Hercules, CA). Transfected cells were immediately transferred to 10-cm culture dishes, each containing 8 mL of culture medium. G418 (0.8–1.0 mg/mL) (Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 16–24 h after transfection. Culture medium, supplemented with G418, was replaced twice per week. Three weeks after transfection, sparsely grown G418-resistant colonies were independently isolated using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan), and were expanded.

Preparation of supernatants from FGR-JFH1 replicon cells. Culture media was collected from Huh7, IMY-N9, and HepG2 cell lines harboring the FGR-JFH1 replicon and was passed through a 0.45-μm filter. Filtrated culture media was then pooled and concentrated 50-fold using Amicon Ultra-15 (100,000 Molecular weight cut off; Millipore, Bedford, MA), and stored at −80 °C until use.

Assay of infection of naïve Huh7 cells. Infection of naïve Huh7 cells were assayed by immunofluorescence and colony formation assays. For the immunofluorescence assay naïve Huh7.5.1 cells were seeded at 1×10^4 cells/well in an 8-well chamber slide (Becton Dickinson, Franklin Lakes, NJ), cultured overnight and then inoculated with diluted culture media containing infectious HCV particles (1×10^6 HCV-RNA copies). At 72 h after inoculation, the cells were fixed in acetone/methanol (1:1) for 10 min at −20 °C, and the infected foci were visualized by immunofluorescence as follows.

An anti-core HCV protein monoclonal antibody 2H9 [2] was added to the cells at 50 μg/mL in BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) diluted in BlockAce. The cells were then washed, treated with DAPI solution (Sigma, Saint Louis, MO) at 0.1 μg/mL and examined by Biozero fluorescence microscopy (Keyence, Osaka, Japan).

Colony formation assays were performed as described previously [9]. Briefly, naïve Huh7 cells were inoculated with culture supernatants from replicon-expressing cell lines for 2 h, and then cultured with complete medium. Inoculated cells were cultured for 3 weeks in medium supplemented with G418 (0.3 mg/mL). Cell survival was assessed by staining with crystal violet.

Titration of infectivity. The infectivity titer of the culture supernatants was determined on Huh7.5.1 cells by end point dilution and immunofluorescence as described above. Briefly, each sample was serially diluted 10-fold in DMEM-10% FBS and 100 μL was used to inoculate Huh7.5.1 cells. Infection was examined 72 h post-inoculation by immunofluorescence using a mouse monoclonal anti-core antibody and secondary anti-mouse IgG-Alexa 488 conjugated antibodies. Infectious foci were counted and the titer was calculated and expressed as focus forming units per mL (FFU/mL).

Sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer containing 10 mM Tris, pH7.5, 150 mM NaCl, and 0.1 mM EDTA. Gradients were centrifuged in an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100 μL drop from each fraction following a gradient run.

Quantification of HCV-core protein and RNA. The level of the HCV-core protein in culture supernatants or sucrose density gradient fractions, was assayed using an immunoassay as described elsewhere [10]. Viral RNA was isolated from harvested culture media, or sucrose density gradient fractions, using the QiaAmp Viral RNA Extraction kit (Qiagen, Tokyo, Japan). The copy number of HCV RNA was determined by real-time detection reverse transcription-polymerase chain reaction (RTD-PCR), using an ABI Prism 7500fast sequence detector system (Applied Biosystems, Tokyo, Japan) [11].

Results

Production of infectious HCV from human liver-derived cell lines

We first determined if it was possible to produce infectious HCV from cell lines other than Huh7. We selected the HepG2 and IMY-N9 cell lines to establish human liver-derived cell lines that enable replication of the JFH-1 genome [6]. Since full-genomic JFH-1 did not transiently replicate in these cells (data not shown), we established FGR-JFH1 replicon cells that stably replicate the JFH-1 genome. In the culture media obtained from these full-genomic replicon cells, HCV-RNA titers were detected by RTD-PCR. The titer of HCV-RNA was highest in the supernatant from an IMY-N9 cell clone and lowest from a HepG2 cell clone (Table 1). When naïve Huh7.5.1 cells were inoculated with culture supernatants from the replicon cells, infected cells could be detected by immunofluorescence using an anti-HCV-core protein antibody (Fig. 1A). These data suggested that HepG2 and IMY-N9 cells are able to produce infectious HCV.

We then compared the specific infectivity of the replicon containing culture supernatants from the different cells. Specific infectivity was calculated by dividing the infectious titer, calculated by immunofluorescence of infectious foci, of the culture media by the titer obtained for HCV-RNA. Using these calculations the culture media from Huh7 and HepG2 cells showed almost the same specific infectivity whereas that from IMY-N9 cell was relatively higher (Table 1). Thus the infectious HCV in the culture media might differ according to the cell line from which it was obtained.

To clarify the differences observed in specific infectivity, we next examined the ability of the various cellular supernatants to induce colony formation. For this assay naïve Huh7 cells were inoculated with culture media of the same HCV-RNA titer as that of the FGR-JFH1 virus and were cultured in G418-containing medium. Cell survival was assayed by staining with crystal violet, and the number of colonies formed was counted. Consistent with the specific infectivity results, the supernatant of the IMY-N9 replicon cell showed higher colony formation compared with that of Huh7 and HepG2 replicon cells (Fig. 1B and C). Thus IMY-N9 cells produce infectious HCV with a relatively higher infectivity than the other cell lines suggesting that the supernatant derived from the different replicon producing cells may differ.

Characterization of the FGR-JFH1 virus from different liver-derived cells

To further characterize potential differences between the viruses produced by the different cell lines we next characterized the FGR-JFH1 virus in the media of the different cell lines by sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient and centrifuged. Twenty-four fractions were collected and the HCV-core protein and RNA was assayed in each fraction. The peak fraction of the HCV-core protein and that of the RNA coincided at a density of 1.15 g/mL in all supernatants. However, the supernatant of the IMY-N9 cells showed different profiles for both the HCV-core protein and RNA compared to those of Huh7. Thus the IMY-N9 cells had a different ratio of

Table 1
Infectivity of the supernatant of replicon cell lines.

Producing cell	HCV-RNA (copies/mL)	Infectious titer (FFU/mL)	Specific infectivity (FFU/RNA copy)
Huh7	$1.36 \pm 0.02 \times 10^8$	$1.30 \pm 0.32 \times 10^4$	9.56×10^{-5}
IMY-N9	$2.80 \pm 0.04 \times 10^8$	$3.75 \pm 0.38 \times 10^4$	1.34×10^{-4}
HepG2	$8.80 \pm 0.75 \times 10^7$	$7.70 \pm 1.41 \times 10^3$	7.96×10^{-5}

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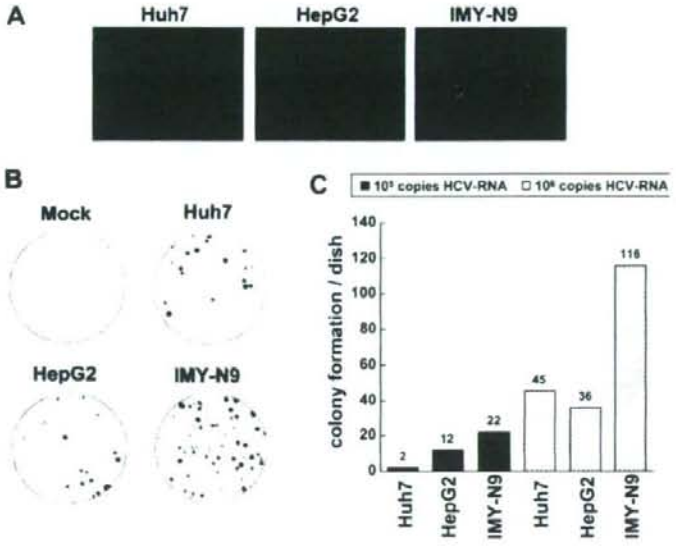

Fig. 1. Naïve Huh7 cell infection assay of JFH-1 full-genomic replicon cell culture supernatants. (A) JFH-1 full-genomic replicon (FGR-JFH1) cells were established in Huh7, HepG2, and IMY-N9 cell lines. Supernatants derived from Huh7 (*left*), HepG2 (*middle*), and IMY-N9 (*right*) cells (1×10^6 HCV-RNA copies) were inoculated into naïve Huh7.5.1 cells (1×10^4) for 48 h, and infected cells were then detected by immunofluorescence using an anti-core antibody (clone 2H9) (green). (B) Naïve Huh7 cells (5×10^5) were inoculated with mock, Huh7, HepG2, and IMY-N9-derived supernatants (10^6 HCV-RNA copies per 10-cm dish) of FGR-JFH1 cells for 2 h. Inoculated cells were cultured for 3 weeks in complete medium supplemented with G418 (0.3 mg/mL), and G418-resistant cells were stained using crystal violet. (C) The number of G418-resistant colonies obtained in (B) was calculated when 10^5 or 10^6 copies of HCV-RNA were tested. Mean values of colony number were indicated in duplicate experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HCV-core protein and RNA at a density of 1.15 g/mL (RNA/Core ratio; Huh7: 511, IMY-N9: 133 copies/fmol) and also showed a secondary peak at lower density (approximately 1.05 g/mL). For all supernatants the peak of infectivity exhibited at a density of 1.10 g/mL that was slightly lower than that of the HCV-core protein and RNA peaks. Furthermore infectivity was barely detectable in the lower density fractions (Fig. 2) suggesting that the HCV-core protein and RNA that was detected at lower density was irrelevant for infectivity of the different supernatants.

We considered the possibility that the core protein and RNA in the lighter fractions may be due to cellular debris containing a replication complex. To determine if this might be the case we therefore analyzed the supernatants from Huh7 and IMY-N9 envelope-deleted replicon cells (FGR-JFH1/deltaE12). The HCV-core protein and RNA were detected in the supernatants of these cells although the titers were very low. These supernatants were not infective for naïve Huh7 cells (data not shown). Furthermore, analysis of the concentrated supernatants of these cell lines by sucrose density gradient analysis detected both the HCV-core protein and RNA, and the major peaks of HCV-RNA were detected in the lower density (approximately 1.10 g/mL) fractions (Fig. 3). However, the profiles of HCV-core protein and RNA did not coincide for either cell line.

Discussion

Infectious HCV can be produced in cell culture by using the JFH-1 genome. This system permits investigation of various aspects of the HCV life cycle such as the steps of entry into cells, replication, and secretion. Infectious HCV derived from JFH-1 is robustly produced in Huh7 cell lines [2,3], and the infectious particles have been characterized. However the difficulty in robustly producing infectious HCV from other cell lines prevents a comparative study of HCV production among different cell lines. In this study, we compared infectious HCV production in Huh7 with that of other cell lines, and characterized the viruses produced.

First, we established Huh7, IMY-N9, and HepG2 FGR-JFH1 replicon cells. These cell lines were able to replicate the JFH-1 subgenomic replicon [6]. The HCV-core protein and RNA were detected in all of the supernatants and all of these supernatants showed infectivity for naïve Huh7. Infectivity was evaluated by transient infection and colony formation assays. These assays indicated that the infectious supernatant from IMY-N9 cell had higher infectivity than the other cell lines for naïve Huh7 cells.

Next, we characterized each supernatant by sucrose density gradient analysis, which revealed both similarities and differences among the infectious supernatants. All samples showed typical peaks at 1.15 g/mL buoyant density for HCV-core protein and RNA, and infectious fractions showed an almost identical buoyant density of 1.10 g/mL. However, the supernatant from the IMY-N9 cells showed a difference in the core/RNA ratio at a density of 1.15 g/mL and higher secondary peak of HCV-core protein and RNA at a lower density (approximately 1.05 g/mL). Since the fractions at lower density did not correlate with infectivity, it is believed that the component at lower density does not contain infectious HCV particles but rather cellular debris that contains HCV proteins, RNA, and lipids [12]. HCV can associate with lipoprotein [13,14], and is secreted with VLDL [15]. Thus, the observed differences in the HCV-producing cells may derive from differences in lipoprotein synthesis. However, it is also possible that the components migrating at lower density contain virus particles. The deletion mutant of FGR-JFH1 (FGR-JFH1/deltaE12) did replicate in Huh7 and IMY-N9 cells, and these replicon cells secreted the HCV-core protein into the culture media, although at low levels. HCV-RNA was also detected in the same culture

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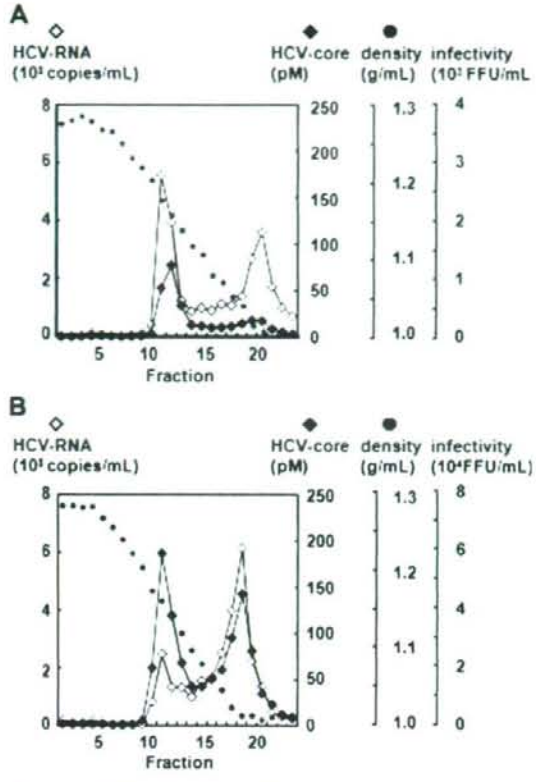

Fig. 2. Density gradient analysis of infectious HCV derived from Huh7 and IMY-N9 cells. Concentrated supernatants of Huh7 cells (A) and IMY-N9 cells (B) were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer. The gradients were centrifuged in a SW41 rotor at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The buoyant density (closed circles), HCV-core protein (closed diamonds), HCV-RNA (open diamonds) and infectivity for naïve Huh7.5.1 cells (shown in gray) was detected in each fraction as described in Materials and methods.

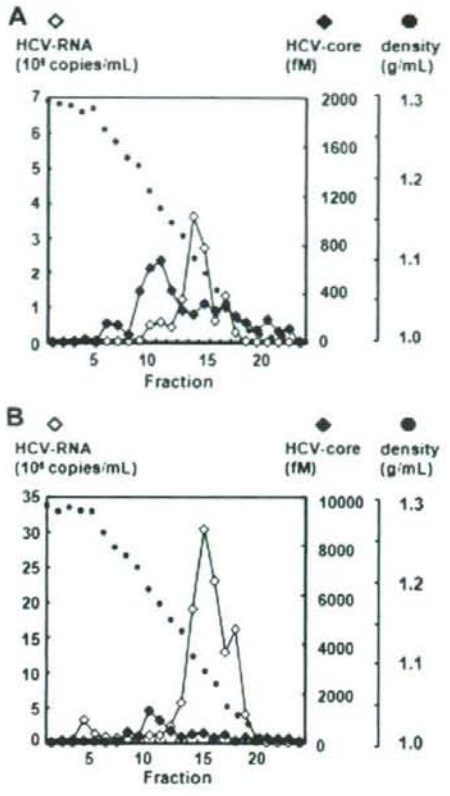

Fig. 3. Density gradient analysis of supernatants derived from Huh7 and IMY-N9 cells transfected with FGR-JFH1/deltaE12 RNA. Concentrated supernatants from Huh7 (A) and IMY-N9 (B) cells were analyzed by sucrose density gradient as described in the legend to Fig. 2. The buoyant density (closed circles), HCV-core protein (closed diamonds) and HCV-RNA (open diamonds) was analyzed in each fraction.

medium, and the profile of this HCV-RNA differed from that of the HCV-core protein in sucrose density gradient analysis. Thus, the peak fractions containing the HCV-core protein and RNA from the supernatant of FGR-JFH1/deltaE12 cells were different from the peak fractions from that of FGR-JFH1 cells. Therefore it is possible that all of the peaks of HCV-core protein and RNA observed in the supernatant of FGR-JFH1 replicon cells may correlate to virus particles with different densities. However, the reason why they centrifuge at different densities is unclear. Interestingly, the supernatants from cells transfected with envelope-deleted replicon RNA exhibit non-identical HCV-core protein and RNA profiles on a sucrose density gradient. Envelope-deleted replicon RNA may have a decreased ability to form nucleocapsids although a detailed examination is necessary to establish this point.

We previously developed a method for infectious HCV production using the FGR-JFH1 [9], and have now succeeded in producing infectious HCV in the supernatant of cultured liver-derived cell lines harboring FGR-JFH1 RNA. Infectious HCV particles are useful for vaccine production and are considered good antigens for the generation of useful antibodies. Selection of an appropriate cell line is important for the production of HCV particles for vaccine development. The technique used in this study seemed to be appropriate for producing infectious HCV in various cell lines [8].

A second advantage of using HepG2 and IMY-N9 cells for the production of virus particles is that these parental cell lines, unlike the Huh7 cell line, do not express the CD81 molecule on the cell surface, however, the expression on cell clones used in this study was not confirmed. This means that the FGR-JFH1 replicon of these cell lines may have a single cycle of HCV production, encompassing replication, assembly, budding and secretion, and do not show HCV permissiveness. These cells should therefore be useful for the discovery of drugs targeted against HCV assembly and secretion.

Acknowledgments

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The DNA Damage Sensors Ataxia-Telangiectasia Mutated Kinase and Checkpoint Kinase 2 Are Required for Hepatitis C Virus RNA Replication▽

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Cellular responses to DNA damage are crucial for maintaining genome integrity, virus infection, and preventing the development of cancer. Hepatitis C virus (HCV) infection and the expression of the HCV nonstructural protein NS3 and core protein have been proposed as factors involved in the induction of double-stranded DNA breaks and enhancement of the mutation frequency of cellular genes. Since DNA damage sensors, such as the ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), poly(ADP-ribose) polymerase 1 (PARP-1), and checkpoint kinase 2 (Chk2), play central roles in the response to genotoxic stress, we hypothesized that these sensors might affect HCV replication. To test this hypothesis, we examined the level of HCV RNA in HuH-7-derived cells stably expressing short hairpin RNA targeted to ATM, ATR, PARP-1, or Chk2. Consequently, we found that replication of both genome-length HCV RNA (HCV-O, genotype 1b) and the subgenomic replicon RNA were notably suppressed in ATM- or Chk2-knockdown cells. In addition, the RNA replication of HCV-JFH1 (genotype 2a) and the release of core protein into the culture supernatants were suppressed in these knockdown cells after inoculation of the cell culture-generated HCV. Consistent with these observations, ATM kinase inhibitor could suppress the HCV RNA replication. Furthermore, we observed that HCV NS3-NS4A interacted with ATM and that HCV NS5B interacted with both ATM and Chk2. Taken together, these results suggest that the ATM signaling pathway is critical for HCV RNA replication and may represent a novel target for the clinical treatment of patients with chronic hepatitis C.

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV infection has now become a serious health problem, with at least 170 million people currently infected worldwide (28). HCV is an enveloped virus with a positive single-stranded 9.6-kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (12, 13, 27).

Studies have shown that various viruses with distinct replication strategies—including the DNA viruses Epstein-Barr virus, herpes simplex virus 1, adenovirus, and simian virus 40 and the retrovirus human immunodeficiency virus type 1 (HIV-1)—can activate DNA damage response pathways and utilize these damage responses to facilitate their own viral reproduction and promote the survival of infected cells (2, 16, 17). In the case of HCV, it has been proposed that HCV infection causes double-stranded DNA (dsDNA) breaks and enhances the mutation frequency of cellular genes and that these effects are mediated by nitric oxide (18, 19). In addition, the HCV core, E1, and NS3 proteins have been suggested to be potent reactive oxygen species inducers, leading to DNA damage (19). Furthermore, we previously demonstrated that HCV NS5B-expressing PH5CH8 immortalized human hepatocyte cells were susceptible to DNA damage in the form of dsDNA breaks (23). Thus, HCV seems to be associated with the dsDNA damage response pathways.

Since the DNA damage sensors, such as ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), poly(ADP-ribose) polymerase 1 (PARP-1), and checkpoint kinase 2 (Chk2; a direct downstream target of ATM), play central roles in response to genotoxic stress (10), we hypothesized that these sensors might affect HCV replication.

To investigate the possible involvement of these cellular factors in HCV replication, we examined the level of HCV RNA in cells rendered defective for DNA damage sensors by RNA interference or by pharmacological inhibition.

MATERIALS AND METHODS

Cell culture. 293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). The HuH-7-derived O cells harboring a replicative genome-length HCV RNA and the HuH-7-derived sO cells harboring the subgenomic replicon RNA of HCV-O were cultured in DMEM with 10% FBS and G418 (300 μg/ml geneticin; Invitrogen) as described previously (11, 14). Oc and sOc cells, which were created by eliminating HCV RNA from O cells and sO cells by interferon (IFN) treatment (11, 14), respectively, were also cultured in DMEM with 10% FBS.

RNA interference. Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding se-

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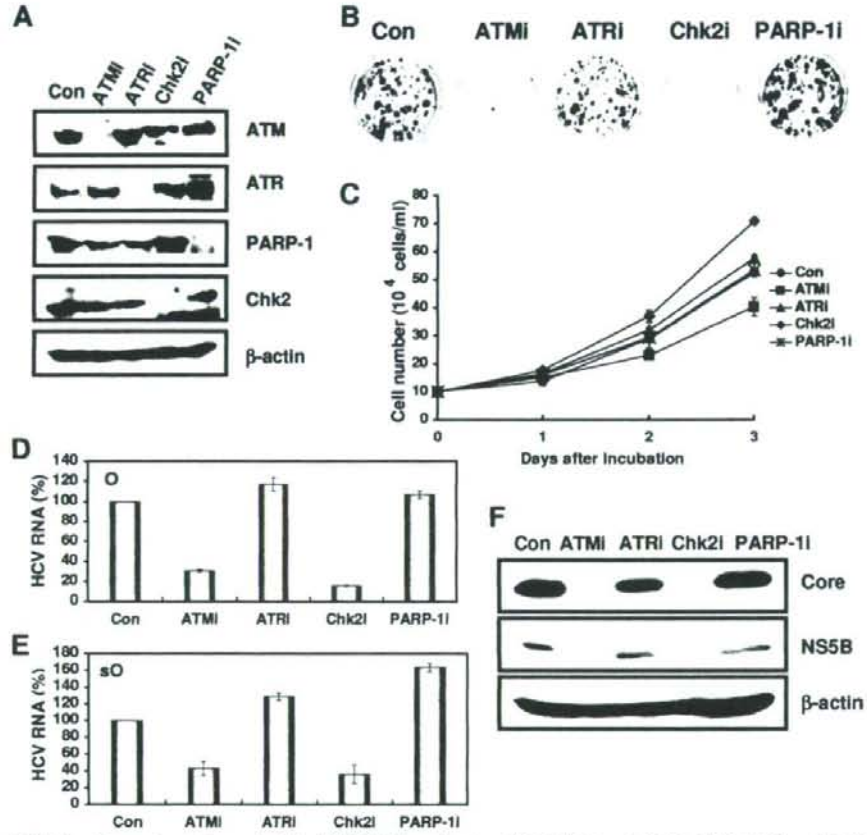

FIG. 1. The ATM signaling pathway is required for HCV RNA replication. (A) Inhibition of ATM, ATR, Chk2, or PARP-1 expression by shRNA-producing lentiviral vectors. The results of the Western blot analysis of cellular lysates with anti-ATM, anti-ATR, anti-Chk2, anti-PARP-1, or anti-β-actin antibody in Oc cells expressing shRNA targeted to ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in Oc cells transduced with a control lentiviral vector (Con) are shown. (B) ECF in ATM-, ATR-, Chk2-, or PARP-1-knockdown cells. In vitro transcribed ON/C-5B K1609E RNA (2 μg) was transfected into the ATM-, ATR-, Chk2, or PARP-1-knockdown Oc cells or the Oc cells transduced with a control lentiviral vector (Con). G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA. Experiments were done in duplicate, and a representative result is shown. (C) The cell growth curve of ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i)-knockdown Oc cells or the Oc cells transduced with a control lentiviral vector (Con). Results from three independent experiments are shown. (D) The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR (Roche). Experiments were done in triplicate, and columns represent the mean percentage of HCV RNA. (E) The level of subgenomic replicon (sO cells) RNA was monitored by real-time LightCycler PCR. Results from three independent experiments are shown as described in panel D. (F) The HCV core or NS5B protein expression level in ATM-, ATR-, Chk2-, or PARP-1-knockdown cells. The results of Western blot analysis of cellular lysates with anti-HCV core protein, anti-HCV NS5B, or anti-β-actin antibody in O cells expressing shRNA targeted to ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in O cells transduced with a control lentiviral vector (Con) are shown.

quences targeted to Chk2 in lentiviral vector: 5'-GATCCCCGGGGGAGAGCTGTT TGACATTCAAGAGATGTCAAACAGCTCTCCCCCTTTTTGGAAA-3' (sense) and 5'-AGCTTTTCCAAAAAGGGGGAGAGCTGTTTGACATCTCTTGAATGT CAAACAGCTCTCCCCCGGG-3' (antisense). The oligonucleotides above were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (5), generating pSUPER-Chk2i. To construct pLV-Chk2i, the BamHI-SalI fragments of the pSUPER-Chk2i were subcloned into the BamHI-SalI site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transduced cells (4). pLV-ATMi, pLV-ATRi, and pLV-PARP-1i were constructed as described previously (1).

Lentiviral vector production. The vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector system has been described previously (24). The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 (30) and the VSV-G envelope plasmid pMDG2 as well as the lentiviral vector into 293FT cells with FuGene6 (Roche Diagnostics, Mannheim, Germany).

Quantitative reverse transcription-PCR analysis. Quantitative reverse transcription-PCR analysis for HCV RNA was performed by real-time LightCycler PCR as described previously (11).

Western blot analysis. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting analysis using anti-ATM (2C1; GTX70103 [GeneTex, San Antonio, TX]), anti-ATR (GTX70133; GeneTex), anti-Chk2 (NT; ProSci, Poway, CA), anti-Chk2 (DCS-273; Medical and Biological Laboratories, Nagoya, Japan), anti-phospho-Chk2 (Thr68) (Cell Signaling,

Danvers, MA), anti-PARP-1 (C-2-10; Calbiochem, Merck Biosciences, Darmstadt, Germany), anti-hemagglutinin (HA) (HA-7; Sigma, St. Louis, MO), anti-core protein (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan), anti-NS3 and anti-NS5B (no. 14; a generous gift from M. Kohara, the Tokyo Metropolitan Institute of Medical Science, Japan), anti-NS5A (no. 8926; a generous gift from A. Takamizawa, The Research Foundation for Microbial Diseases of Osaka University, Japan), and anti-β-actin (Sigma) Antibodies.

Immunofluorescence and confocal microscopic analysis. Cells were fixed in 3.5% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in 0.1% NP-40 in PBS at room temperature. Cells were incubated with anti-ATM antibody (5C2; GTX70107 [GeneTex] or PM026 [MBL]), anti-HA antibody (3F10), anti-NS5B antibody and/or anti-NS3 antibody at a 1:300 dilution in PBS containing 3% bovine serum albumin at 37°C for 30 min. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) or anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch) at a 1:300 dilution in PBS containing bovine serum albumin at 37°C for 30 min. Following extensive washing in PBS, cells were mounted on slides using a mounting medium of 90% glycerin–10% PBS with 0.01% *p*-phenylenediamine added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (LSM510; Zeiss, Jena, Germany).

Immunoprecipitation. Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.5% NP-40, 10 mM NaF, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared with 30 μl of protein G-Sepharose (GE Healthcare Biosciences, Uppsala, Sweden). Precleared supernatants were incubated with 5 μg of anti-HA antibody (3F10; Roche), 10 μl of anti-NS5B antibody, 5 μg of anti-Chk2 antibody (DCS-273; MBL), 5 μg of anti-FLAG antibody (M2; Sigma), or 5 μg of anti-ATM antibody (2C1) (GTX70103; GeneTex) at 4°C for 1 h. Following absorption of the precipitates on 30 μl of protein G-Sepharose resin for 1 h, the resin was washed four times with 700 μl of lysis buffer. Proteins were eluted by boiling the resin for 5 min in 2× Laemmli sample buffer. The proteins were then subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting analysis using anti-ATM, anti-Chk2, anti-HCV core protein (CP-9 and CP-11 mixture), anti-NS5A, anti-NS5B, anti-HA (HA-7; Sigma), or anti-NS3 antibody.

RESULTS

ATM and Chk2 are required for HCV RNA replication. To determine the potential role of DNA damage sensors in HCV replication, we first used lentiviral vector-mediated RNA interference to stably knockdown ATM, ATR, PARP-1 (1), or Chk2 in the following human hepatoma HuH-7-derived cell lines: O cells harboring a replicative genome-length HCV RNA (HCV-O, genotype 1b) (11), Oc cells derived from O cells (created by eliminating genome-length HCV RNA from O cells by IFN treatment) (11), sO cells harboring the subgenomic replicon of HCV-O (14), or RSc cells that cell culture-generated HCV (HCVcc) (JFH1, genotype 2a) (29) could infect and effectively replicate (3). To express shRNAs targeted to ATM, ATR, PARP-1 (1), or Chk2, we used a VSV-G-pseudotyped HIV-1-based vector system (24). We used puromycin-resistant pooled cells 10 days after the lentiviral transduction in all experiments. Western blot analysis of the lysates demonstrated very effective knockdown of ATM, ATR, Chk2, and PARP-1 in Oc cells (Fig. 1A). The effective knockdown of ATM, ATR, Chk2, or PARP-1 in O cells or sO cells was also confirmed by Western blot analysis (data not shown). In this context, the efficiency of colony formation (ECF) in ATM- or Chk2-, but not ATR- or PARP-1-, knockdown Oc cells transfected with the genome-length HCV-O RNA with an adapted mutation at amino acid position 1609 in the NS3 helicase region (ON/C-5B K1609E RNA) (11) was notably reduced compared with the control cells (Fig. 1B) even though Chk2-knockdown cells had a slightly faster growth rate than the control cells (Fig. 1C), suggesting that both ATM and Chk2 are crucial for HCV RNA replication. To further confirm this

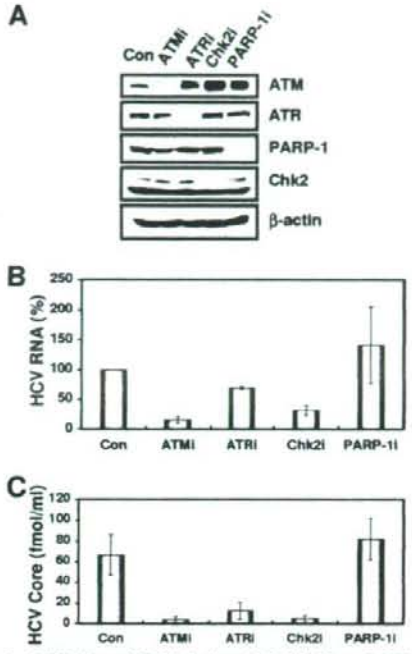

FIG. 2. ATM affects HCV infection. (A) Inhibition of ATM, ATR, Chk2, or PARP-1 expression by shRNA-producing lentiviral vectors. The results of Western blot analysis of cellular lysates with anti-ATM, anti-ATR, anti-PARP-1, anti-Chk2, or anti-β-actin antibody in RSc cured cells expressing shRNA targeted to ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in RSc cells transduced with a control lentiviral vector (Con) are shown. (B) The level of genome-length HCV (JFH1) RNA was monitored by real-time LightCycler PCR after inoculation of the HCVcc. Results from three independent experiments are shown as described in the legend of Fig. 1D. (C) The levels of the core protein in the culture supernatants were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories). Experiments were done in triplicate, and columns represent the mean core protein levels.

observation, we quantitatively examined the level of HCV RNA in the O cell- or sO cell-derived knockdown cells. Consequently, we found that replication of both genome-length HCV RNA (HCV-O) and its subgenomic replicon RNA (sO) were notably suppressed in ATM- or Chk2-knockdown cells but not in ATR- or PARP-1-knockdown cells (Fig. 1D and E). Consistent with this finding, the expression levels of core and NS5B proteins were also significantly decreased in the cell lysates of ATM- or Chk2-knockdown O cells (Fig. 1F). We next examined the replication level of HCV-JFH1 in ATM-, ATR-, Chk2-, or PARP-1-knockdown RSc cells (Fig. 2A). The results revealed that RNA replication of HCV-JFH1 and release of core protein into the culture supernatants were suppressed in only ATM- or Chk2-knockdown RSc cells after inoculation with HCVcc (Fig. 2B and C). Interestingly, the release of core protein into the culture supernatant was also significantly suppressed in ATR-knockdown RSc cells, while HCV RNA replication was slightly suppressed in these cells

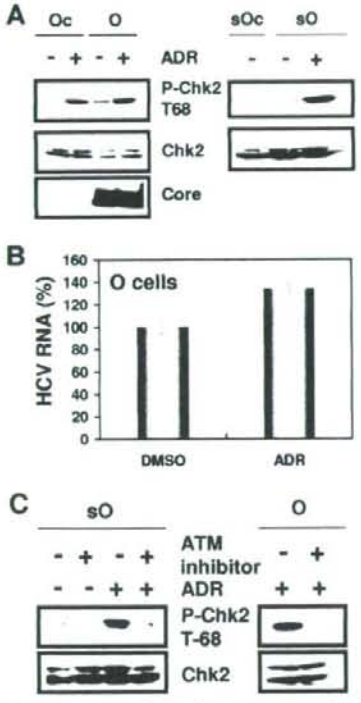

FIG. 3. ATM-dependent DNA damage response in HCV RNA-replicating cells. (A) Stimulation of Chk2 phosphorylation in the HCV RNA-replicating cells. The Oc, O, or sO cells were treated with 100 nM adriamycin (Sigma) for 2 h. The results of Western blot analysis of cellular lysates with anti-phospho-Chk2 (Thr68) (P-Chk2 T68), anti-Chk2, or anti-core protein antibody are shown. (B) Effect of adriamycin on HCV RNA replication. The O cells were treated with 100 nM adriamycin for 24 h. The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR. Results from three independent experiments are shown as described in the legend of Fig. 1D. DMSO, dimethyl sulfoxide. (C) Effect of ATM kinase inhibitor on Chk2 phosphorylation. The sO or O cells were pretreated with 10 μM ATM kinase inhibitor (KU-55933) (Calbiochem) for 2 h, followed by treatment with 100 nM adriamycin for 2 h. The results of Western blot analysis of cellular lysates with anti-phospho-Chk2 (Thr68) or anti-Chk2 antibody are shown.

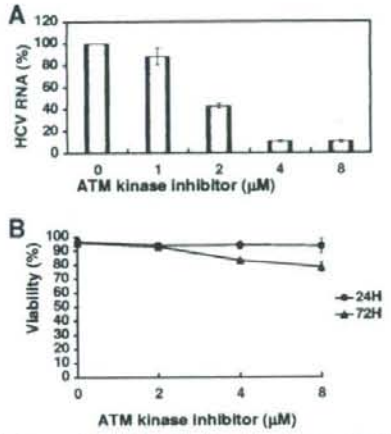

FIG. 4. Suppression of HCV RNA replication by ATM kinase inhibitor. (A) The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR after treatment with the indicated concentration of ATM kinase inhibitor for 72 h. Results from three independent experiments are shown as described in the legend of Fig. 1D. (B) Cell viabilities after treatment with the indicated concentration of ATM kinase inhibitor for 24 h or 72 h are shown.

(Fig. 2B and C), suggesting that ATR participates in the production of HCV virion.

In contrast, highly efficient knockdown of PARP-1 had no observable effects on the ECF (Fig. 1B), HCV RNA replication (Fig. 1D and E and 2B), or core protein expression in the cell lysate or in the supernatant (Fig. 1F and 2C), suggesting that our finding was not due to a nonspecific event. Thus, we have demonstrated for the first time that DNA damage sensors, ATM and Chk2, are required for HCV RNA replication.

ATM kinase activity in HCV RNA-replicating cells. Although it has been proposed that HCV causes dsDNA breaks (18, 19), little is known about whether HCV activates or inhibits the ATM-dependent damage response pathway. In this regard, it is worth noting that we observed weak but significant Chk2 phosphorylation at threonine 68, the specific marker for ATM activation (20, 21), in the HCV RNA-replicating cells (O and sO cells) but not in the HCV-negative Oc and sOc cells (created by eliminating replicon RNA from sO cells by IFN treatment) (Fig. 3A), suggesting that the persistent HCV RNA replication stimulated the ATM-dependent DNA damage response. Furthermore, a 2-h treatment with 100 nM adriamycin, a dsDNA break inducer, markedly induced Chk2 phosphorylation in Oc, O, and sO cells (Fig. 3A). Importantly, Chk2 phosphorylation was not inhibited even in the HCV RNA-replicating cells (O and sO cells) (Fig. 3A), suggesting that the persistent HCV RNA replication and the HCV proteins are not able to suppress the ATM-dependent DNA damage response. To examine whether such a DNA damage response activates HCV RNA replication, we quantified the level of HCV RNA in the O cells treated with 100 nM adriamycin for 24 h. The results show that HCV RNA replication was increased (approximately 1.3-fold) after treatment with adriamycin (Fig. 3B), suggesting that the DNA damage response activates HCV RNA replication.

Suppression of HCV RNA replication by a small-molecule inhibitor of the ATM kinase. We next examined the effect of a specific small-molecule inhibitor of the ATM kinase (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one [KU-55933]) (16) on HCV RNA replication. As expected, the ATM kinase inhibitor effectively inhibited Chk2 phosphorylation after adriamycin treatment in both sO and O cells (Fig. 3C). In this context, the ATM kinase inhibitor could efficiently suppress genome-length HCV RNA replication with an in vitro 50% effective concentration (EC_{50}) of approximately 2 μM at 72 h after treatment with adriamycin (Fig. 4A). Although this ATM kinase inhibitor did not affect cell viability at 24 h after the treatment, there was a slight decrease in the cell viability at 72 h after treatment (Fig. 4B). Thus, this or other ATM kinase inhibitors may be

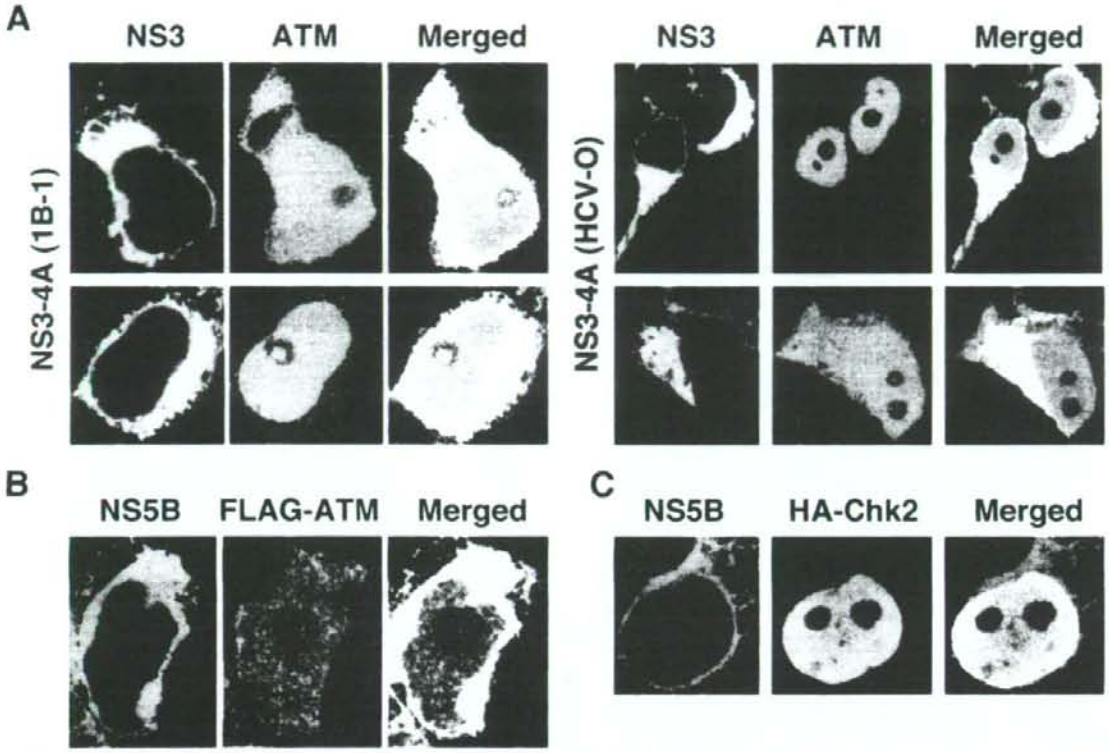

FIG. 5. Subcellular localization of ATM and Chk2 in HCV NS3-4A- or NS5B-expressing cells. (A) ATM partially colocalized with HCV NS3-4A. 293FT cells cotransfected with 300 ng of pCX4bsr/NS3-4A (1B-1) (8) or pCX4bsr/NS3-4A (O) (8) and 300 ng of pcDNA3-FLAG-ATMwt (6) were examined by confocal laser scanning microscopy. Cells were stained with anti-NS3 and anti-ATM (5C2) antibodies and then visualized with FITC (NS3) or Cy3 (ATM). (B) ATM partially colocalized with HCV NS5B. 293FT cells cotransfected with 300 ng of pCX4bsr/NS5B (1B-1) (23) and 300 ng of pcDNA3-FLAG-ATMwt (6). Cells were stained with anti-NS5B (no. 14) and anti-ATM (PM026) antibodies and then visualized with FITC (ATM) or Cy3 (NS5B). (C) Chk2 partially colocalized with HCV NS5B. 293FT cells cotransfected with 300 ng of pCX4bsr/NS5B (1B-1) (23) and 300 ng of pcDNA3-HA-Chk2wt (20, 21). Cells were stained with anti-NS5B and anti-HA (3F10) antibodies and then visualized with FITC (HA-Chk2) or Cy3 (NS5B). Images were visualized using confocal laser scanning microscopy (LSM510; Carl Zeiss). The right panels exhibit two-color overlay images (Merged). Colocalization is shown in yellow.

useful for the clinical treatment of patients with chronic hepatitis C.

Interaction of HCV NS3-4A with ATM. Since HCV NS3 has been proposed to be a viral factor involved in the induction of dsDNA breaks (18, 19), we first examined the subcellular localization of NS3-NS4A ([NS3-4A] 1B-1 or HCV-O strain) and ATM by confocal laser scanning microscopy. In most of the observed cells, ATM partially colocalized with NS3-4A in the perinuclear region and in dispersed points throughout the cytoplasm (Fig. 5A). In particular, we observed prominent colocalization of ATM with NS3-4A in some cells (Fig. 5A). Next, using anti-FLAG and anti-ATM antibodies, we immunoprecipitated lysates from 293FT cells in which FLAG-tagged ATM and either NS3-4A (HCV-O) or NS3 (HCV-O) were overexpressed and then performed immunoblotting analysis using either anti-ATM or anti-NS3 antibody to determine whether ATM binds to NS3-4A or NS3. The results revealed that ATM preferentially bound to NS3-4A over NS3 alone (Fig. 6A). Similarly, we found that ATM bound to NS3-4A using the O cell lysates (Fig. 6B), while HA-tagged Chk2 did not bind to NS3-4A in immunoprecipitation analysis using lysates from 293FT cells in which NS3-4A and HA-tagged Chk2 were overexpressed (Fig. 6C). Although NS3-4A has protease activity, ATM was not cleaved by the NS3-4A protease (Fig. 6D). Taking these results together, we conclude that ATM is able to interact with NS3-4A.

Interaction of HCV NS5B with ATM and Chk2. We next examined the subcellular localization of ATM and/or Chk2 in HCV NS5B-expressing cells by confocal laser scanning microscopy since we previously demonstrated that HCV NS5B-expressing PH5CH8 immortalized human hepatocyte cells were susceptible to DNA damage in the form of dsDNA breaks (23). ATM partially colocalized with NS5B in dispersed points throughout the cytoplasm (Fig. 5B), similar to the subcellular localization of HCV NS3-4A and ATM. Furthermore, Chk2 also partially colocalized with NS5B in the perinuclear region and in dispersed points in the nucleus (Fig. 5C). To determine whether endogenous ATM binds to NS5B, lysates from Oc or

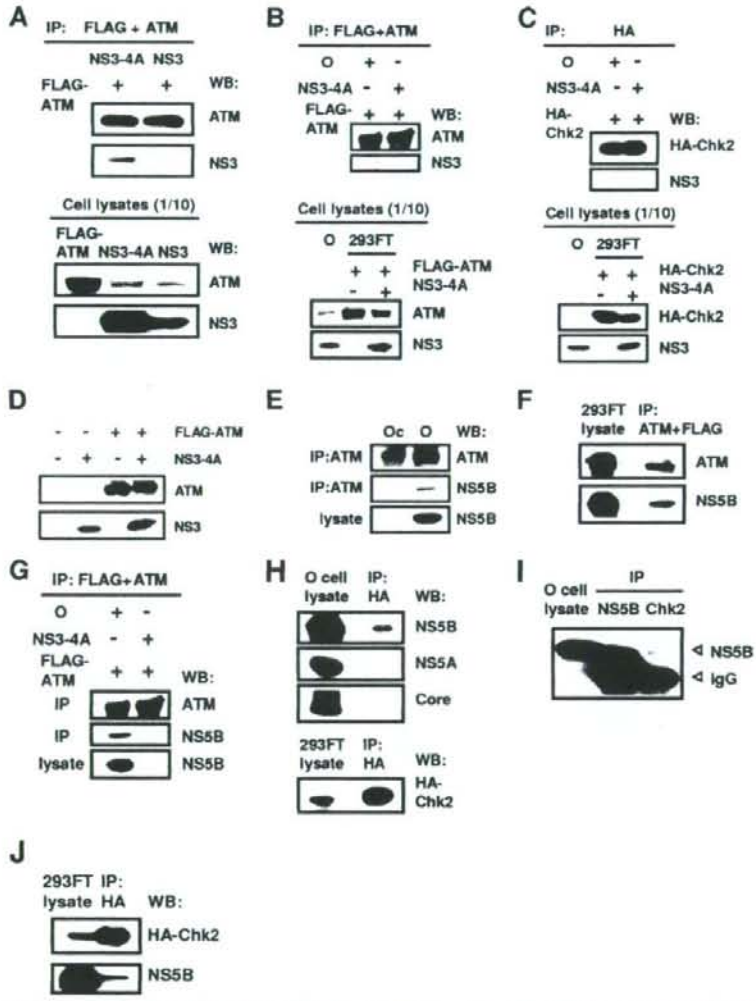

FIG. 6. Interaction of HCV NS3-4A and NS5B with the ATM signaling pathway. (A and B) ATM bound to HCV NS3-4A. (A) 293FT cells were transfected with 4 μg of pCX4bsr/NS3-4A (O), 4 μg of pCX4bsr/NS3 (O), or 4 μg of pcDNA3-FLAG-ATMwt. The cell lysates of expressed FLAG-ATM were mixed with lysates expressing either NS3-4A or NS3. The cell lysates were immunoprecipitated with both anti-FLAG (M2) and anti-ATM (2C1) antibodies, followed by immunoblotting analysis using either anti-ATM (2C1) or anti-HCV NS3 antibody. The results of Western blot analysis of 1/10 of the cellular lysates with anti-ATM or anti-NS3 antibody are also shown. (B) 293FT cells were cotransfected with 4 μg of pcDNA3-FLAG-ATMwt and/or 4 μg of pCX4bsr/NS3-4A (O). The cell lysates of expressed FLAG-ATM alone were mixed with the O cell lysates. Immunoprecipitation and Western blot analysis were performed as described in panel A. (C) Chk2 did not bind to NS3-4A. 293FT cells were cotransfected with 4 μg of pcDNA3-HA-Chk2wt and/or 4 μg of pCX4bsr/NS3-4A (O). The cell lysates of expressed HA-Chk2 alone were mixed with the O cell lysates. The cell lysates were immunoprecipitated with anti-HA antibody (3F10), followed by Western blot analysis using either anti-HA (HA-7) or anti-HCV NS3 antibody. The results of Western blot analysis of 1/10 of the cellular lysates with anti-HA or anti-NS3 antibody are also shown. (D) ATM was not cleaved by HCV NS3-4A protease. 293FT cells were cotransfected with 4 μg of pCX4bsr/NS3-4A (O) and/or 4 μg of pcDNA3-FLAG-ATMwt. The results of Western blot analysis of cellular lysates with anti-ATM or anti-NS3 antibody are shown. (E to G) ATM bound to HCV NS5B. (E) The lysates of O or Oc cells were immunoprecipitated with anti-ATM antibody (2C1), followed by immunoblotting analysis using either anti-ATM or anti-HCV NS5B antibody (no. 14). The results of Western blot analysis of 1/10 of the cellular lysates with anti-NS5B antibody are also shown. (F) 293FT cells were cotransfected with 4 μg of pCX4bsr/NS5B (1B-1) and 4 μg of pcDNA3-FLAG-ATMwt. The cell lysates were immunoprecipitated with both anti-FLAG and anti-ATM antibodies, followed by immunoblotting analysis using either anti-ATM or anti-HCV NS5B antibody. (G) Western Blot analysis was performed with anti-NS5B antibody, reusing the same blotted membrane that was used for panel B. (H to J) Chk2 bound to HCV NS5B. (H) 293FT cells were cotransfected with 4 μg of pcDNA3-HA-Chk2wt. The cell lysates of expressed HA-Chk2 were mixed with the O cell lysates and were immunoprecipitated with anti-HA antibody (3F10), followed by immunoblotting analysis using anti-HCV NS5B, anti-HCV NS5A (no. 8926), anti-HCV core protein (CP-9 and CP-11 mixture), or anti-HA (HA-7) antibody. The results of Western blot analysis of 1/10 of the cellular lysates with the same antibodies are also shown. (I) The lysates of O cells were immunoprecipitated with anti-NS5B or anti-Chk2 antibody (DCS-273), followed by immunoblotting analysis using anti-HCV NS5B antibody. The result of Western blot analysis of 1/10 of the cellular lysates with anti-NS5B antibody is also shown. (J) 293FT cells were cotransfected with 4 μg of pCX4bsr/NS5B (1B-1) and 4 μg of pcDNA3-HA-Chk2wt. The cell lysates were immunoprecipitated with anti-HA antibody (3F10), followed by immunoblotting analysis using either anti-HA (HA-7) or anti-HCV NS5B antibody. IP, immunoprecipitation; WB, Western blotting; IgG, immunoglobulin G.

O cells were immunoprecipitated with anti-ATM antibody, and then immunoblotting analysis using either anti-ATM or anti-NS5B antibody was performed. The results revealed that endogenous ATM bound to endogenous NS5B (Fig. 6E). Furthermore, we confirmed that ATM bound to NS5B in immunoprecipitation analysis using lysates from 293FT cells, in which NS5B (1B-1 strain) and FLAG-tagged ATM were overexpressed (Fig. 6F). Similarly, we confirmed that FLAG-tagged ATM bound to NS5B derived from O cell lysates in immunoprecipitation analysis using lysates from 293FT cells in which FLAG-tagged ATM was overexpressed (Fig. 6G). Finally, to determine which HCV protein binds to Chk2, the 293FT cell lysates of overexpressed HA-Chk2 were mixed with the O cell lysates and were immunoprecipitated with anti-HA antibody, followed by Western blot analysis using anti-HCV NS5B, anti-HCV NS5A, anti-HCV core protein, or anti-HA antibody. Consistent with the immunofluorescence result that Chk2 partially colocalized with NS5B (Fig. 5C), we observed that HA-tagged Chk2 bound to NS5B (Fig. 6H). Importantly, we found that endogenous Chk2 bound to endogenous NS5B derived from O cells (Fig. 6I). In addition, HA-tagged Chk2 bound to NS5B in immunoprecipitation analysis using lysates from 293FT cells in which NS5B (1B-1 strain) and HA-tagged Chk2 were overexpressed (Fig. 6J). Thus, Chk2 also interacted with NS5B as well as ATM. Taking these results together, we conclude that HCV targets ATM and Chk2 DNA damage sensors and that the ATM signaling pathway is required for HCV RNA replication.

DISCUSSION

ATM has been implicated as a target of most DNA viruses, harboring their genomes in the form of dsDNA which can activate or inhibit the ATM signaling pathway (17). In this study, we have demonstrated for the first time that the ATM signaling pathway is required for HCV RNA replication even though HCV does not have a dsDNA genome, unlike DNA viruses. In this regard, Machida et al. previously proposed that HCV infection and the expression of HCV NS3 and core protein induced dsDNA breaks (18, 19). Furthermore, NS3 has DNA helicase activity by which it unwinds dsDNA, suggesting that NS3 affects host dsDNA (22, 25). Thus, HCV infection might trigger the activation of ATM without a dsDNA genome. In fact, we observed weak but significant phosphorylation of Chk2 at threonine 68, the specific marker for ATM activation, in the HCV RNA-replicating cells (O and sO cells) but not in the HCV-negative Oc and sOc cells (Fig. 3A), suggesting that the ATM-dependent DNA damage response is constantly stimulated in persistent HCV RNA-replicating cells. Furthermore, we demonstrated that ATM preferentially bound to NS3-4A over NS3 alone (Fig. 5B) and that ATM partially colocalized with NS3-4A in the perinuclear region, where HCV is known to form a replication complex and replicate itself, and in dispersed points throughout the cytoplasm (Fig. 5A), indicating the interaction of ATM with NS3-4A. Interestingly, Lai et al. very recently reported that NS3-4A impaired DNA repair and enhanced sensitivity to ionizing radiation through interaction with ATM (15). However, we observed an equivalent level of Chk2 phosphorylation at threonine 68, a direct downstream target of ATM (20, 21), in both HCV RNA-replicating cells (O cells) and HCV-negative cells (Oc cells) after treatment with adriamycin (Fig. 3A), suggesting that Chk2 phosphorylation by ATM is not impaired by HCV RNA replication. In this regard, Gaspar and Shenk also showed that human cytomegalovirus could inhibit a DNA damage response by mislocalizing ATM and phosphorylated Chk2 at threonine 68 to a cytoplasmic virus assembly zone, indicating that human cytomegalovirus blocked at the level of Chk2 (9). On the other hand, dsDNA triggers IFN immune defenses through retinoic acid-induced gene I, the mitochondrial antiviral signaling protein, or the DNA-dependent activator of IFN-regulatory factor (7, 26); and NS3-4A protease, which is known to cleave the mitochondrial antiviral signaling protein, can block it (26), suggesting that interaction of NS3-4A with ATM is partially involved in such a common antiviral signaling pathway. On the other hand, we previously demonstrated that HCV NS5B-expressing PH5CH8 immortalized human hepatocyte cells were susceptible to DNA damage in the form of dsDNA breaks (23). In this regard, we have found that HCV NS5B could bind to both ATM and Chk2 (Fig. 5B and C and 6E to J). Together, these results indicate that HCV might hijack ATM and Chk2 and utilize ATM and Chk2 for HCV RNA replication, thereby resulting in impairment of DNA repair, enhancement of mutation frequency, and development of hepatocellular carcinoma.

Finally, consistent with our finding that ATM was required for HCV RNA replication, an ATM kinase inhibitor efficiently suppressed genome-length HCV RNA replication at an EC_{50} of approximately 2 μM at 72 h after the treatment (Fig. 4A). Similarly, Lau et al. reported that the same ATM kinase inhibitor could suppress HIV-1 replication at an EC_{50} of approximately 2.3 μM (16). Importantly, the EC_{50} for HIV-1 replication is similar to that for HCV replication. Thus, this or other ATM kinase inhibitors may represent a novel approach for the clinical treatment of patients with chronic hepatitis C as well as AIDS patients.

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Trans-encapsidation of hepatitis C virus subgenomic replicon RNA with viral structure proteins

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ABSTRACT

A trans-packaging system for hepatitis C virus (HCV) subgenomic replicon RNAs was developed. HCV subgenomic replicon was efficiently encapsidated by the HCV structural proteins that were stably expressed *in trans* under the control of a mammalian promoter. Infectious HCV-like particles (HCV-LPs), established a single-round infection, were produced and released into culture medium in titers of up to 10^3 focus forming units/ml. Expression of NS2 protein with structural proteins (core, E1, E2, and p7) was shown to be critical for the infectivity of HCV-LPs. Anti-CD81 treatment decreased the number of infected cells, suggesting that HCV-LPs infected cells in a CD81-dependent manner. The packaging cell line should be useful both for the production of single-round infectious HCV-LPs to elucidate the mechanisms of HCV assembly, particle formation and infection to host cells, and for the development of HCV replicon-based vaccines.

Hepatitis C virus (HCV) is a positive-strand RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family. The HCV genome comprises about 9600 nucleotides that encode a single polyprotein of around 3000 amino acids [1–3], which is processed by cellular and viral encoded proteases into at least 10 different structural and nonstructural proteins [4–6]. The JFH-1 strain of HCV, classified as genotype 2a strain, is the first HCV strain that can produce HCV particles in Huh7 cells [7,8]. The synthesis of HCV-like particles (HCV-LPs) using a recombinant baculovirus containing the cDNA of HCV structural proteins has been reported [9]. HCV-LP production by mammalian expression systems using vesicular stomatitis virus [10] and semliki forest virus [11] were also reported although the amount of VLP production is not as high as that of baculovirus system.

Subgenomic replicon system is a useful tool as gene expression vectors and is desirable for the development of vaccines. In the case of flaviviruses, several systems have been described for packaging flavivirus replicons, including Kunjin virus replicons [12–14], yellow fever virus replicons [15], tick-borne encephalitis virus replicons [16], and West Nile virus replicons [17,18]. In some cases, these packaging systems have utilized cell lines expressing the flavivirus structural proteins under the control of eukaryotic promoters [16,19]. These virus-like particle (VLP)-generating systems have been useful for packaging viral genomes encoding various foreign genes [14,15,20,18], the study of virus tropism and various aspects of viral assembly and entry [17].

Subgenomic replicons of JFH-1 replicate efficiently in Huh7 cells and do not require cell culture-adaptive mutations [21]. The construction of a system to package HCV replicon into HCV-LPs would not only be useful to investigate as-yet unclear steps of HCV life cycles such as genome packaging and virion assembly but also offers the possibilities of a new approach for vaccine development. In this study, we constructed subgenomic replicon cell lines constitutively expressing JFH-1 structural proteins under the control of elongation factor-1α (EF) promoter, and found stable expression of structural proteins and release of HCV-LPs from the cell line. A sucrose density gradient centrifugation of the culture medium resulted in partial purification of the HCV-LPs. Infectivity of HCV-LPs produced by this system was confirmed by colony formation assay and immunofluorescence analysis. Anti-CD81 antibody treatment decreased the infectivity of HCV-LPs, suggesting that VLPs infected to cells in CD81-dependent fashion. This is the first report that HCV structural proteins of HCV can trans-package its subgenomic replicon. The system described here should be useful to elucidate the mechanisms of HCV assembly, particle formation, and infection to host cells.

Materials and methods

Plasmid construction. Core to p7 coding region of JFH-1 was amplified using pJFH-1 [21] as a template and sense primer

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5′-GAGAATTCGTAGACCGTGCACCATG-3′ and antisense primer 5′-AAGAATTCTTAGGCATAAGCCTGCCGGGGCA-3′. Core to NS2 coding region of JFH-1 was amplified using pJFH-1 as a template and sense primer 5′-GAGAATTCGTAGACCGTGCACCATG-3′ and antisense primer 5′-AAGAATTCTTAAAGGAGCTTCCACCCCTTGG-3′. Amplified fragments were inserted into EcoRI site of pEF4 (Invitrogen) to generate pEFJFH/c-p7 and pEFJFHc-NS2, respectively.

Establishment of cell lines capable of packaging JFH-1 replicon RNA into VLPs. Huh7 cells were transfected using Lipofectamine (Invitrogen) with either pEFJFH/c-p7 or pEFJFHc-NS2 and were cultured with 0.2 mg/ml of zeocin (Invitrogen). Zeocin-resistant colonies were collected 3 weeks after transfection. The cell lines, Huh/c-p7 and Huh/c-NS2 (expressing pEFJFH/c-p7 and pEFJFHc-NS2, respectively) were then electroporated with 1 μg of JFH-1 subgenomic replicon (SGR-JFH1) RNA and were cultured with 0.375 mg/ml of G418 (Nacalai Tesque). Expression of core, E2 and NS5A was confirmed by Western blotting using anti-HCV core and anti-HCV E2 monoclonal antibodies [22] and anti-HCV NS5A polyclonal antibody [23]. The total RNA of culture media for each cell line (Huh/c-p7/SGR and Huh/p-NS2/SGR) was extracted using the QIAampViral RNA Mini spin column (Qiagen). Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems), as described previously [24,25]. The HCV core antigen in the culture media was measured by immunoassay (Ortho HCV-Core ELISA Kit; Ortho-Clinical Diagnotics), following the manufacturer's instructions. Culture medium was centrifuged at 8000g for 30 min to remove all cellular debris, after which the supernatant was concentrated to 1 ml by centrifugation using Amicon Ultracel 100k (Amicon). The concentrated medium was then layered on top of a continuous 10–60% (wt/vol) sucrose gradient in phosphate buffered saline (PBS) and then centrifuged at 40,000 rpm at 4 ℃ for 16 h (SW41E rotor, Beckman). Fractions (1 ml each) were collected from the top of the tube (12 fractions in total) and the density for each fraction was determined. The concentrations of replicon RNAs and core proteins of each fraction were measured as described above.

Infectivity of HCV-LPs. To determine whether these cell lines produced infectious HCV-LPs, we performed a colony formation assay using neomycin-resistant gene of SGR-JFH1 RNA. Naive Huh7 cells were infected with pooled fractions of 1.12–1.20 g/ml of both cell lines and were cultured for 3 weeks with G418 at 0.375 mg/ml. Formed colonies were stained with crystal violet and counted.

We also performed an immunofluorescence study in order to analyze the infectivity of the HCV-LPs. Following 3 days of incubation, the cells were fixed and immunostained for NS5A with anti-NS5A rabbit polyclonal antibody as described previously (Murakami et al., in press). Ffu (focus forming units) was calculated essentially based on the method as described previously [7,26]. Virus titration was performed by seeding Huh-7 cells in 96-well plates at 1×10^4 cells/well. Samples were serially diluted 5-fold in complete growth medium and used to infect the seeded cells (six wells per dilution). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI).

Results

Establishment of cell lines capable of packaging JFH-1 replicon RNA into VLPs

Stable cell lines expressing JFH-1 structural proteins were generated by transfecting with either pEFJFH/c-p7 or pEFJFHc-NS2. Zeocin-resistant colonies were collected 3 weeks after transfection and the expression of JFH-1 structural proteins was confirmed by Western blotting using anti-HCV core and anti-HCV E2 monoclonal antibodies [22] (Fig. 1A, lanes 1 and 2). The cell lines, Huh/c-p7 and Huh/c-NS2 (expressing pEFJFH/c-p7 and pEFJFHc-NS2, respectively) were then electroporated with 1 μg of SGR-JFH1 RNA. Six G418-resistant colonies were selected 3 weeks after electroporation and were termed Huh/c-p7/SGR (1-6) and Huh/c-NS2/SGR (1-6) cells. Expression of core, E2 and NS5A of Huh/c-p7/SGR-1, and Huh/c-NS2/SGR-3 was confirmed by Western blotting (Fig. 1A, lanes 3 and 4).

To investigate whether HCV-LPs were secreted from Huh/c-p7/SGR and Huh/c-NS2/SGR cells, we analyzed the culture medium of these cell lines 6 days postinfection. As shown in Fig. 1B, HCV replicon RNA and core protein were secreted from both cell lines. Fifty milliliters of culture medium from one Huh/c-NS2/SGR-1 and Huh/c-p7/SGR-3 cell line was concentrated, layered on top of a continuous 10–60% (wt/vol) sucrose gradient in PBS and then centrifuged at 40,000 rpm at 4 ℃ for 16 h. Fractions were collected from the top of the tube and the concentrations of replicon RNAs and core proteins of each fraction were measured. HCV RNA and core protein were predominantly detected in the 1.15–1.20 g/ml fractions, with a peak fraction of 1.16 g/ml fraction (Fig. 2A). HCV-LPs were

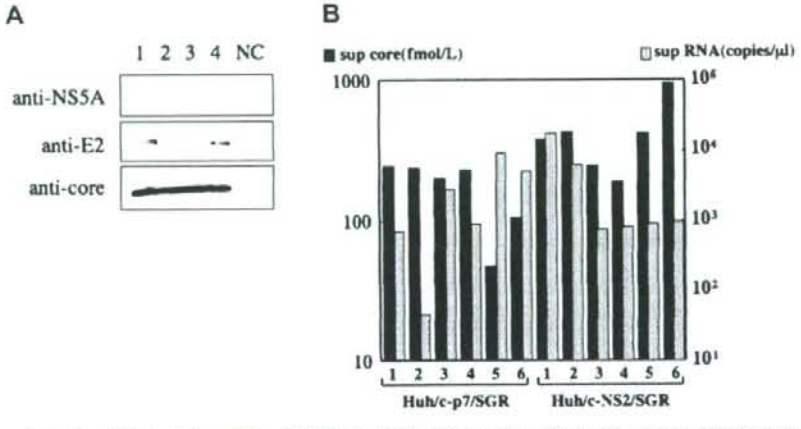

Fig. 1. (A) Western blot analysis of established cell lines. Huh/c-p7/SGR (1), Huh/c-NS2/SGR (2), Huh/c-p7/SGR-1 (3), and Huh/c-NS2/SGR-3 (4) cells were analyzed using anti-core, anti-E2, and anti-NS5A antibodies, respectively. Huh7 cells were used as a negative control. (B) Screening of G418-resistant cell lines. HCV replicon RNA and core protein of culture media of six colonies from Huh/c-p7/SGR or Huh/c-NS2/SGR cells were measured by real-time RT-PCR and ELISA, respectively. Black bars represented the concentration of core protein (fmol/l), dotted bars represented the concentration of replicon RNA (copies/μl).

observed by electron microscopy and these resembled previously reported particles (Fig. 2B)[27]. The secretion of HCV-LPs from these cell lines was maintained at almost the same level for more than 1 year (data not shown).

Infectivity of HCV-LPs

To determine whether these cell lines produced infectious HCV-LPs, we performed a colony formation assay using neomycin-resistant gene of SGR-JFH1 RNA. If HCV-LPs were infectious, SGR-JFH1 that was encapsidated in the particles would be introduced into infected cells, thus would confer neomycin resistance to the cells. To exclude the possibility that subgenomic replicon RNA in culture medium was captured by inoculated cells, Huh7 cells were also inoculated with concentrated culture medium of SGR-JFH1 cells. As shown in Fig. 3A, Huh7 cells infected with the fraction of Huh/c-NS2/SGR cells formed visible colonies 10–14 days after infection. Calculated colony forming units (cfu) of the culture medium of Huh/c-NS2/SGR cells were in the order of $5.54 \pm 2.92 \times 10^1$ cfu/ml similar to those of culture medium of JFH-1-infected cells [28]. The cells inoculated with concentrated medium of SGR-JFH1 cells formed no colonies (Fig. 3A). On the other hand, cells infected with Huh/c-p7/SGR formed no colonies, suggesting that NS2 protein was required for the infectivity of HCV-LPs. Infectivity of HCV-LPs from other cell lines of Huh/c-NS2/SGR, shown in Fig. 1, were also confirmed by colony formation assay, whereas HCV-LPs from other cell lines of Huh/c-p7/SGR showed no infectivity (data not shown).

In order to analyze the infectivity of the HCV-LPs, an immunofluorescence study was also performed. Huh7 cells infected with the Huh/c-NS2/SGR culture medium peak fraction (Fig. 2A) were positive for NS5A at 72 h postinfection (Fig. 3B), whereas the cells infected with the Huh/c-p7/SGR culture medium peak fraction were negative for NS5A (Fig. 3B), suggesting that the expression of NS2 protein in infected cells was critical for the infectivity of the HCV-LPs. The infectivity of the Huh/c-NS2/SGR culture medium was calculated to be $3.4 \pm 0.6 \times 10^2$ ffu/ml. The Cfu of this culture medium was determined to be approximately 16% of ffu, likely because only a portion of introduced replicon could render neomycin resistance to the infected cells. The cells infected with JFH-1 showed spread of infection 72 h postinfection. On the other hand, the cells infected with the Huh/c-NS2/SGR culture medium peak fraction showed very limited or no spread of infection (Fig. 3B). Moreover, no NS5A-positive cells were observed when we inoculated new Huh7 cells with the concentrated culture medium from Huh7 cells that were infected the Huh/c-NS2/SGR culture medium peak fraction (Fig. 3B, reinfection), suggesting that HCV-LPs produced by Huh/c-NS2/SGR cells supported only a single-round of infection.

We also measured the infectivity of the 12 sucrose density gradient fractions of the culture medium of Huh/c-NS2/SGR cells. The density of the peak of infectivity was lower than the peak densities of the core protein and replicon RNA (Fig. 2A), however this result agreed with a previous observation [29].

Neutralization of HCV-LPs infection by CD81-specific antibody

CD81 was shown to be involved in HCV entry. To determine whether HCV-LPs formed in Huh/c-NS2/SGR cells were infected in a CD81-dependent fashion, we incubated Huh7 cells with the peak fractions of Huh/c-NS2/SGR and Huh/c-p7/SGR cells in the presence of 10 μg/ml of CD81 specific monoclonal antibody or non-specific mouse antibody and cultured in the presence of 0.375 mg/ml of G418. After 3 weeks postinfection, colonies were fixed and the numbers of colonies were counted. CD81-specific antibody reduced the number of colonies from 132.3 ± 32.3 to 13.0 ± 11.5 ffu/

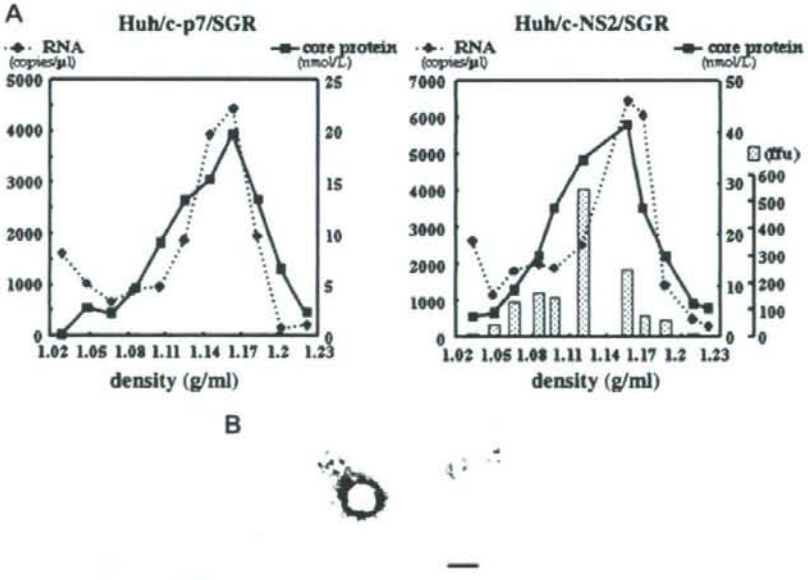

Fig. 2. (A) Sucrose density gradient analysis of culture supernatants of Huh/c-p7/SGR and Huh/c-NS2/SGR cells. Fifty milliliters of culture media collected from Huh/c-p7/SGR or Huh/c-NS2/SGR cells was concentrated to 1 ml and fractionated by ultracentrifugation at 40,000 rpm for 16 h by continuous 10–60% (wt/vol) sucrose gradient in PBS. Fractions (1 ml each) were collected from the top of the tube (12 fractions in total). HCV replicon RNA and core protein were measured by real-time RT-PCR and ELISA. The infectivity of each fraction of culture supernatant of Huh/c-NS2/SGR cells (right, lower panel) was determined by immunostaining of NS5A. (B) Electron microscopy analysis. Samples were prepared from the 1.12–1.20 g/ml fractions of culture media collected from Huh/c-NS2/SGR cells. Bar: 50 nm.

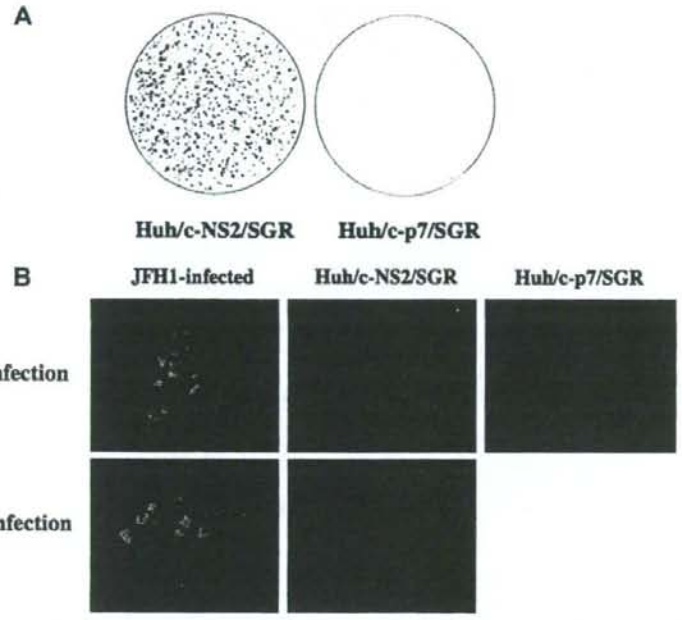

Fig. 3. (A) G418-resistant colony formation. Naive Huh7 cells were infected with 1.12–1.20 g/ml fractions of either Huh/c-p7/SGR or Huh/c-NS2/SGR cells and were cultured for 3 weeks with G418 at 0.375 mg/ml working concentration before staining with crystal violet. Experiments were performed in triplicate, and representative staining examples are shown. (B) Immunostaining experiments. Samples were serially diluted 5-fold in complete growth medium and used to infect the seeded cells (six wells per dilution). Huh7 cells in 96-well plates infected with the peak fraction of culture medium. Three days postinfection, infected cells were fixed, permeabilized with 0.3% Triton X-100 in Block Ace (Yukijirushi) and stained with anti-NS5A rabbit polyclonal antibody and Alexa488-conjugated goat anti-rabbit IgG as described previously (Murakami et al., in press). NS5A protein was shown in green. Nuclei were labeled with DAPI and were shown in blue. Re-infection shows the immunostaining of naive Huh7 cells infected with either culture media of JFH1-infected cells or that of Huh/c-NS2/SGR cells.

ml (Fig. 4), confirming that the infection of HCV-LPs to target cells is CD81-dependent and an important role of CD81 in HCV entry.

Discussion

Here we describe the development of cell lines selected to persistently harbor noncytopathic subgenomic replicons of HCV encoding neomycin resistant gene and the HCV core to NS2 cassette. The HCV-LPs secreted by this cell line are not proliferative and exhibit morphological, biophysical and antigenic properties similar to those of the putative HCV virions [27]. Jeong et al. suggested that HCV-LP is a potent immunogen for the induction of HCV-specific humoral and cellular immune responses by using baboon as a primate model [30]. Recently, replicon-based vectors of positive-stranded RNA viruses were recognized as a desirable choice of highly efficient and safe vaccines. Recent comparative analyses of vaccine potential of Kunjin virus replicons delivered as plasmid DNA, as naked RNA, and as VLPs showed a significantly better induction of immune responses to an encoded immunogen after VLP delivery than with other delivery modalities [31]. These studies suggested that HCV-LPs encapsidating its subgenomic replicon RNA are an attractive candidate for a hepatitis C vaccine. We are now constructing cell lines that secrete HCV-LPs of genotype 1a and 1b strains with this trans-packaging system and analyzing the HCV-LPs infectivity. We also showed that the expression of NS2 region is essential for infectious

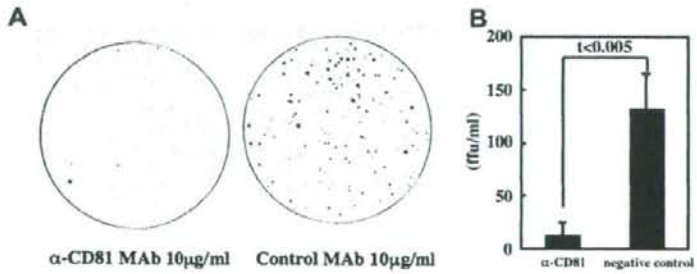

Fig. 4. Neutralization of HCV-LPs infection by CD81-specific antibody. Naive Huh7 cells were infected with peak fraction of either Huh/c-p7/SGR or Huh/c-NS2/SGR cells in the presence of 10 μg/ml of CD81 specific monoclonal antibody or nonspecific mouse antibody, then cultured 3 weeks with 0.375 mg/ml of G418. Colonies were stained with crystal violet and colony numbers were counted. (A) Colony formation. Experiments were performed in triplicate, and representative staining examples are shown. (B) Cfu of culture media per 1 ml was calculated and means ± SD was shown.

HCV-LPs production. NS2 is dispensable for RNA replication, since subgenomic replicons that lack the entire core to NS2 coding region replicate autonomously. The HCV NS2/3 protein is a highly hydrophobic protease responsible for the cleavage of the viral polypeptide between nonstructural proteins NS2 and NS3. However, many aspects of the NS2/3 protease's role in the viral life cycle and mechanism of action remain unknown. By using intergenotypic chimeras, Pietschmann et al. showed that NS2 plays an important role in the HCV morphogenesis by interacting with other NS proteins during the process of virion assembly [32]. Jones et al. reported that NS2 was required for infectious virus production and acts early in virion morphogenesis prior to the accumulation of infectious intracellular virus and indicated that the NS2 protease domain may form important interactions with other NS proteins during the process of virion assembly [33]. The results presented here also showed the importance of NS2 protein expression for the production of infectious particles, coincided with these previous observations. The mechanism NS2 plays in the process of virion morphogenesis is still unclear and remains to be determined.

In summary, we have generated a stable packaging cell line allowing production of large amounts of HCV-LPs in which the subgenomic replicon was encapsidated. The packaging cell line proved to be useful both for the production of HCV-LPs and for the encapsidation of HCV replicons for a single-round of infection.

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Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice

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The establishment of clonal infection of hepatitis C virus (HCV) in a small-animal model is important for the analysis of HCV virology. A previous study developed models of molecularly cloned genotype 1a and 2a HCV infection using human hepatocyte-transplanted chimeric mice. This study developed a new model of molecularly cloned genotype 1b HCV infection. A full-length genotype 1b HCV genome, HCV-KT9, was cloned from a serum sample from a patient with severe acute hepatitis. The chimeric mice were inoculated intrahepatically with *in vitro*-transcribed HCV-KT9 RNA. Inoculated mice developed viraemia at 2 weeks post-infection, and this persisted for more than 6 weeks. Passage experiments indicated that the sera of these mice contained infectious HCV. Interestingly, a similar clone, HCV-KT1, in which the poly(U/UC) tract was 29 nt shorter than in HCV-KT9, showed poorer *in vivo* infectivity and replication ability. An *in vitro* study showed that no virus was produced in the culture medium from HCV-KT9-transfected cells. In conclusion, this study developed a genetically engineered genotype 1b HCV-infected mouse. This mouse model will be useful for the study of HCV virology, particularly the mechanism underlying the variable resistance of HCV genotypes to interferon therapy.

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INTRODUCTION

Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus, infects and replicates efficiently only in the hepatocytes of humans and chimpanzees. There are many genotypes of HCV distributed worldwide (Simmonds *et al.*, 1993); among them genotype 1b is the major genotype in Asia, including Japan, and is known to be one of the most resistant genotypes to interferon (IFN) therapy (Fried *et al.*, 2002). Until recently, studies of HCV replication have long been hampered by the lack of a virus culture system. The development of HCV replicon systems has allowed the

The GenBank/EMBL/DDBJ accession numbers for the sequences of HCV-KT9 and HCV-KT1 determined in this work are AB435162 and AB426117, respectively.

study of the mechanisms of replication of HCV (Lohmann *et al.*, 1999). However, these replicons lack structural proteins, do not replicate efficiently without adaptive mutations and do not produce infectious virions. Recently, it was reported that the genotype 2a full-length JFH-1 genome replicated efficiently in Huh7 cells without adaptive mutations and produced virions that were infectious for both naïve cells and chimpanzees, as well as for a human hepatocyte-transplanted chimeric mouse (Wakita *et al.*, 2005; Zhong *et al.*, 2005; Lindenbach *et al.*, 2006). To date, five full-length genotype 1b clones, HCV-N (Beard *et al.*, 1999), Con-1 (Bukh *et al.*, 2002), HCV-J4 (Okamoto *et al.*, 1992), HCV-CG1b (Thomson *et al.*, 2001) and HCV-BK (Takamizawa *et al.*, 1991), have been demonstrated to be infectious by intrahepatic inoculation of transcribed HCV RNA into the liver of chimpanzees. Among these, only the HCV-CG1b genome is reported to produce HCV particles when transfected into Huh7 cells (Heller *et al.*, 2005).

Although the chimpanzee is a useful animal model for the study of HCV infection, there are ethical restrictions on the use of this animal. Instead, Mercer *et al.* (2001) developed a useful small-animal model for the study of HCV infection using chimeric urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) mice (which are immunodeficient and undergo liver failure) with engrafted human hepatocytes. This HCV-infected mouse model is reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (Kneteman *et al.*, 2006). We have previously described methods to improve the replacement levels of human hepatocytes in this mouse model (Tateno *et al.*, 2004) and we have developed a reverse genetics system for hepatitis B virus (Tsuge *et al.*, 2005) and HCV (Hiraga *et al.*, 2007). In the present study, we report the establishment of an infectious genotype 1b HCV clone that infects and replicates efficiently in human hepatocyte chimeric mice.

METHODS

Cloning of infectious genotype 1b HCV isolate. Serum samples were obtained from a 43-year-old physician who developed severe acute hepatitis after needle stick exposure from a patient with chronic hepatitis C. On admission, the serum total bilirubin concentration was 10.0 mg dl^{-1} and the prothrombin time was 40 %. The patient tested positive for HCV antibodies by a third-generation radioimmunoassay (Ortho-Clinical Diagnostics) and for HCV RNA by RT-PCR. Serum HCV RNA was quantified using an Amplicor Monitor HCV test (Roche Diagnostics). The HCV RNA titre was 2.5×10^6 copies ml^{-1} on admission and then decreased gradually. Fig. 1 shows the serial changes in alanine aminotransferase (ALT) as a measure of liver function and HCV RNA levels in this patient. Serum samples obtained in the early phase of infection were used for cloning the full-length genome.

RNA extraction, cDNA synthesis, plasmid construction and RNA transcription. Total RNA was extracted from 100 µl serum samples using SepaGene RV-R (Sanko Junyaku) and reverse transcribed with random hexamers and ReverTra Ace reverse transcriptase (Toyobo) according to the manufacturer's instructions. PCR primers were designed based on the sequence of HCV-Con1 (GenBank accession no. AJ238799; Bukh *et al.*, 2002). Five overlapping cDNA segments (nt 1–2292, 2269–6715, 6696–9094, 7564–9404 and 9361–9605; nucleotide numbers are those of HCV-Con1) were amplified by PCR with TaKaRa LA *Taq* polymerase (Takara Biochemicals) using the above cDNA. Amplified products were separated by agarose gel electrophoresis. Nucleotide sequences were determined using a Big Dye Terminator Mix Cycle Sequencing kit (Applied Biosystems Japan) with an automated DNA sequencer (model 310; PE Biosystems). We corrected the nucleotide sequences of the obtained clones by site-directed mutagenesis and made them identical to the nucleotide sequences obtained by direct sequencing. Naturally occurring restriction enzyme cutting sites were utilized to clone each segment. We utilized the vector pBR322 and created a multiple-cloning site under the control of the T7 promoter by ligating a linker at restriction enzyme cutting sites as they appeared in order from 5′ to 3′ in the HCV sequences (Fig. 2a). Each segment of HCV was cloned into this vector to generate the full-length clones. The HCV-KT9 clone was established using the 3′-terminal fragment with the longest poly(U/UC) tract length (115 nt), which should have a high replication ability (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). A clone with a shorter poly(U/UC) tract length (86 nt), HCV-KT1, was also generated. A polymerase-deficient mutant with an amino acid substitution in the GDD motif (GDD→GND; HCV-KT9-GND) was generated using a Quick Change Site-Directed Mutagenesis kit (Stratagene). After digesting the plasmid with *Xba*I (New England BioLabs) at the 3′ end of the HCV cDNA, HCV RNA was transcribed using T7 RNA polymerase (MEGAscript; Ambion) at 37 °C for 3 h in a 100 µl reaction mixture, according to the manufacturer's instructions. The RNA was analysed using denaturing agarose gel electrophoresis and kept at −80 °C until use.

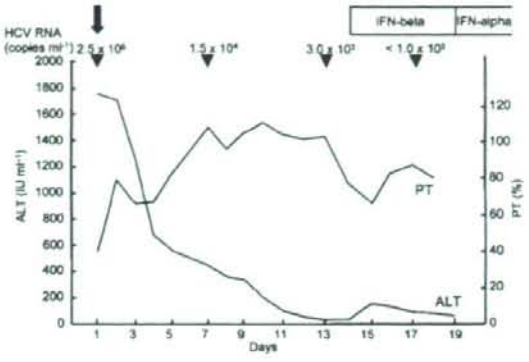

Fig. 1. Clinical course of a patient with severe acute hepatitis C. Alanine aminotransferase (ALT) and prothrombin time (PT) are shown from the day of admission (day 1). The patient was treated daily with 10^6 U IFN-β intravenously for 5 days, followed by 10^6 U IFN-α intramuscularly three times a week for 6 months. HCV RNA was measured on days 1, 7, 13 and 17 (arrowheads). A serum sample was taken on day 1 (arrow) and used to clone the full-length HCV genome.

Construction of a phylogenetic tree. A phylogenetic tree was constructed based on the entire nucleotide sequences of 26 full-length genotype 1b clones plus HCV-KT9. The total number of synonymous and non-synonymous substitutions among the nucleotide sequences was estimated using the method of Gojobori *et al.* (1982) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

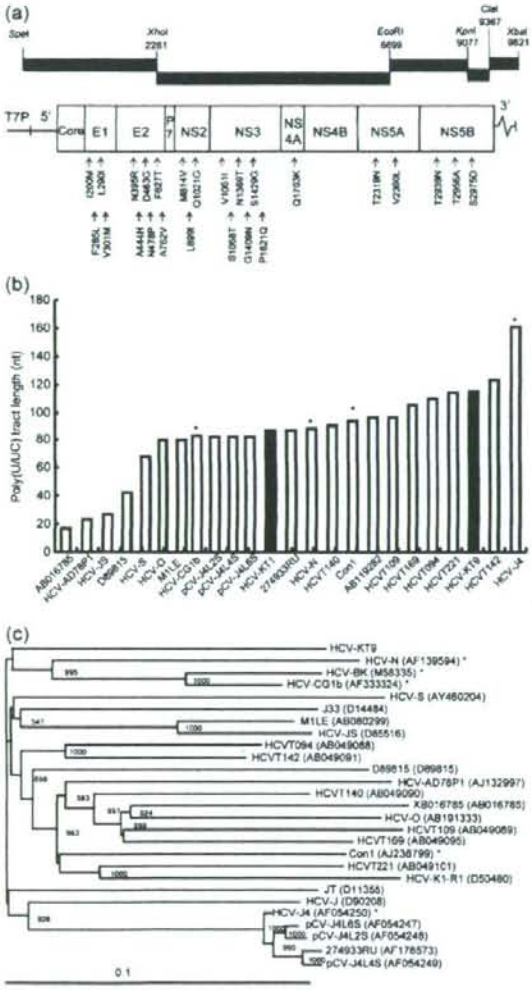

Fig. 2. (a) Schematic diagram of the organization of the cDNA clone HCV-TK9. The T7 RNA promoter (T7P) is located immediately upstream of the HCV genome. Restriction enzyme sites that were used to create clone HCV-KT9 are labelled according to their nucleotide position within the HCV sequence. Amino acid sequences unique to HCV-KT9 compared with 26 other HCV genotype 1b isolates are indicated at the bottom of the figure, with the position of the repaired amino acid residues noted within the polyprotein. (b) Length of the poly(U/UC) tracts of HCV-KT1, HCV-KT9 and 22 other HCV genotype 1b clones reported previously. Asterisks indicate clones confirmed to be infectious by experiments using chimpanzees. (c) Phylogenetic tree constructed with HCV-KT9 and 26 genotype 1b HCV whole-genome sequences. Bar, number of nucleotide substitutions per site. Asterisks indicate clones confirmed to be infectious in experiments using chimpanzees.

Intrahepatic injection experiments in human hepatocyte chimeric mice. We used methods described previously (Tateno *et al.*, 2004) to generate uPA$^{+/+}$/SCID$^{+/+}$ mice and transplant human hepatocytes. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index and were measured as described previously (Tateno *et al.*, 2004). Intrahepatic injection of RNA, extraction of serum samples and euthanasia were performed under ether anaesthesia. Briefly, 500 µl RNA solution containing 30 µg transcribed HCV RNA was injected into the liver of anaesthetized chimeric mice through a small abdominal incision. RNA extraction from mouse serum samples, quantification of HCV RNA and nested PCR were performed as described previously (Hiraga *et al.*, 2007). All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments and under the approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture, RNA transfection and measurement of HCV core antigen. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10 % fetal calf serum. RNA transfection and measurement of HCV core antigen in the culture medium were performed as described previously (Wakita *et al.*, 2005).

Statistical analysis. The infectious ratio of chimeric mice was compared and the differences assessed using a χ^2 test. Differences in HCV RNA replication ability *in vitro* were analysed statistically by one-way analysis of variance followed by Scheffe's test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of genotype 1b clones HCV-KT9 and HCV-KT1

The entire genome of HCV cDNA was assembled from five DNA fragments (Fig. 2a). We obtained 24 3′-extremity clones with different poly(U/UC) tract lengths. We selected the clone with the longest (U/UC) tract because a previous study indicated that the length of poly(U/UC) tract correlates with HCV replication in an HCV replicon system (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). The length of the poly(U/UC) tract in the longest 3′ clone was 115 nt. The entire genome length of the HCV-KT9 clone using this longest 3′ clone was 9621 nt. We also generated the clone HCV-KT1 with a shorter (86 nt) poly (U/UC) tract to compare the replication abilities of these clones. The lengths of the poly(U/UC) tracts of 22 clones deposited in GenBank are shown in Fig. 2(b). All infectious clones had a poly(U/UC) tract longer than 80 nt. Fig. 2(c) shows a phylogenetic tree constructed using the nucleotide sequences of the 26 full-length genotype 1b clones published to date. Interestingly, the sequence of HCV-KT9 was closest to that of HCV-CG1b (GenBank accession no. AF333324), which has been reported to be infectious, and formed a cluster with two other infectious clones, HCV-N (Beard *et al.*, 1999) and HCV-BK (Takamizawa *et al.*, 1991). We compared the amino acid