's instructions. To establish stable cell lines constitutively producing HCV particles, pHHJFH1/Zeo was transfected into Huh7.5.1 cells within 35-mm dishes. At 24 h posttransfection (p.t.), the cells were then divided into 100-mm dishes at various cell densities and incubated with DMEM containing 0.4 mg of zeocin/ml for approximately 3 weeks. Selected cell colonies were picked up and amplified. The expression of HCV proteins was confirmed by measuring secreted core proteins. The stable cell line established was designated H751JFH1/Zeo.

***In vitro* synthesis of HCV RNA and RNA transfection.** RNA synthesis and transfection were performed as previously described (26, 49).

RNA preparation, Northern blotting, and RNase protection assay (RPA). Total cellular RNA was extracted with a TRIzol reagent (Invitrogen), and HCV RNA was isolated from filtered culture supernatant by using the QIAamp viral RNA minikit (Qiagen, Valencia, CA). Extracted cellular RNA was treated with DNase (TURBO DNase; Ambion, Austin, TX) and cleaned up by using an RNeasy minikit, which includes another step of RNase-free DNase digestion (Qiagen). The cellular RNA (4 μg) was separated on 1% agarose gels containing formaldehyde and transferred to a positively charged nylon membrane (GE Healthcare, Piscataway, NJ). After drying and cross-linking by UV irradiation, hybridization was performed with [α-^{32}P]dCTP-labeled DNA using Rapid-Hyb buffer (GE Healthcare). The DNA probe was synthesized from full-length JFH-1 cDNA using the Megaprime DNA labeling system (GE Healthcare). Quantification of positive- and negative-strand HCV RNA was performed using the RPA with biotin-16-uridine-5′-triphosphate (UTP)-labeled HCV-specific RNA probes, which contain 265 nucleotides (nt) complementary to the positive-strand (+) 5′UTR and 248 nt complementary to the negative-strand (−) 3′UTR. Human β-actin RNA probes labeled with biotin-16-UTP were used as a control to normalize the amount of total RNA in each sample. The RPA was carried out using an RPA III kit (Ambion) according to the manufacturer's procedures. Briefly, 15 μg of total cellular RNA was used for hybridization with 0.3 ng of the β-actin probe and 0.6 ng of either the HCV (+) 5′UTR or (−) 3′UTR RNA probe. After digestion with RNase A/T1, the RNA products were analyzed by electrophoresis in a 6% polyacrylamide–8 M urea gel and visualized by using a chemiluminescent nucleic acid detection module (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Reverse transcriptase PCR (RT-PCR), sequencing, and rapid amplification of cDNA ends (RACE). Aliquots (5 μl) of RNA solution extracted from filtered culture supernatant were subjected to reverse transcription with random hexamer and Superscript II reverse transcriptase (Invitrogen). Four fragments of HCV cDNA (nt 129 to 2367, nt 2285 to 4665, nt 4574 to 7002, and nt 6949 to 9634), which covers most of the HCV genome, were amplified by nested PCR. Portions (1 or 2 μl) of each cDNA sample were subjected to PCR with TaKaRa LA *Taq* polymerase (Takara, Shiga, Japan). The PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min. The amplified products were separated by agarose gel electrophoresis and used for direct DNA sequencing. To establish the 5′ ends of the HCV transcripts from pHHJFH1, a synthetic 45-nt RNA adapter (Table 1) was ligated to RNA extracted from the transfected cells 1 day p.t. using T4 RNA ligase (Takara). The viral RNA sequences were then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) with a primer, RT (Table 1). The resultant cDNA sequences were subsequently amplified by PCR with 5′RACEouter-S and 5′RACEouter-R primers, followed by a second cycle of PCR using 5′RACEinner-S and 5′RACEinner-R primers (Table 1). To establish the terminal 3′-end sequences, extracted RNA sequences were polyadenylated using a poly(A) polymerase (Takara), reverse transcribed with CAC-T35 primer (Table 1), and amplified with the primers 3X-10S (Table 1) and CAC-T35. The amplified 5′ and 3′ cDNA sequences were then separated by agarose gel electrophoresis, cloned into the pGEM-T Easy vector (Promega), and sequenced.

Western blotting. The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) after separation by SDS-PAGE. After blocking, the membranes were probed with a mouse monoclonal anti-HCV core antibody (2H9) (49), a rabbit polyclonal anti-NS5B antibody, or a mouse monoclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Chemicon, Temecula, CA), followed by incubation with a peroxidase-conjugated secondary antibody and visualization with an ECL Plus Western blotting detection system (Amersham, Buckinghamshire, United Kingdom).

Quantification of HCV core protein. HCV core protein was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Sucrose density gradient analysis. Samples of cell culture supernatant were processed by low-speed centrifugation and passage through a 0.45-μm-pore-size filter. The filtrated supernatant was then concentrated ~30-fold by ultrafiltration by using an Amicon Ultra-15 filter device with a cutoff molecular mass of 100,000 kDa (Millipore), after which it was layered on top of a continuous 10 to 60% (wt/vol) sucrose gradient, followed by centrifugation at 35,000 rpm at 4°C for 14 h with an SW41 rotor (Beckman Coulter, Fullerton, CA). Fractions of 1 ml were collected from the bottom of the gradient. The core level and infectivity of HCV in each fraction were determined.

Quantification of HCV infectivity. Infectious virus titration was performed by a 50% tissue culture infectious dose ($TCID_{50}$) assay, as previously described (23, 26). Briefly, naive Huh7.5.1 cells were seeded at a density of 10^4 cells/well in a 96-well flat-bottom plate 24 h prior to infection. Five serial dilutions were performed, and the samples were used to infect the seeded cells (six wells per dilution). At 72 h after infection, the inoculated cells were fixed and immunostained with a rabbit polyclonal anti-NS5A antibody (14), followed by an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen).

Labeling of *de novo*-synthesized viral RNA and immunofluorescence staining. Labeling of *de novo*-synthesized viral RNA was performed as previously described with some modifications (40). Briefly, cells were plated onto an eight-well chamber slide at a density of 5 × 10^4 cells/well. One day later, the cells were incubated with actinomycin D at a final concentration of 10 μg/ml for 1 h and washed twice with HEPES-saline buffer. Bromouridine triphosphate (BrUTP) at 2 mM was subsequently transfected into the cells using FuGENE 6 transfection reagent, after which the cells were incubated for 15 min on ice. After the cells were washed twice with phosphate-buffered saline (PBS), they were incubated in fresh DMEM supplemented with 10% FBS at 37°C for 4 h. The cells were then fixed with 4% paraformaldehyde for 20 min and permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. Immunofluorescence staining of NS5A and *de novo*-synthesized HCV RNA was performed as previously described (26, 40). The nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) solution (Sigma-Aldrich). Confocal microscopy was performed

TABLE 1. Oligonucleotides used for RT-PCR and RACE of the JFH-1 genome

Method or segment	Oligonucleotide	Sequences (5′–3′)
5′RACE	RT	GTACCCCATGAGGTCGGCAAAG
	45-nt RNA adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA
	5′RACEouter-S	GCTGATGGCGATGAATGAACACTG
	5′RACEouter-R	GACCGCTCCGAAGTTTTCCTTG
	5′RACEinner-S	GAACACTGCGTTTGCTGGCTTTGATG
	5′RACEinner-R	CGCCCTATCAGGCAGTACCACAAG
3′RACE	CAC-T35	CACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
	3X-10S	ATCTTAGCCCTAGTCACGGC
nt 129-2367	44S (1st PCR)	CTGTGAGGAACTACTGTCTT
	2445R	TCCACGATGTTCTGGTGAAG
	17S (2nd PCR)	CGGGAGAGCCATAGTGG
	2367R	CATTCCGTGGTAGAGTGCA
nt 2285-4665	2099S (1st PCR)	ACGGACTGTTTTAGGAAGCA
	4706R	TTGCAGTCGATCACGGAGTC
	2285S (2nd PCR)	AACTTCACTCGTGGGGATCG
	4665R	TCGGTGGCGACGACCAC
nt 4574-7002	4547S (1st PCR)	AAGTGTGACGAGCTCGCGG
	7027R	CATGAACAGGTTGGCATCCACCAT
	4594S (2nd PCR)	CGGGGTATGGGCTTGAACGC
	7003R	GTGGTGCAGGTGGCTCGCA
nt 6949-9634	6881S (1st PCR)	ATTGATGTCCATGCTAACAG
	3X-75R	TACGGCACTCTCTGCAGTCA
	6950S (2nd PCR)	GAGCTCCTCAGTGAGCCAG
	3X-54R	GCGGCTCACGGACCTTTCAC

using a Zeiss confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany).

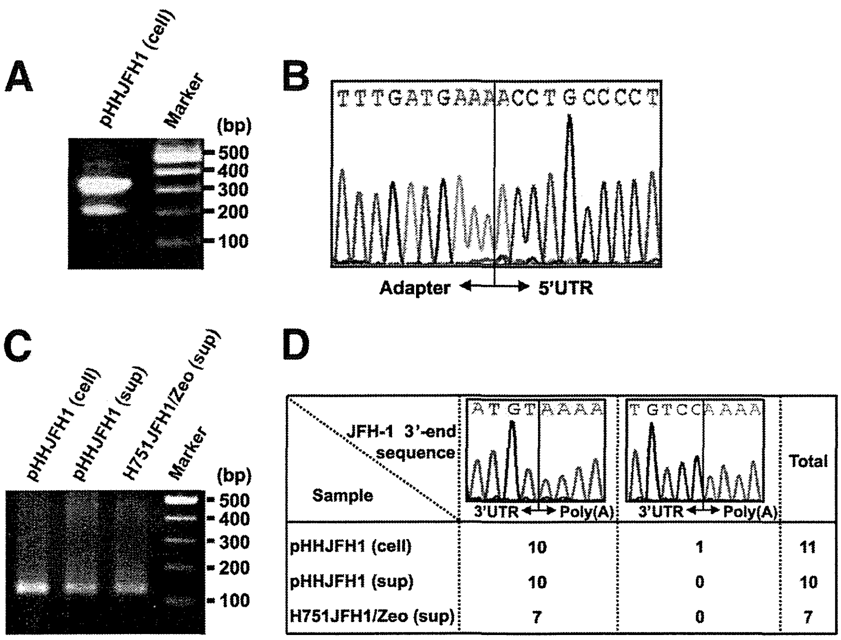
Luciferase assay. Huh7.5.1 cells were seeded onto a 24-well cell culture plate at a density of 3 × 10^4 cells/well 24 h prior to inoculation with 100 μl of supernatant from the transfected cells. The cells were incubated for 72 h, followed by lysis with 100 μl of lysis buffer. The luciferase activity of the cells was determined by using a luciferase assay system (Promega). All luciferase assays were done at least in triplicate. For the neutralization experiments, a mouse monoclonal anti-CD81 antibody (JS-81; BD Pharmingen, Franklin Lakes, NJ) and a mouse monoclonal anti-FLAG antibody (Sigma-Aldrich) were used.

Flow cytometric analysis. Cells detached by treatment with trypsin were incubated in PBS containing 1% (vol/vol) formaldehyde for 15 min. A total of 5 × 10^5 cells were resuspended in PBS and treated with or without 0.75 μg of anti-CD81 antibody for 30 min at 4°C. After being washed with PBS, the cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) at 1:200 for 30 min at 4°C, washed repeatedly, and resuspended in PBS. Analyses were performed by using FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ).

RESULTS

Analysis of the 5′ and 3′ ends of HCV RNA sequences generated from Pol I-driven plasmids. To examine whether the HCV transcripts generated from Pol I-driven plasmids had correct nucleotides at the 5′ and 3′ ends, we extracted RNA from Huh-7 cells transfected with pHHJFH1, which carries a genome-length HCV cDNA with a Pol I promoter/terminator, as well as from the culture supernatants. After this, the nucleotide sequences at both ends were determined using RACE and sequence analysis. A 328-nt fragment corresponding to cDNA from the 5′ end of HCV RNA was detected in the cell samples (Fig. 1A). Cloning of amplified fragments confirmed that the HCV transcripts were initiated from the first position of the viral genome in all of the clones sequenced (Fig. 1B). Similarly, a 127-nt amplification fragment was detected in each sample by 3′RACE (Fig. 1C), and the same 3′-end nucleotide sequence was observed in all clones derived from the culture supernatant (Fig. 1D, left). An additional two nucleotides (CC) were found at the 3′ end of the HCV transcript in a limited number of sequences (1 of 11 clones) derived from the cell sample (Fig. 1D, right), which were possibly derived from the Pol I terminator sequence by incorrect termination. These results indicate that most HCV transcripts generated from the Pol I-based HCV cDNA expression system are faithfully processed, although it is not determined whether the 5′ terminus of the viral RNA generated from Pol I system is triphosphate or monophosphate. It can be speculated that viral RNA lacking modifications at the 5′ and 3′ ends is preferentially packaged and secreted into the culture supernatant.

Production of HCV RNA, proteins, and virions from cells transiently transfected with Pol I-driven plasmids. To examine HCV RNA replication and protein expression in cells transfected with pHHJFH1, pHHJFH1/GND, or virion production-defective mutants, pHHJFH1/ΔE and pHHJFH1/R783A/R785A, which possess an in-frame deletion of E1/E2 region and substitutions in the p7 region, respectively (19, 42, 49), RPA and Western blotting were performed 5 days p.t. (Fig. 2A, B, and D). Positive-strand HCV RNA sequences were more abundant than negative-strand RNA sequences in these cells. Positive-strand RNA, but not negative-strand RNA, was detected in cells transfected with the replication-defective mutant pHHJFH1/GND (Fig. 2A and B). Northern blotting showed that genome-length RNA was generated in pHHJFH1-transfected cells but not in pHHJFH1/GND-transfected cells (Fig. 2C).



JFH-1 3'-end sequence / Sample	ATGTAAAA (3'UTR ↔ Poly(A))	TGTCCAAAA (3'UTR ↔ Poly(A))	Total
pHHJFH1 (cell)	10	1	11
pHHJFH1 (sup)	10	0	10
H751JFH1/Zeo (sup)	7	0	7

FIG. 1. Determination of the nucleotide sequences at the 5′-and 3′ ends of HCV RNA produced by the Pol I system. (A and B) 5′RACE and sequence analysis. A synthesized RNA adapter was ligated to RNA extracted from cells transfected with pHHJFH1. The positive-strand HCV RNA was reverse transcribed, and the resulting cDNA was amplified by nested PCR. The amplified 5′-end cDNA was separated by agarose gel electrophoresis (A), cloned, and sequenced (B). (C and D) 3′RACE and sequence analysis. RNA extracted from pHHJFH1-transfected cells, the culture supernatant of transfected cells, and the culture supernatant of H751JFH1/Zeo cells were polyadenylated, reverse transcribed, and amplified by PCR. The amplified 3′-end cDNA was separated by agarose gel electrophoresis (C), cloned, and sequenced (D).

As shown in Fig. 2D, the intracellular expression of core and NS5B proteins was comparable among cells transfected with pHHJFH1, pHHJFH1/ΔE, and pHHJFH1/R783A/R785A. Neither viral protein was detected in pHHJFH1/GND-transfected cells, suggesting that the level of viral RNA generated transiently from the DNA plasmid does not produce enough HCV proteins for detection and that ongoing amplification of the HCV RNA by the HCV NS5B polymerase allows a high enough level of viral RNA to produce detectable levels of HCV proteins.

To assess the release of HCV particles from cells transfected with Pol I-driven plasmids, core protein was quantified in culture supernatant by enzyme-linked immunosorbent assay (ELISA) or sucrose density gradient centrifugation. Core protein secreted from pHHJFH1-transfected cells was first detectable 2 days p.t., with levels increasing up to ~4 pmol/liter on day 6 (Fig. 3A). This core protein level was 4- to 6-fold higher than that in the culture supernatant of pHHJFH1/ΔE- or pHHJFH1/R783A/R785A-transfected cells, despite comparable intracellular core protein levels (Fig. 2D). Core protein was not secreted from cells transfected with pHHJFH1/GND (Fig. 3A). In another experiment, a plasmid expressing the secreted form of human placental alkaline phosphatase (SEAP) was cotransfected with each Pol I-driven plasmid. SEAP activity in culture supernatant was similar among all transfection groups, indicating comparable efficiencies of transfection (data not shown). Sucrose density gradient analysis of the concentrated supernatant of pHHJFH1-transfected cells indicated that the distribution of core protein levels peaked in the fraction of 1.17 g/ml density, while the peak of infectious titer was observed in the fraction of 1.12 g/ml density (Fig. 3B), which is consistent with the results of previous studies based on JFH-1-RNA transfection (23).

We next compared the kinetics of HCV particle secretion in the Pol I-driven system and RNA transfection system. Huh-7 cells, which have limited permissiveness for HCV infection (2), were transfected with either pHHJFH1 or JFH-1 RNA, and then cultured by passaging every 2 or 3 days. As shown in Fig. 3C, both methods of transfection demonstrated similar kinetics of core protein levels until 9 days p.t., after which levels gradually fell. However, significantly greater levels of core protein were detected in the culture of pHHJFH1-transfected cells compared to the RNA-transfected cells on day 12 and 15 p.t. This is likely due to an ongoing production of positive-strand viral RNA from transfected plasmids since RNA degradation generally occurs more quickly than that of circular DNA.

Establishment of stable cell lines constitutively producing HCV virion. To establish cell lines with constitutive HCV production, pHHJFH1/Zeo carrying HCV genomic cDNA and the Zeocin resistance gene were transfected into Huh7.5.1 cells. After approximately 3 weeks of culture with zeocin at a concentration of 0.4 mg/ml, cell colonies producing HCV core protein were screened by ELISA, and three clones were identified that constitutively produced the viral protein (H751JFH1/Zeo cells). Core protein levels within the culture supernatant of selected clones (H751-1, H751-6, and H751-50) were 2.0 × 10^4, 2.7 × 10^3, and 1.4 × 10^3 fmol/liter, respectively. Clone H751-1 was further analyzed. Indirect immunofluorescence with an anti-NS5A antibody showed fluorescent staining of NS5A in the cytoplasm of almost all H751JFH1/