

件名: Re: request for plasmid JFH1AAA99

送信日時: 2009年 3月 28日 土曜日 4:26 PM

差出人: Thomas Baumert <Thomas.Baumert@viro-ulp.u-strasbg.fr>

宛先: "kunitada.shimotono@it-chiba.ac.jp" <kunitada.shimotono@it-chiba.ac.jp>

Dear Kunitada:

I thank you again for your visit to our laboratory last year. It was wonderful to meet you.

Today I am approaching you regarding construct pJFH1AAA99 (impaired virus production phenotype) which has been described in your landmark paper Miyanari et al. Nat. Cell Biol. 2007.

Would it be possible to obtain an aliquot of this plasmid to study the mechanisms of viral assembly. We would acknowledge the gift of the plasmid in any way you prefer including co-authorship of our future publication.

As you know from a previous transfer, we have signed already a MTA with Dr. Takaji Wakita covering the use of HCV-JFH1. A copy of this MTA has been faxed to you in 2007 (if needed we can fax it again).

We would greatly appreciate your support.

Looking forward to see you at the next HCV meeting in Nice France (I am part of the local organizing committee).

Best wishes and kindest regards,

Thomas

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## The DNA Damage Sensors Ataxia-Telangiectasia Mutated Kinase and Checkpoint Kinase 2 Are Required for Hepatitis C Virus RNA Replication<sup>†</sup>

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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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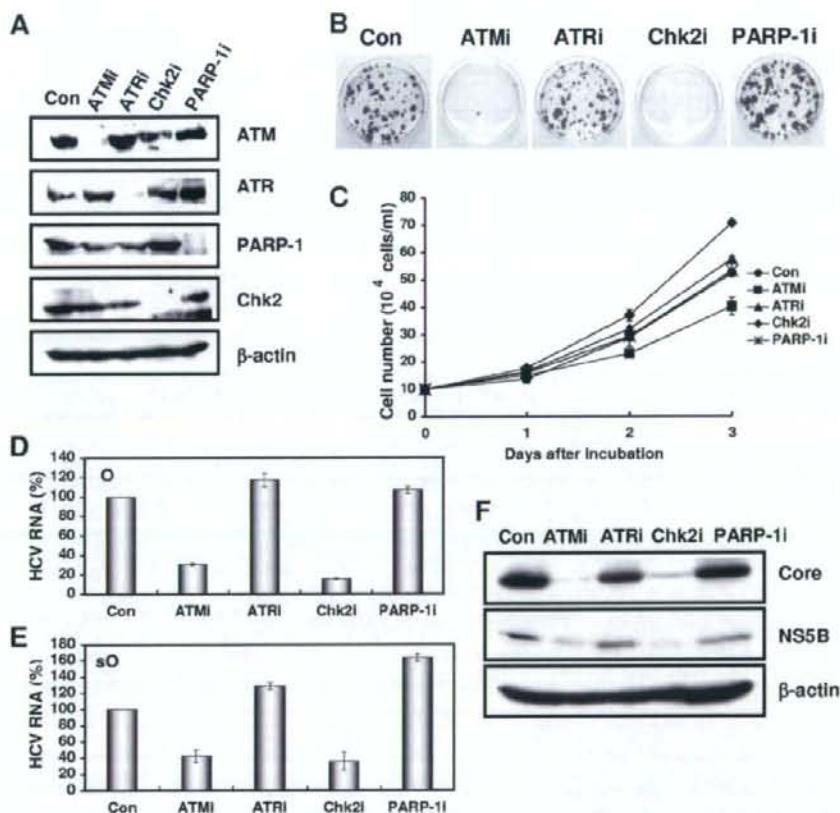


FIG. 1. The ATM signaling pathway is required for HCV RNA replication. (A) Inhibition of ATM, ATR, Chk2, or PARP-1 expression by shRNA-producing lentiviral vectors. The results of the Western blot analysis of cellular lysates with anti-ATM, anti-ATR, anti-Chk2, anti-PARP-1, or anti- $\beta$ -actin antibody in Oe cells expressing shRNA targeted to ATM (ATMi), ATR (ATRI), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in Oe cells transfected with a control lentiviral vector (Con) are shown. (B) ECF in ATM-, ATR-, Chk2-, or PARP-1-knockdown cells. In vitro transcribed ONC-5B K1609E RNA (2  $\mu$ g) was transfected into the ATM-, ATR-, Chk2-, or PARP-1-knockdown Oe cells or the Oe cells transfected 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 Oe cells or the Oe cells transfected 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- $\beta$ -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 transfected with a control lentiviral vector (Con) are shown.

quences targeted to Chk2 in lentiviral vector: 5'-GATCCCGGGGAGAGCTGTTTGACATTCAGAGATGTCACACAGCTCTCCCTTTTGGAAA-3' (sense) and 5'-AGCTTTTCCAAAAGGGGGAGAGCTGTTTGACATTCCTTGAATGTCACACAGCTCTCCCGGGG-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-Sall fragments of the pSUPER-Chk2i were subcloned into the BamHI-Sall site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transfected 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- $\Delta$ R8.91 (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 (GTX70153; GeneTex), anti-Chk2 (NT; ProSci, Poway, CA), anti-Chk2 (DCS-273; Medical and Biological Laboratories, Nagoya, Japan), anti-phospho-Chk2 (Thr68) (Cell Signaling,

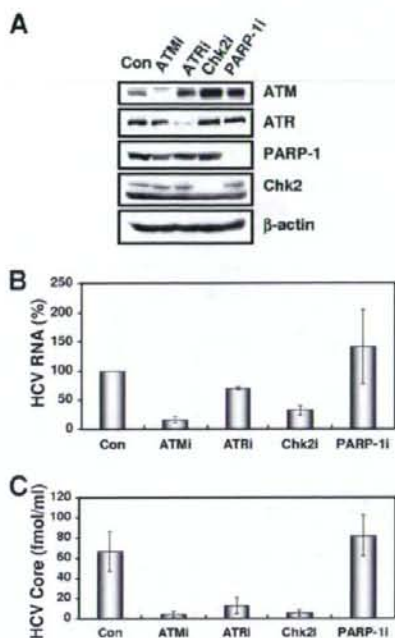
Danvers, MA), anti-PARP-1 (C-2-10; Calbiochem, Merck Biosciences, Darmstadt, Germany), anti-hemagglutinin (HA) (HA-7; Sigma, St. Louis, MO), anti-core protein (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan), anti-NS3 and anti-NS5B (no. 14; a generous gift from M. Kohara, the Tokyo Metropolitan Institute of Medical Science, Japan), anti-NSSA (no. 8926; a generous gift from A. Takamizawa, The Research Foundation for Microbial Diseases of Osaka University, Japan), and anti- $\beta$ -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  $\mu$ l of protein G-Sepharose (GE Healthcare Biosciences, Uppsala, Sweden). Precleared supernatants were incubated with 5  $\mu$ g of anti-HA antibody (3F10; Roche), 10  $\mu$ l of anti-NS5B antibody, 5  $\mu$ g of anti-Chk2 antibody (DCS-273; MBL), 5  $\mu$ g of anti-FLAG antibody (M2; Sigma), or 5  $\mu$ g of anti-ATM antibody (2C1) (GTX70103; GeneTex) at 4°C for 1 h. Following absorption of the precipitates on 30  $\mu$ l of protein G-Sepharose resin for 1 h, the resin was washed four times with 700  $\mu$ l of lysis buffer. Proteins were eluted by boiling the resin for 5 min in 2 $\times$  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-NSSA, 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- $\beta$ -actin antibody in RSc cells expressing shRNA targeted to ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in RSc cells transfected 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



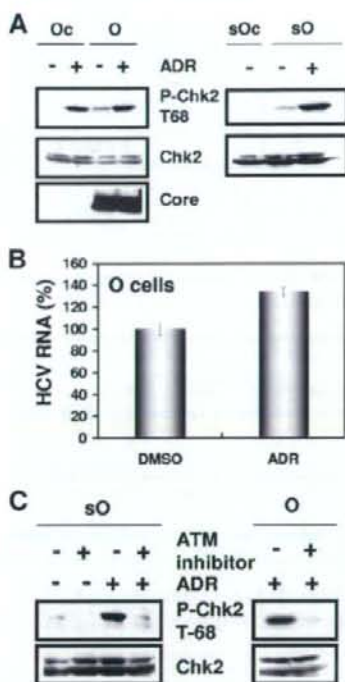


FIG. 3. ATM-dependent DNA damage response in HCV RNA-replicating cells. (A) Stimulation of Chk2 phosphorylation in the HCV RNA-replicating cells. The Oc, O, or sO cells were treated with 100 nM adriamycin (Sigma) for 2 h. The results of Western blot analysis of cellular lysates with anti-phospho-Chk2 (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  $\mu$ M 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. 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

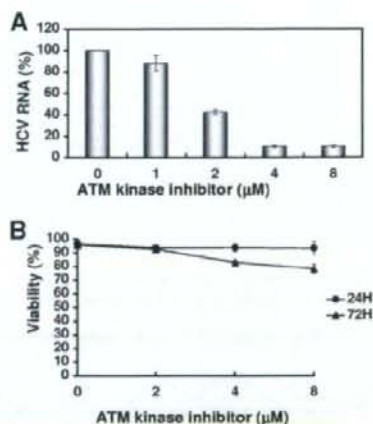
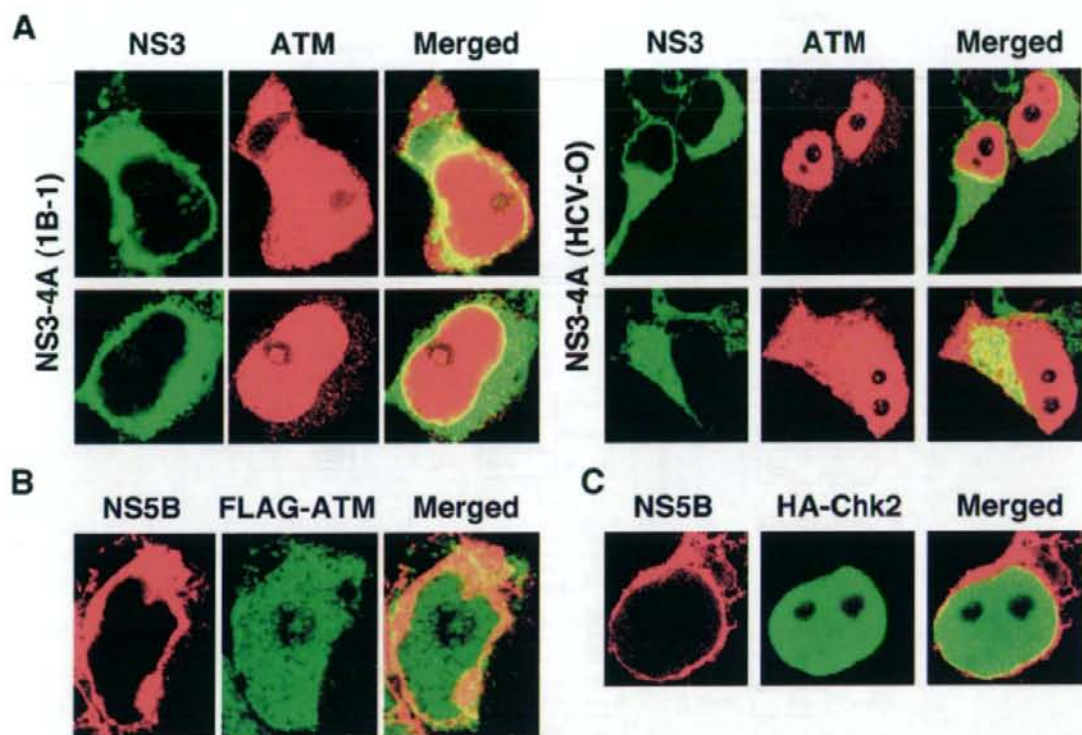


FIG. 4. Suppression of HCV RNA replication by ATM kinase inhibitor. (A) The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR after treatment with the indicated concentration of ATM kinase inhibitor for 72 h. Results from three independent experiments are shown as described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

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  $\mu$ M 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.

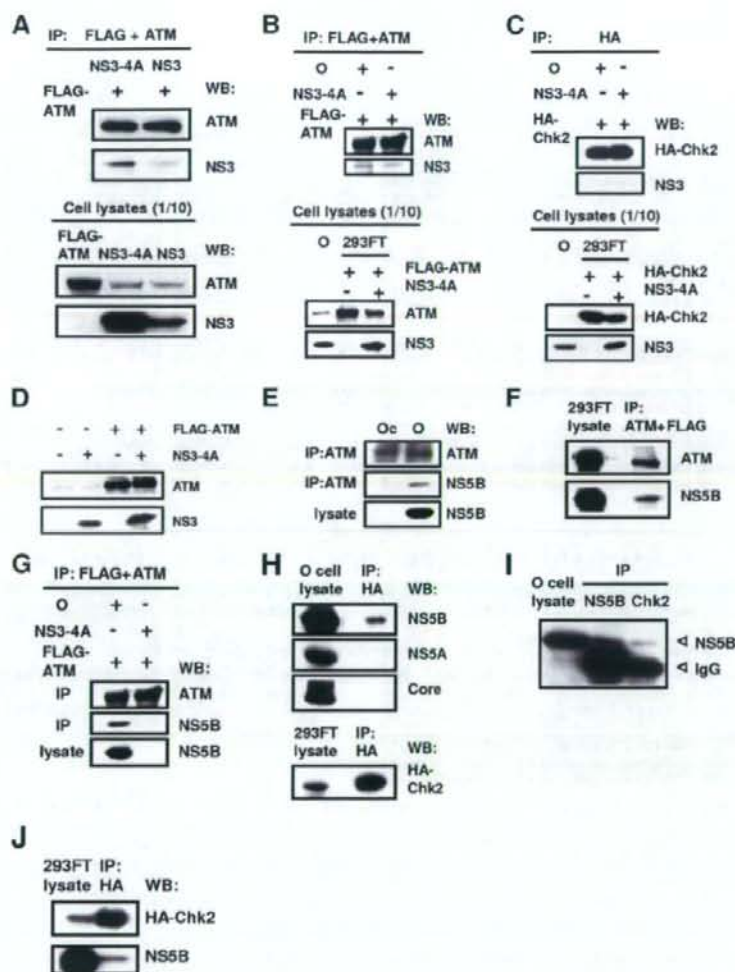
useful for the clinical treatment of patients with chronic hepatitis C.

**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  $\mu$ g of pCX4bsr/NS3-4A (O), 4  $\mu$ g of pCX4bsr/NS3 (O), or 4  $\mu$ g 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  $\mu$ g of pcDNA3-FLAG-ATMwt and/or 4  $\mu$ g 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  $\mu$ g of pcDNA3-HA-Chk2wt and/or 4  $\mu$ g 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  $\mu$ g of pCX4bsr/NS3-4A (O) and/or 4  $\mu$ g 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  $\mu$ g of pCX4bsr/NS5B (1B-1) and 4  $\mu$ g 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  $\mu$ g 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  $\mu$ g of pCX4bsr/NS5B (1B-1) and 4  $\mu$ g 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  $\mu$ M 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  $\mu$ M (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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## A new living cell-based assay system for monitoring genome-length hepatitis C virus RNA replication

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### ABSTRACT

We previously developed a cell-based luciferase reporter assay system for monitoring genome-length hepatitis C virus (HCV) RNA replication (OR6 assay system). Here, we aimed to develop a new living cell-based reporter assay system using enhanced green fluorescent protein (EGFP). Genome-length HCV RNAs encoding EGFP were introduced into a subline of HuH-7 cells and G418 selection was performed. One cloned cell line, OGF7, was successfully selected from among the several G418-resistant cell lines obtained, and the robust expression of HCV RNA and proteins in OGF7 cells was confirmed. The fluorescent intensity of OGF7 cells was decreased by interferon- $\alpha$  treatment in a dose-dependent manner, and it correlated well with the HCV RNA concentration. We demonstrated that the interferon- $\alpha$  sensitivity in the OGF7 assay system measuring the fluorescent intensity was equivalent to that of the OR6 assay system, and that the OGF7 assay system was useful for quantitative evaluation of anti-HCV reagents. The OGF7 assay system is expected to be the most time-saving and inexpensive assay system for high-throughput screening of anti-HCV reagents.

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### 1. Introduction

Persistent hepatitis C virus (HCV) infection frequently causes active liver disease in the form of chronic hepatitis (Choo et al., 1989; Kuo et al., 1989), liver cirrhosis, and hepatocellular carcinoma (Ohkoshi et al., 1990; Saito et al., 1990). HCV infection has now become a serious health problem, with at least 170 million people currently infected worldwide (Thomas, 2000). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae* (Kato et al., 1990; Tanaka et al., 1995). The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and virally encoded proteases (Hijikata et al., 1991, 1993; Kato, 2001). NS5B possessing an RNA-dependent RNA polymerase (RdRp) activity is the central enzyme in replication of the HCV genome (Kato, 2001).

In the recent past, interferon (IFN) was used as the main treatment for patients with chronic hepatitis C. Currently, the com-

bination of pegylated-IFN (PEG-IFN) and ribavirin is the standard therapy worldwide, although only 50% of patients show a sustained virological response to this therapy (Hayashi and Takehara, 2006). Several clinical drugs have been proposed as adjuvants to IFN, including cyclosporine A (CsA) (Wataashi et al., 2003), mizoribine (Naka et al., 2005), and statins (Ikeda et al., 2006; Ye et al., 2003). Currently, NS3 proteinase/helicase activity and NS5B RdRp activity have been considered as targets for the development of anti-HCV reagents (e.g., the NS3 protease inhibitor BILN 2061 (Lamarre et al., 2003)). To date, however, we have not obtained HCV-specific drugs possessing more effective anti-HCV activity than PEG-IFN. Therefore, a more convenient high-throughput screening system is still required to explore more effective anti-HCV reagents.

We previously developed a cell-based genome-length HCV RNA replication system using *Renilla* luciferase as a reporter in order to monitor the HCV RNA replication level (OR6 assay system) (Ikeda et al., 2005; Naka et al., 2005). Other groups have also developed cell-based subgenomic HCV replicon systems using secreted alkaline phosphatase (Yi et al., 2002) or beta-lactamase (Murray et al., 2003) as a reporter. However, these assay systems are still quite time- and cost-intensive methods for measuring enzyme activity.

In the present study, we report a new living cell-based reporter assay system that is able to monitor the level of genome-length HCV RNA replication and to reduce both the time required and the expense.

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## 2. Materials and methods

### 2.1. Reagents

IFN- $\alpha$ , IFN- $\gamma$ , and CsA were purchased from Sigma–Aldrich (St. Louis, MO). IFN- $\beta$  was a gift from Toray Industries (Tokyo, Japan). Fluvastatin (FLV) was purchased from Calbiochem (San Diego, CA).

### 2.2. Cell culture

Genome-length HCV RNA replicating cells and OR6c cells were maintained as described previously (Ikeda et al., 2005). OR6c cells are cured OR6 cells (Naka et al., 2005) from which genome-length HCV RNA was eliminated by IFN- $\alpha$  treatment as described previously (Ikeda et al., 2005).

### 2.3. Construction of plasmids and RNA synthesis

The plasmids used in this study (Fig. 1A and B) were constructed on the basis of the plasmid pON/C-5B/KE (Ikeda et al., 2005). The plasmid pON/C-5B/KE contains neomycin phosphotransferase (Neo<sup>R</sup>) downstream of HCV internal ribosome entry site (IRES) and the full-length HCV-O polyprotein-coding sequence downstream of the encephalomyocarditis virus (EMCV) IRES, and K1609E mutation (Ikeda et al., 2005), was introduced into the NS3 helicase region as the adaptive mutation. The plasmid pOGN/C-5B/KE (Fig. 1A(1)) was constructed from the plasmid pON/C-5B/KE by inserting the PCR product of enhanced green fluorescent protein (EGFP; Clontech Laboratories, Inc., Mountain View, CA) into the *AscI* recognition site of the 5'-end of the Neo<sup>R</sup> gene. The plasmids pON/GC-5B/KE (Fig. 1A(2)) and pON/C-5B G2390/KE (Fig. 1A(3)) were constructed from the plasmid pON/C-5B/KE by inserting the PCR product of EGFP into the *XhoI* recognition site of the 5'-end of the core-coding sequence and at aa position 2390 (Moradpour et al., 2004) in the NS5A-coding sequence, respectively. Both recognition sites were introduced by PCR mutagenesis with primers containing these recognition sites according to the previously described method (Dansako et al., 2005). To construct the plasmids pOGN/C-5B G2390/KE (Fig. 1B(4)) and pON/GC-5B G2390/KE (Fig. 1B(6)), the *EcoRI*-*SpeI* fragments of the plasmids pOGN/C-5B/KE and pON/GC-5B/KE, respectively, were replaced with the *EcoRI*-*SpeI* region of the plasmid pON/C-5B G2390/KE. The *EcoRI* recognition site is located at the 5'-end of HCV IRES, and the *SpeI* recognition site is located at the 5'-end of the NS3 region within the plasmid pON/C-5B/KE, respectively. To construct the plasmids pOGN/GC-5B/KE (Fig. 1B(5)) and pOGN/GC-5B G2390/KE (Fig. 1B(7)), the *EcoRI*-*RsrII* fragment of the plasmid pOGN/C-5B/KE was replaced with the *EcoRI*-*RsrII* region of the plasmids pON/GC-5B/KE and pON/GC-5B G2390/KE, respectively. The *RsrII* recognition site is located in the 3'-end of the Neo<sup>R</sup> region within the plasmid pON/C-5B/KE. The obtained plasmids were linearized by *XbaI* and were used for RNA synthesis with T7 MEGAscript (Ambion, Austin, TX) as previously described (Kato et al., 2003).

### 2.4. RNA transfection and selection of G418-resistant cells

The transfection of genome-length HCV RNA synthesized *in vitro* into OR6c cells was performed by electroporation, and the cells were selected in the presence of G418 (0.3 mg/ml; Invitrogen) for 3 weeks as described previously (Kato et al., 2003).

### 2.5. Visualization of the fluorescence by EGFP

The fluorescence of EGFP was directly visualized by a fluorescence microscope (Axiovert 25CFL; Carl Zeiss) or a confocal

laser-scanning microscope (LSM510; Carl Zeiss). The cells were fixed with 4% paraformaldehyde and were photographed under a fluorescence microscope or a confocal laser-scanning microscope as described previously (Dansako et al., 2003).

### 2.6. Integration analysis

Genomic DNA was extracted from the cultured cells by using a DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). The HCV 5'-untranslated region (UTR) and the IFN- $\beta$  gene were detected according to a method described previously (Kato et al., 2003). To test the efficiency of the PCR analysis and the quality of the genomic DNAs, a set of primers was used for the PCR detection of an intronless IFN- $\beta$  gene (1 copy per haploid genome; the expected PCR product is 341 bp).

### 2.7. Northern blot analysis

Total RNA was extracted from the cultured cells by using an RNeasy Mini Kit (QIAGEN). HCV RNA and  $\beta$ -actin were detected according to a method described previously (Ikeda et al., 2005).

### 2.8. Measurement of the fluorescent intensity in living cells replicating a genome-length HCV RNA with EGFP

The cells replicating a genome-length HCV RNA with EGFP ( $5 \times 10^4$ ) were plated onto 12-well plates. By using a fluorometer (Fluoroskan Ascent; Thermo Fisher Scientific K.K., Yokohama, Japan), the fluorescent intensity in living cells was measured at 24, 48, and 72 h. In several experiments, the fluorescent intensity in living cells was measured only at 72 h after the treatment with reagents. After the measurements of the fluorescent intensity, the cells were subjected to Western blot analysis for HCV proteins and quantitative RT-PCR analysis for HCV RNA.

### 2.9. Western blot analysis

The preparation of cell lysates, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the immunoblotting analysis were performed as previously described (Hijikata et al., 1993). Production of core, E1, NS3, NS5A, and NS5B proteins in the O and OGF7 cells was analyzed by immunoblotting using anti-core (CP11; Institute of Immunology, Tokyo, Japan), anti-E1 (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-NS3 (Novocastra Laboratories, Newcastle, UK), anti-NS5A (a generous gift from Dr. A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University), and anti-NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science) antibodies, respectively. Production of EGFP-Neo<sup>R</sup> fusion protein was also detected by anti-GFP antibody (JL-8; Clontech).  $\beta$ -Actin antibody (AC-15; Sigma) was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Sciences, Boston, MA).

### 2.10. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed by using a real-time LightCycler PCR as described previously (Ikeda et al., 2005).



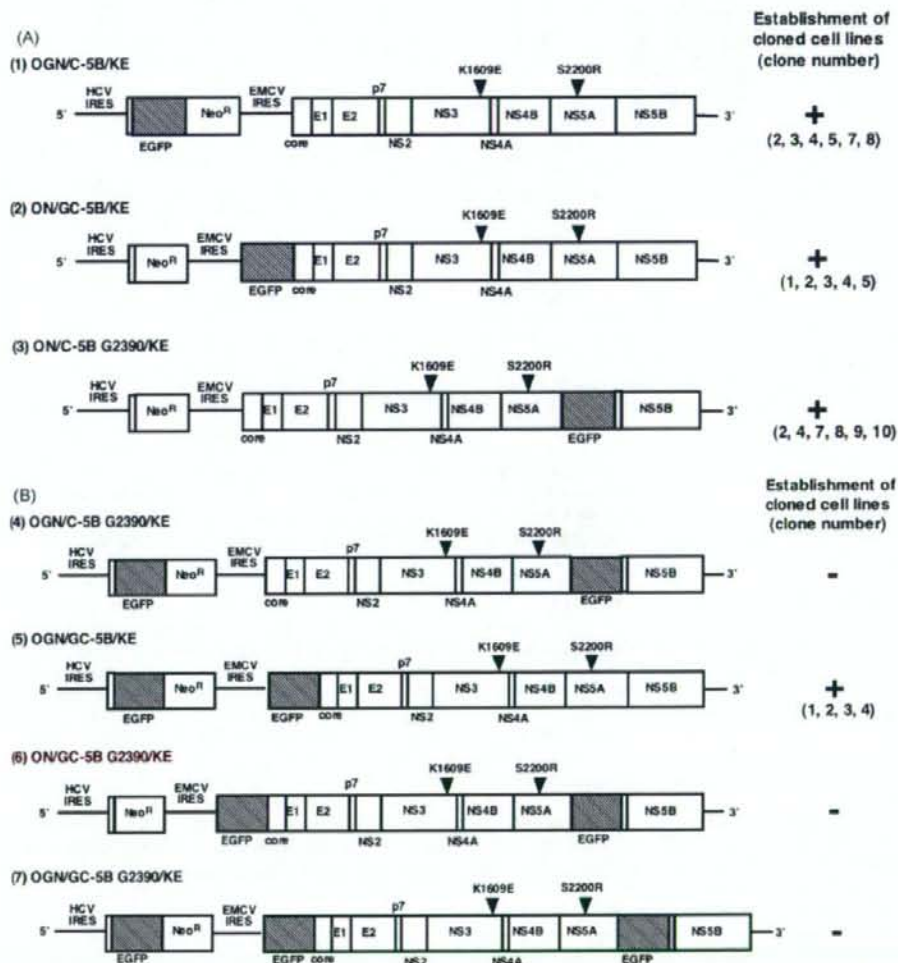
### 3. Results

#### 3.1. Establishment of the cloned cell lines replicating a genome-length HCV RNA with EGFP

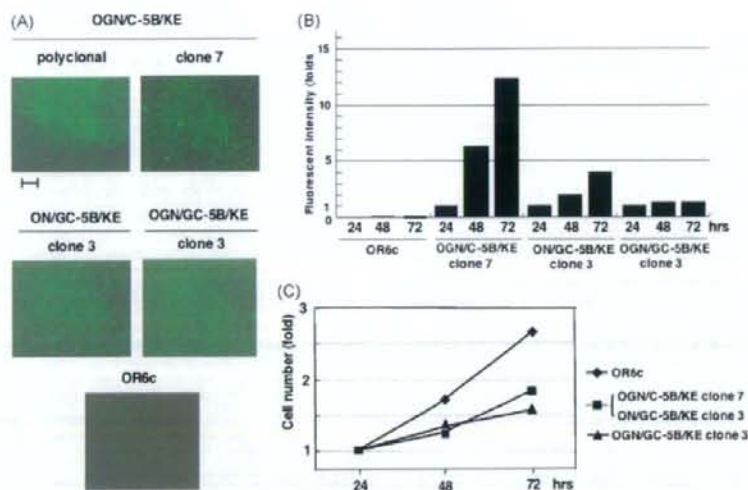
We previously developed a dicistronic genome-length HCV RNA (O strain of genotype 1b) replication system that stably expresses *Renilla luciferase* as a reporter in order to monitor the level of HCV RNA replication (OR6 assay system) (Ikeda et al., 2005; Naka et al., 2005). To further facilitate mass screening of potential candidates for anti-HCV reagents, we attempted to develop a novel assay system for monitoring the level of HCV RNA replication without lysis of cells. For this purpose, we chose EGFP as a reporter, and we first tried to establish cloned cell lines that efficiently replicate genome-length HCV RNA encoding EGFP. All of the constructed plasmids (Fig. 1) were used as templates for RNA synthesis *in vitro*,

and then the transcribed RNAs were transfected into OR6c cells by the electroporation method, as described in Section 2. After 3 weeks of G418 selection, we obtained several G418-resistant colonies from the OGN/C-5B/KE RNA, ON/GC-5B/KE RNA, ON/C-5B G2390/KE RNA, or OGN/GC-5B/KE RNA-introduced cells, and most of the G418-resistant colonies were successfully established as cell lines (Fig. 1). In contrast, no G418-resistant colonies were obtained from the OGN/C-5B G2390/KE RNA, ON/GC-5B G2390/KE RNA, or OGN/GC-5B G2390/KE RNA-introduced cells (Fig. 1).

To select a cloned cell line showing the highest expression level of EGFP and HCV protein, we first performed Western blot analysis for the detection of EGFP and HCV NS3 protein. The results revealed that OGN/C-5B/KE clone 7, ON/GC-5B/KE clone 3, and OGN/GC-5B/KE clone 3 showed marginally higher expression levels of EGFP and HCV NS3 protein than the other clones (data not shown). Because, in the examination by fluorescence microscopy,



**Fig. 1.** Schematic presentation of various genome-length HCV RNAs (HCV-O strain) containing an EGFP-encoding sequence. (A) Genome-length HCV RNAs containing one copy of the EGFP-encoding sequence. The basic construct is described in our previous study (Ikeda et al., 2005). The EGFP-encoding region is depicted as a shaded box. Neomycin phosphotransferase is indicated as Neo<sup>R</sup>. K1609E and S2200R are adaptive mutations found in previous studies (Ikeda et al., 2005; Kato et al., 2003). (B) Genome-length HCV RNAs containing two or three copies of EGFP-encoding sequence.



**Fig. 2.** Fluorescent intensities of G418-resistant cell lines. (A) Visualization of the fluorescence of G418-resistant cell lines under a fluorescence microscope. The panels show the fluorescence of expressed EGFP. Bar, 200  $\mu$ m. (B) Time course of the fluorescent intensity of G418-resistant cell lines. The fluorescent intensity was measured at 24, 48, and 72 h after cell seeding by a fluorometer as described in Section 2. For calculating the fluorescent intensity in each cell line, the intensity at 24 h after cell seeding was assigned a value of 1. OR6c cells were used as a negative control. (C) Growth curve of G418-resistant cell lines. The cells were plated onto 6-well plate ( $1 \times 10^5$  cells per well), and the kinetics of cell proliferation during 72 h in culture were determined by Trypan blue treatment. OR6c cells were used as a control.

the fluorescence of EGFP in these selected cell lines was roughly equivalent to that in OGNC-5B/KE polyclonal cells (Fig. 2A), we next examined the time course of the fluorescent intensities of these cell lines by using a fluorometer, and observed a remarkable, twelve-fold increase in the fluorescent intensity of OGNC-5B/KE clone 7 cells at 72 h after the start of cell culture in comparison with the intensity at 24 h (Fig. 2B). The fluorescent intensity of ON/GC-5B/KE clone 3 cells was slightly increased at 72 h (approximately four-fold). In contrast to these cell lines, the fluorescent intensity of OGNGC-5B/KE clone 3 cells did not change during the cell culture. Growth curve analysis of these G418-resistant cell lines revealed that these cell clones had a similar kinetics for cell proliferation, although the growth rate of these cell clones was significantly lower than that of OR6c cells (Fig. 2C). These results suggest that the efficiency of genome-length HCV RNA replication in OGNC-5B/KE clone 7 cells is higher than that in the other clones. Therefore, we finally selected OGNC-5B/KE clone 7 (herein designated OGF7) for further characterization.

First, to exclude the possibility that the HCV RNA sequence had become integrated into the genomic DNA, we assayed for the HCV 5'-UTR sequence in the genomic DNA isolated from OGF7 cells by PCR. As a positive control, we used a cloned cell line (Mori et al., 2008) in which the HCV 5'-UTR sequence was integrated into the genomic DNA. The HCV 5'-UTR sequence was not detected in the genomic DNA isolated from OGF7 cells, genome-length HCV RNA-replicating O cells (Ikeda et al., 2005), or OR6c cells (Fig. 3A), although an expected product (266 bp or 205 bp) was detected in the positive control (Fig. 3A, lane PC). These results suggest that the HCV RNA sequence (at least HCV 5'-UTR sequence) is not integrated into the genomic DNA in OGF7 cells. Consistent with these results, an approximately 12 kb RNA of the genome-length HCV RNA encoding EGFP in OGF7 cells was also detected by Northern blot analysis, and its accumulation level was almost the same as that in the O cells (Fig. 3B). In addition, we confirmed by Western blot analysis that OGF7 cells efficiently expressed not only HCV proteins but also the EGFP-Neo<sup>R</sup> fused protein, and the expression levels of HCV proteins in the OGF7 cells were also equivalent to those in the O cells

(Fig. 3C). In summary, these results indicate that the OGF7 cell line harboring replicative genome-length HCV RNA encoding EGFP as a reporter was stably established.

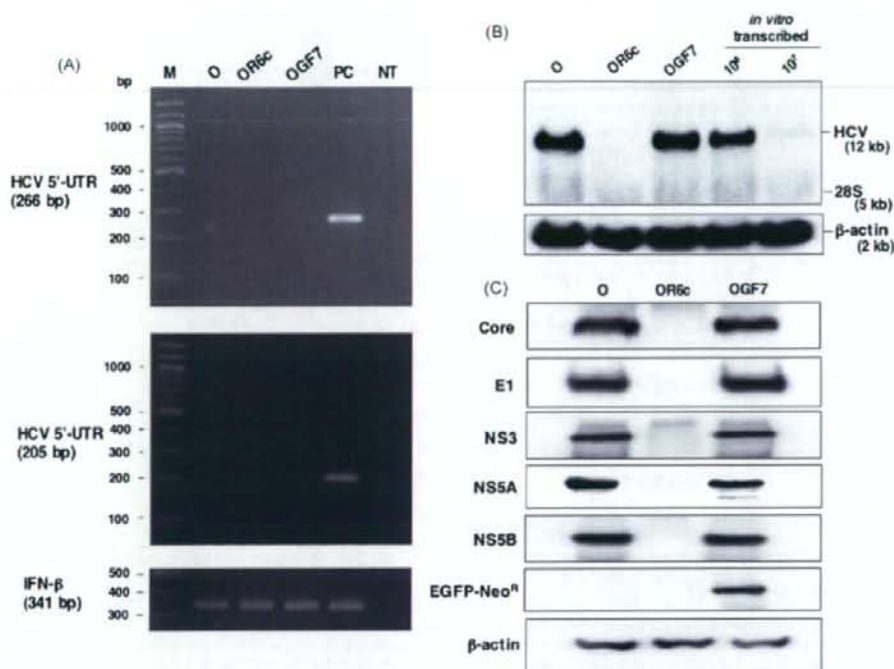
### 3.2. OGF7 living cells are useful for direct monitoring of the level of HCV RNA

First, we examined whether or not the expression level of EGFP in OGF7 cells was sufficient to allow direct visualization by confocal laser-scanning microscopy. As a consequence, we could detect the fluorescence in addition to the core protein expressed in OGF7 cells (Fig. 4). Furthermore, we confirmed that the detected fluorescence was derived from the EGFP expressed in OGF7 cells, because both the fluorescence and the core protein disappeared after IFN- $\alpha$  treatment (Fig. 4). These results suggest that the replication of genome-length HCV RNA encoding EGFP-Neo<sup>R</sup> fused protein occurs efficiently in OGF7 cells. We next examined whether or not the IFN sensitivity of the EGFP level was associated with that of the HCV RNA level in OGF7 cells. The levels of EGFP and HCV RNA were examined by the fluorometer and real-time LightCycler PCR, respectively. The results revealed that the level of reduction in the fluorescent intensity by IFN- $\alpha$  treatment was equivalent to the level of reduction in the HCV RNA level (Fig. 5A and B). In addition, we confirmed by Western blot analysis that the reduction pattern of the EGFP-Neo<sup>R</sup> fusion protein by IFN- $\alpha$  treatment was also similar to those of the core and NS3 proteins (Fig. 5C). These results indicate that the expression level of EGFP is sufficient for monitoring of the level of HCV RNA, and suggest that the direct measurement of the fluorescent intensity of the living OGF7 cells was an effective means of monitoring the level of HCV RNA replication.

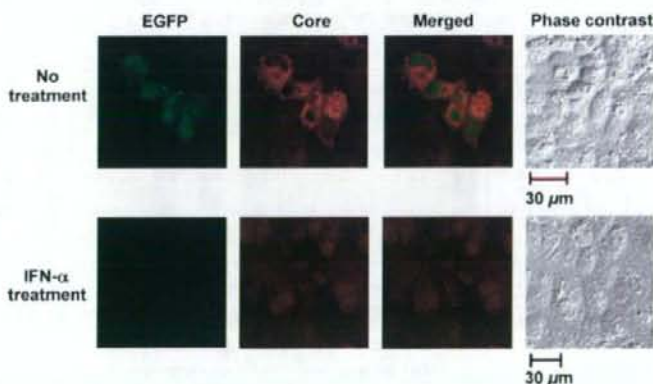
### 3.3. The OGF7 system is useful as a quantitative assay system for various anti-HCV reagents

To clarify whether or not the OGF7 system is useful as a quantitative antiviral assay system, we first compared the IFN- $\alpha$  sensitivity of the OGF7 fluorescent reporter system with that

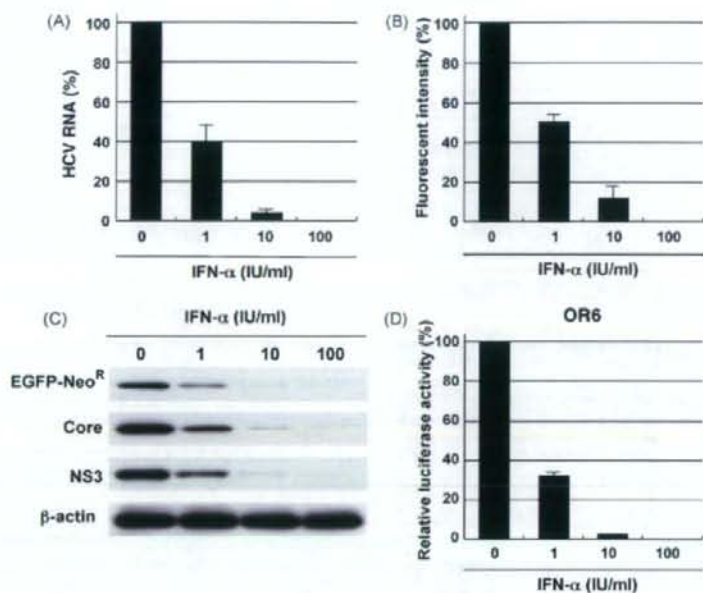




**Fig. 3.** Characterization of OGF7 cells replicating genome-length HCV RNA encoding EGFP as a reporter. Genome-length HCV RNA-replicating O cells (Ikeda et al., 2005) and OR6c cells (cured OR6 cells) were used for the comparison. (A) HCV genome-derived sequences were not integrated into the genomic DNA from OGF7 cells. Genomic DNA from the OGF7 cells was subjected to PCR for the detection of the HCV 5'-UTR and the IFN-β gene. Genomic DNAs from the O and OR6c cells were also used as negative controls. As a positive control, we used genomic DNA from a cell line (Mori et al., 2008) into which the HCV 5'-UTR sequence had been accidentally integrated (lane PC). PCR without genomic DNA was also performed (lane NT). PCR products (266 and 205 bp for HCV 5'-UTR, or 341 bp for the IFN-β gene) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. The 100 bp DNA ladder was used as a size marker (lane M). (B) Northern blot analysis. Total RNAs (3 μg each) from the O, OR6c, and OGF7 cells were analyzed by Northern blot analysis using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific RNA probe (lower panel), respectively. In vitro-synthesized ORN/C-5B/KE (Ikeda et al., 2005) RNA (10<sup>7</sup> and 10<sup>8</sup> genome equivalents) spiked into normal cellular RNA was used for the comparison of expression levels. (C) Western blot analysis. Production of core, E1, NS3, NS5A, and NS5B proteins in the O and OGF7 cells was analyzed by immunoblotting using anti-core, anti-E1, anti-NS3, anti-NS5A, and anti-NS5B antibodies, respectively. Production of EGFP-Neo<sup>R</sup> fusion protein and β-actin was also detected by anti-GFP and anti-β-actin antibodies, respectively.



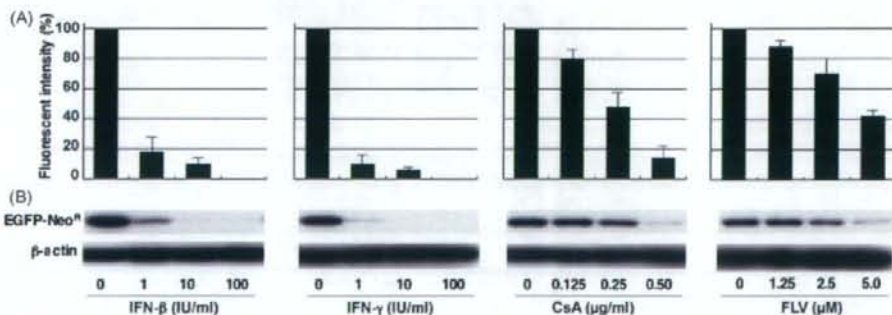
**Fig. 4.** The EGFP and core protein expressed in OGF7 cells disappeared following IFN-α treatment. OGF7 cells were examined by confocal laser-scanning microscopy. Cells were treated with IFN-α (500 IU/ml for 6 h). The cells were visualized with a fluorescence microscope, and then the cells were stained with anti-core antibody (CP11; Institute of Immunology, Tokyo, Japan) and Cy3-conjugated anti-mouse secondary antibody (Jackson Immuno Research, West Grove, PA) according to a method described previously (Naka et al., 2006). The merged panels show the two-color overlay images. Bar, 30 μm.



**Fig. 5.** Effect of IFN- $\alpha$  on genome-length HCV RNA replication in OGF7 and OR6 cells. OGF7 and OR6 cells were treated with IFN- $\alpha$  (0, 1, 10, and 100 IU/ml) for 72 h. After the measurements of the fluorescent intensity of OGF7 cells, the cells were subjected to quantitative RT-PCR analysis for HCV RNA and Western blot analysis. (A) Quantitative RT-PCR analysis of HCV RNA in OGF7 cells. Total RNA extracted from the cells was subjected to real-time LightCycler PCR analysis. The relative level of HCV RNA (%) calculated at each point, when the level of HCV RNA in untreated cells was assigned a value of 100%, is shown here. The experiments were performed in at least triplicate. (B) Fluorescent intensity of OGF7 cells. The fluorometer was used for the measurement of the fluorescent intensity of OGF7 cells. The relative level of the fluorescent intensity calculated, when the fluorescent intensity of untreated cells was taken as 100%, is shown here. The data indicate means from triplicate experiments. (C) Western blot analysis. The production of EGFP-Neo<sup>R</sup>, core, and NS3 in OGF7 cells was analyzed by immunoblotting using anti-EGFP, anti-core, and anti-NS3 antibodies, respectively.  $\beta$ -Actin was used as a control for the amount of protein loaded per lane. (D) Renilla luciferase reporter assay using OR6 cells. The relative level of the luciferase activity calculated, when the luciferase activity of untreated cells was assigned a value of 100%, is shown here. The experiments were performed in at least triplicate.

of the OR6 luciferase reporter system. The results revealed that the profile of IFN- $\alpha$  sensitivity obtained by the OGF7 fluorescent system (Fig. 5B) was similar to that obtained using the OR6 luciferase system (Fig. 5D). Although the OGF7 system was slightly less sensitive than the OR6 system, the small difference may have been due to the different cell clones used. Because the results suggested that the OGF7 system is useful as a quantitative antiviral assay system, we proceeded to examine the

activities of other anti-HCV reagents using the OGF7 system. The results revealed that the fluorescent intensity of OGF7 cells was decreased by the treatments of IFN- $\beta$ , IFN- $\gamma$ , CsA, and FLV in a dose-dependent manner (Fig. 6A), and that the level of the EGFP-Neo<sup>R</sup> fusion protein was also decreased by these anti-HCV reagents in a dose-dependent manner (Fig. 6B). These results suggest that the OGF7 system is useful as a quantitative anti-HCV assay system.



**Fig. 6.** Effects of IFN- $\beta$ , IFN- $\gamma$ , CsA, and FLV on genome-length HCV RNA replication in OGF7 cells. OGF7 cells were treated with IFN- $\beta$  (0, 1, 10, and 100 IU/ml), IFN- $\gamma$  (0, 1, 10, and 100 IU/ml), CsA (0, 0.125, 0.25, and 0.5  $\mu$ g/ml) and FLV (0, 1.25, 2.5, and 5.0  $\mu$ M). (A) Fluorescent intensity of OGF7 cells. After 72 h of treatment, the fluorescent intensity of OGF7 cells was measured by a fluorometer. The relative level of the fluorescent intensity calculated, when the fluorescent intensity of untreated cells was assigned a value of 100%, is shown here. The data indicate means from triplicate experiments. (B) Western blot analysis. The production level of EGFP-Neo<sup>R</sup> was analyzed by immunoblotting using anti-EGFP antibody.  $\beta$ -Actin was used as a control for the amount of protein loaded per lane.



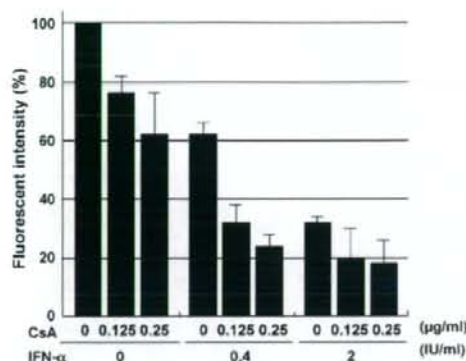


Fig. 7. Effect of IFN- $\alpha$  in combination with CsA on genome-length HCV RNA replication in OGF7 cells. OGF7 cells were co-treated with IFN- $\alpha$  (0, 0.4, and 2.0 IU/ml) and CsA (0, 0.125, and 0.25  $\mu$ g/ml), and at 72 h after treatment, the fluorescent intensity of OGF7 cells was measured by a fluorometer. The relative level of the fluorescent intensity calculated, when the fluorescent intensity of untreated cells was taken as 100%, is shown here. The data indicate means from triplicate experiments.

### 3.4. The OGF7 assay system is also useful as a system for evaluating the efficacy of co-treatment with various anti-HCV reagents

Since we demonstrated that the OGF7 system could be used effectively as either a quantitative anti-HCV assay system or OR6 assay system, we further examined whether or not the OGF7 system could be used to evaluate the efficacy of co-treatment with various anti-HCV reagents. The results showed that co-treatment with IFN- $\alpha$  and CsA was more effective than treatment with IFN- $\alpha$  alone (Fig. 7).

Together, the above results led us to conclude that the OGF7 living cell system is the most time-saving and low-cost anti-HCV assay system currently available.

## 4. Discussion

In the present study, we developed a new living cell-based reporter assay system (OGF7 assay system) for monitoring HCV RNA replication. We demonstrated that this OGF7 assay system was useful for the quantitative evaluation of anti-HCV reagents. Our study suggests that this new assay system is the most time-saving and inexpensive assay system for high-throughput screening of anti-HCV reagents.

To date, several cloned cell lines harboring HCV RNA (Con1 strain of genotype 1b) with EGFP have been reported (Liu et al., 2006; McCormick et al., 2006; Moradpour et al., 2004). However, regarding the Con1 strain, established cell lines are limited to the subgenomic replicon RNA, although several cloned cell lines harboring genome-length HCV RNA (JFH-1 strain of genotype 2a) with EGFP have been recently reported (Kim et al., 2007; Jones et al., 2007; Schaller et al., 2007). Since a quantitative reporter assay system for monitoring the level of HCV RNA replication has not been developed in these studies, we have tried to establish cell lines in which a genome-length HCV RNA encoding two or three copies of EGFP is efficiently replicating. However, from this study we have learned the limitation of RNA genome size. Although we tested seven different kinds of constructs for HCV RNA replication, most of the G418-resistant colonies were obtained from one copy type of EGFP (RNA genome size 11.8 kb) (Fig. 1). Although we obtained G418-resistant colonies from only OGN/GC-5B/KE con-

struct containing two copies of EGFP (RNA genome size 12.5 kb), the fluorescent intensities of these colonies did not increase in a culture time-dependent manner, suggesting that the HCV RNA replication is not efficient in these cloned cells. These findings suggest that the genome size limitation in HCV RNA replication is approximately 12 kb. This suggestion is consistent with the previous finding (Ikeda et al., 2005) obtained in the process of development of the OR6 assay system. However, specific combination (Q1112R and K1609E) of adaptive NS3 mutations, which drastically enhanced the efficiency of genome-length HCV RNA replication (Abe et al., 2007), may overcome the genome size limitation (approximately 12 kb) in HCV RNA replication. When this genome size limitation is solved, a new cell line in which a genome-length HCV RNA encoding both EGFP-Neo<sup>R</sup> fused protein and another fluorescent reporter (e.g., EYFP)-NS5A fused protein replicate efficiently may be developed. Such a system would allow us to monitor the levels of HCV RNA and HCV proteins simultaneously.

We demonstrated that the established OGF7 cells were useful as a quantitative antiviral assay system (OGF7 assay system), because the anti-HCV activities of IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , CsA, and FLV were clearly shown in a dose-dependent manner just as in the evaluation using the OR6 assay system (Ikeda et al., 2005, 2006; Naka et al., 2005; Yano et al., 2007). Furthermore, since the OGF7 assay system allows us to measure, at different times, the same well containing OGF7 cells treated with the reagent, the OGF7 assay system can be considered superior to the OR6 assay system. Finally, since the OGF7 assay system is based on the simple measurement of the fluorescent intensity of living cells, this system has great advantages regarding time and cost for the antiviral assay of a number of reagents. Therefore, the OGF7 assay system is the most convenient method for high-throughput mass screening of a large compound library. Although we used 12-well plates for the assay in this study, we confirmed that we could monitor the level of HCV RNA replication on 24-well plates (data not shown). If the replication level of HCV RNA were to become higher than that in OGF7 cells due to additional adaptive mutation(s), such system might be capable of monitoring the replication level of HCV RNA in the living cells on 48- or 96-well plates. Such a system containing an OGF7 assay could be used to identify more effective and specific anti-HCV reagents in the future.

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## New efficient replication system with hepatitis C virus genome derived from a patient with acute hepatitis C<sup>\*</sup>

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### ABSTRACT

We report for the first time a new RNA replication system with a hepatitis C virus (HCV) strain (AH1) derived from a patient with acute hepatitis C. Using an HCV replicon RNA library constructed with the AH1 strain (genotype 1b), we first established a cloned cell line, sAH1, harboring the HCV replicon. Cured cells obtained with interferon treatment of sAH1 cells were used for transfection with genome-length HCV RNA possessing four mutations found in sAH1 replicon. Consequently, one cloned cell line, AH1, supporting efficient replication of genome-length HCV RNA was obtained. By the comparison of AH1 cells with the O cells supporting genome-length HCV RNA (HCV-O strain) replication, we found different anti-HCV profiles of interferon- $\gamma$  and cyclosporine A between AH1 and O cells. Reporter assay analysis suggests that the diverse effects of interferon- $\gamma$  are due to the difference in HCV strains, but not the cellular environment.

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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 because at least 170 million people worldwide are currently infected with HCV [1]. HCV is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues [2,3]. 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, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [3].

As a striking breakthrough in HCV research, in 1999, an HCV replicon system enabling robust HCV subgenomic RNA (Con-1 strain of genotype 1b) replication in specific human HuH-7 hepatoma cells has been developed [4]. After the first Con-1 replicon, several HCV replicon (genotypes 1a, 1b, and 2a) systems using HuH-7-derived cells have been developed. These replicon systems have become powerful tools for basic studies of HCV replication, HCV–host cell interactions, and screening of anti-HCV reagents, [5,6]. Furthermore, genome-length HCV RNA replication systems have been developed [7–9], since HCV replicons lacking HCV structural proteins are insufficient for further HCV research. We also established a genome-length HCV RNA-replicating cell line (HCV-

O strain of genotype 1b; called O cell line) [10] using cured cells derived from sO cells [11], in which HCV replicon RNA (HCV-O strain) with an adaptive mutation (S2200R) is replicating. However, to date, established genome-length HCV RNA-replicating stable cell lines are limited to five HCV strains, H77 (1a), HCV-N (1b), Con-1 (1b), HCV-O (1b), and JFH1 (2a) [7–10,12], and there is no RNA replication system with an HCV strain derived from a patient with acute hepatitis C. Furthermore, there have been few reports comparing these HCV strains.

To clarify these problems, we have attempted to establish a new stable cell line, in which genome-length HCV RNA derived from a patient with acute hepatitis C is efficiently replicating. We report herein a new efficient RNA replication system with HCV derived from a patient with acute hepatitis C and provide a comparative analysis of RNA replication systems with AH1 and HCV-O strains regarding the sensitivities to anti-HCV reagents, including interferon (IFN)- $\alpha$ .

### Materials and methods

**Cell culture.** Cells supporting HCV replicon or genome-length HCV RNA, and cured cells, from which the HCV RNA had been eliminated by IFN treatment, were maintained as described previously [10].

**Reverse transcription (RT)-nested PCR.** RNA from a serum of patient AH1 [13] with acute hepatitis C was prepared using the ISOGEN-LS extraction kit (Nippon Gene Co., Japan). This RNA sample was used as a template for RT-nested PCR to amplify the HCV RNA. RT-nested PCR was performed separately in two parts; one part (3.5 kb) covered from HCV 5'UTR to NS3, and the other part (6 kb) covered from NS2 to NS5B. For the first part, the antisense primer AH13553R, 5'-CACACCGCTGATGC AGTGC-3' was used for RT. Primers 21 [11] and AH3519R, 5'-TCCGTCGGCC

<sup>\*</sup> The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AB429050.

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TGGAACCCACCTG-3' were employed in the first round of PCR (35 cycles). An internal primer pair (21X [11] and AH3466R: 5'-ATTATCTAGAGCCCTGTGAGACTG GTGATGATGC-3'; containing a XbaI site (underlined)) was used for the second round of PCR (35 cycles). For the second part, the antisense primer 386R [11] was used for RT. Primers 542 and 9388R [11] were employed in the first round of PCR (35 cycles). An internal primer pair (3295X: 5'-ATTATCTAGACTGAGATGGA CACCAAGATCATC-3'; containing a XbaI site (underlined) and 9357RX: 5'-ATTATCTAGAGCCCGTTCACCGGTGGGAGCAG-3'; containing a XbaI site (underlined)) was used for the second round of PCR (35 cycles). These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis for HCV RNA after cloning into the XbaI site of pBR322MC [11]. Superscript II (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

**Plasmid construction.** PCR product (NS3 to NS5B of AH1 strain) with primers 542 and 9388R was further amplified with primers 3501S: 5'-ATTACTAGCTCTCACAGG CCGGACAAGAACC-3'; containing a SpeI site (underlined) and 9162RB: 5'-ATTATCTAGAGCCCGGTTGAAGAGGACTTGC-3'; containing a BsiWI site (underlined). The amplified fragment was digested with SpeI and BsiWI, and ligated into the replicon cassette plasmid pNSS1KZ2RU [11], which was predigested with SpeI and BsiWI. Using this ligation reaction mixture, a replicon RNA library (AH1N/3-5B in Supplementary Fig. 1) was prepared by a previously described method [11]. To make the plasmid pAH1N/C-5B/PL LS, (VA), containing full-length HCV polyprotein of AH1 strain, pON/C-5B containing full-length HCV polyprotein of HCV-O strain [10] was utilized. First, to make a fragment for pAH1N/C-5B (Supplementary Fig. 1), overlapping PCR was used to fuse EMCV IRES to the core protein-coding sequence of the AH1 strain, as described previously [10]. The resulting DNA was digested with PmeI and ClaI, and then replaced with the PmeI–ClaI fragment of pON/C-5B (pON/C-5B/CoreAH) was obtained. Second, the ClaI–AgeI fragment of pHCV-AH1 containing full-length HCV polyprotein of AH1 strain was replaced with the ClaI–AgeI fragment of pON/C-5B/CoreAH (pAH1N/C-5B was obtained). Finally, the SpeI–BsiWI fragment of pAH1N/3-5B clone 2 (see Fig. 1C) was replaced with the SpeI–BsiWI fragment of pAH1N/C-5B (pAH1N/C-5B/PL LS, (VA), was obtained).

**RNA synthesis.** Plasmid DNAs were linearized by XbaI and were used for RNA synthesis with T7 MEGAScript (Ambion) as previously described [11].

**RNA transfection and selection of G418-resistant cells.** The transfection of HCV replicon RNA or genome-length HCV RNA synthesized in vitro into Huh-7-derived cells was performed by electroporation, and the cells were selected in the presence of G418 (0.3 mg/ml; Promega) for 3 weeks as described previously [11].

**Quantification of HCV RNA.** The quantitative RT-PCR (RT-qPCR) analysis for HCV RNA was performed by LightCycler PCR as described previously [10]. Experiments were done in triplicate.

**Integration analysis.** Genomic DNA was extracted from the cultured cells using the DNeasy Blood & Tissue Kit (QIAGEN). The HCV 5'UTR and the IFN- $\beta$  gene were detected according to a PCR method described previously [11].

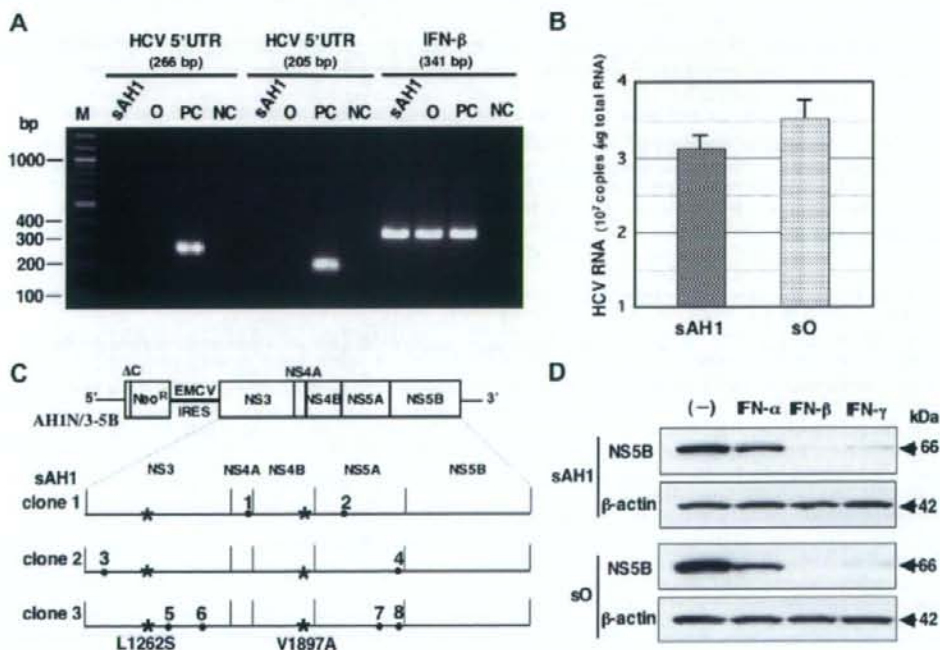
**Western blot analysis.** The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as previously described [11]. The antibodies used in this study were those against Core, E2, NS3, NS4A, NS5A, and NS5B [10].  $\beta$ -Actin antibody (AC-15, Sigma) was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

**Sequence analysis of HCV RNA.** To amplify replicon RNA and genome-length HCV RNA, RT-PCR was performed as described previously [10,11]. The PCR products were subcloned into the XbaI site of pBR322MC, and sequence analysis was performed as described previously [11].

**Northern blot analysis.** Total RNA was extracted from the cultured cells using the RNeasy Mini Kit (QIAGEN). Three micrograms of total RNA was used for the analysis. HCV-specific RNA and  $\beta$ -actin were detected according to a method described previously [10].

**Luciferase reporter assay.** For the dual-luciferase assay, firefly luciferase vectors, pGBP-1(-216)-Luc and p2'-5'-OAS(-159)-Luc [14], were used. The reporter assay was performed as previously described [14]. The experiments were performed in at least triplicate.

**Statistical analysis.** Differences between AH1 and O cell lines were tested using the Student's *t*-test. *P* values <0.05 were considered statistically significant.



**Fig. 1.** Characterization of sAH1 cells harboring HCV replicon. (A) No integration of the HCV sequence in the genomic DNA. Genomic DNA from sAH1 cells was subjected to PCR for the detection of the HCV 5'UTR and the IFN- $\beta$  gene. O cells were used as a negative control. Lane PC, HCV sequence-integrated cells; lane NC, no genomic DNA; lane M, 100 bp DNA ladder. PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (B) Quantitative analysis of intracellular replicon RNA. The levels of replicon RNA were quantified by LightCycler PCR. sO cells harboring HCV-O replicon [11] were used for the comparison. (C) Amino acid substitutions detected in intracellular AH1 replicon RNA. NS3 to NS5B regions of three independent clones sequenced were presented. L1262S and V1897A conserved substitutions are indicated by asterisks. Clone-specific aa substitutions (indicated by the numbers with dots) are as follows: 1, K1691R; 2, M2105I; 3, P1115L; 4, V2360A; 5, K1368R; 6, A1533T; 7, I2285V; 8, D2377H. (D) IFN sensitivity of AH1 replicon. sAH1 cells were treated with IFN- $\alpha$  (Sigma), IFN- $\beta$  (a gift from Toray Industries), and IFN- $\gamma$  (Sigma) (20 IU/ml each) for 5 days. For the comparison, sO cells were treated as well as sAH1 cells. NS5B was detected by Western blot analysis.