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Replicons from genotype 1b HCV-positive sera exhibit diverse sensitivities to anti-HCV reagents

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ABSTRACT

Half of the population of genotype 1 HCV is resistant to current pegylated-interferon-α (PEG-IFN-α) and ribavirin therapy. The resistance to IFN therapy is an urgent problem, especially in patients with genotype 1 HCV infection. However, sensitivities among HCV strains to anti-HCV reagents including IFNs have not been thoroughly addressed. Here, we established three different subgenomic replicons (1B-4, 1B-5, and KAH5 strains) in addition to our previously established replicon (O strain). We comparatively examined the sensitivities of four replicons to IFN-α, IFN-γ, IFN-λ, cyclosporine A, and fluvastatin. Among the replicons, the 1B-4 and KAH5 replicons were the most sensitive and resistant, respectively to IFN-λ (EC_{50}: 1.50 ng/ml vs. 8.50 ng/ml) and fluvastatin (EC_{50}: 2.82 μM vs. 7.87 μM), although these replicons possessed similar features in terms of genetic distance from the O strain, HCV RNA expression levels, and sensitivity to IFN-α (EC_{50}: 1.44 IU/ml vs. 1.37 IU/ml) and cyclosporine A (EC_{50}: 0.71 μg/ml vs. 0.96 μg/ml). These replicons are thus useful tools for examining the mechanism of anti-HCV activity, especially in IFN-λ and statins.

1. Introduction

Hepatitis C virus (HCV) belongs to *Flaviviridae* family and contains a positive single-stranded RNA genome of 9.6 kb (Kato et al., 1990; Tanaka et al., 1996). The viral genome encodes a single polyprotein of approximately 3010 amino acid residues, which is proteolytically processed by 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 (Kato, 2001). HCV infection frequently causes chronic hepatitis C (CH C) and progresses to fatal cirrhosis and hepatocellular carcinoma. The current standard therapy for CH C is pegylated-interferon-α (PEG-IFN-α) and ribavirin. However, the cure rate of the therapy for the treatment of CH C is limited to approximately 50% (Firpi and Nelson, 2007). The major cause of resistance to this therapeutic approach was observed in genotype 1 HCVs. However, the mechanisms of the diverse sensitivity to IFN therapy among genotype 1 HCVs have remained unclear. Therefore, the development of more effective anti-HCV reagents is an urgent issue.

Since the HCV replicon system was developed by Lohmann et al. (1999), several groups have reported candidate anti-HCV reagents. Statin, a 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is one of the well-characterized anti-HCV reagents and its anti-HCV activity has been shown to be due to the inhibition of geranylgeranylation of host proteins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). Cyclosporine A (CsA), an immunosuppressant, is another well-characterized anti-HCV reagent that inhibits HCV RNA replication via its interaction with cyclophilins (CyPs) (Inoue et al., 2007; Nakagawa et al., 2005; Watashi et al., 2003). In addition to type I IFNs (α and β) and type II IFN (γ), recently identified type III IFN (λ) has been reported to possess anti-HCV activity in cell culture (Doyle et al., 2006; Marcello et al., 2006; Robek et al., 2005). Subgenomic HCV replicons have been reported since the breakthrough of the Con1(1b) replicon using different HCV strains: H77 (1a), N (1b), 1B-1 (1b), O (1b), JFH 1 (2a), and AH1 (1b) (Blight et al., 2003; Ikeda et al., 2002, 2005; Kato et al., 2003a,b; Kishine et al., 2002; Lohmann et al., 1999; Mori et al., 2008; Pietschmann et al., 2002). Moreover, a number of groups have examined anti-HCV reagents using the established replicon. However, such studies have been conducted using replicon(s) from only one or two HCV strain(s). To date, there has been no comprehensive study regarding the diverse sensitivities of anti-HCV reagents to genotype 1 HCV replicons from different strains.

To address this issue, we developed three HCV replicons from different genotype 1b HCV positive sera, in addition to our previously reported O strain (Ikeda et al., 2005). Two replicons were

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constructed using HCV-positive sera from healthy carriers (1B-4 and 1B-5) and one replicon was constructed using serum sampled from a case of acute hepatitis C (KAH5). These replicons contained neomycin phosphotransferase (Neo) and *Renilla* luciferase (RL) genes at the first cistron of the replicon with the aim of conducting a stable and highly sensitive reporter assay. In this study of four replicons, we examined the anti-HCV reagents IFN-α, IFN-γ, IFN-λ, CsA, and various statins (pitavastatin (PTV), fluvastatin (FLV), and rosvastatin (RSV)), and we found diverse sensitivities among the replicons. Newly developed replicons will be useful tools for the present study regarding the diverse sensitivities of genotype 1b HCVs to anti-HCV reagents, including IFNs.

2. Materials and methods

2.1. HCV-positive sera and GeneBank accession numbers

Serum O (previously described as 1B-2), 1B-4, and 1B-5 were derived from an HCV-positive healthy carrier and have been described previously (Ikeda et al., 1997). Serum KAH5 was obtained from a patient with acute hepatitis C (AH C) who provided prior informed consent. The nucleotide sequence data for 1B-4, 1B-5, and KAH5 will appear in the DDBJ, EMBL, and GeneBank nucleotide sequence databases under accession nos. AB442219, AB442220, and AB442222, respectively.

2.2. Cell cultures

Three HCV-positive sera (KAH5, 1B-4, and 1B-5 strains) were used for the development of subgenomic replicons with reporter (RL). We first established 9, 4, and 6 replicon harboring clonal cell lines derived from KAH5, 1B-4, and 1B-5 strains, respectively. Then, after characterization for these cell lines, we selected the representative clonal cell lines and designated sKAH5R (clone 6), s1B-4R (clone 2), and s1B-5R (clone 4) as sKAH5R, s1B-4R, and s1B-5R, respectively (Supplemental Figs. 1A, B, and C). sO and O cells were used as subgenomic and genome-length HCV RNA-harboring cells with a Neo gene in the first cistron, as previously described (Kato et al., 2003a; Ikeda et al., 2005). These cells were derived from a hepatoma cell line, HuH-7, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 0.3 mg/ml of G418 (Geneticin; Invitrogen, Carlsbad, CA). The cells were passaged twice weekly at a 5:1 split ratio. The sequences in the original subgenomic replicons were described above and appeared in the database with indicated accession numbers.

2.3. RT-nested PCR

HCV RNAs were prepared from HCV-positive sera (1B-4, 1B-5, and KAH5) using ISOGEN-LS (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's protocol. These RNA samples were used for RT-PCR in order to amplify the NS2 to NS5B region (6.0 kb) of the HCV genomes. RT was performed with the OligodA23 primer, 5′-AAAAAAAAAAAAAAAAAAAAAA-3′. The primer pair 542: 5′-GTAGAGCCCGTCGTCTTCTCTGACATGGA-3′ and 9388R: 5′-ATGGCCTATTGGCCTGGAGTG-3′ was employed in the first-round PCR (35 cycles). The primer pair 3295X: 5′-ATTATTCTAGACTGACATGGAGACCAAGATCATCAC-3′ and 9357RX: 5′-ATTATTCTAGACCCGTTCACCGGTTGGGGAGCAG-3′, containing the XbaI site (underlined) was employed in the second-round PCR (35 cycles). SuperScript III reverse transcriptase (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

2.4. Plasmid construction

To construct an HCV replicon with RL and Neo genes, we used a previously described pRN/3-5B/KE plasmid as a cassette vector (Ikeda et al., 2005). Basically, the NS3 to NS5B region was replaced with RT-PCR products from sera with 1B-4, 1B-5, and KAH5 at SpeI (located in NS3) and BsiWI (located in NS5B) sites. The PCR products were further amplified with the primers NS3 SpeI: 5′-ATCATCACTAGTCTCACAGGCCGGGACAAGAAAC-3, containing the SpeI site (underlined); and NS5B BsiWI: 5′-CTTGGTCCGTACGGCCCAGTTGAAGAGGTACTTGC-3′, containing the BsiWI site (underlined). The amplified fragments were digested with SpeI and BsiWI, and were ligated into the pRN/3-5B/KE cassette vector, which was predigested with SpeI and BsiWI.

2.5. RNA transcription

Plasmid DNAs were linearized by XbaI digestion and were used for RNA synthesis with T7 MEGAscript (Ambion) as previously described (Kato et al., 2003a).

2.6. RNA transfection and G418-resistant cells

Ten micrograms of *in vitro* synthesized HCV replicon RNAs were introduced into HuH-7 derived cells (OR6c cells) by electroporation, and the cells were selected in the presence of G418 (0.3 mg/ml) for 3 weeks as described previously (Mori et al., 2008).

2.7. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (Kato et al., 2003a). The antibodies used in this study were those against Core, NS3, NS5A, and NS5B. β-actin antibody (AC-15, Sigma) was used as a control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science, Boston, MA).

2.8. Quantification of HCV RNA

The RNAs were prepared from an HCV replicon RNA replicating cell line, and 2 μg of each total RNA was used for RT-qPCR with 5′-UTR of an HCV-specific primer pair, as described previously (Ikeda et al., 2005). Experiments were conducted in triplicate.

2.9. Northern blot analysis

Total RNA was extracted from the cultured cells using an RNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). Three micrograms of total RNA were used for the analysis. HCV-specific RNA and β-actin were detected according to a previously described method (Ikeda et al., 2005).

2.10. Reagents

IFN-α and IFN-γ were purchased from Sigma, and CsA was obtained from Calbiochem (San Diego, CA). IFN-λ (IL-29) was purchased from WAKO. PTV was purchased from the Kowa Company, Ltd. (Tokyo, Japan). FLV was purchased from Calbiochem. RSV was obtained from AstraZeneca.

2.11. Luciferase reporter assay

For the luciferase assay, $1.0–1.5 \times 10^4$ HCV replicon-harboring cells were plated onto 24-well plates in triplicate and were cultured

for 24 h. The cells were treated with each anti-HCV reagent for 72 h. Then the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol. All the luciferase assays were repeated at least three times.

2.12. Statistical analysis

Statistical comparison of the luciferase activity in various treatment groups was performed using Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Establishment of four subgenomic replicon-harboring cell lines using different genotype 1b HCV sera

We tried to establish replicon-harboring cells from different HCV-positive sera to assess the sensitivity of anti-HCV reagents among genotype 1b HCV strains. To this end, three sera (1B-4, KAH5, and 1B-5) were used to amplify the NS region of HCV genomes by reverse transcription-polymerase chain reaction (RT-PCR). The dicistronic replicons were designed as shown in Fig. 1A. RL and Neo genes were introduced into the first cistron and translation was driven by the HCV internal ribosomal entry site (IRES) leading to the expression of RL and Neo as a fusion protein. In the second cistron, NS3 to NS5B was translated via the encephalomyocarditis virus (EMCV) IRES (Fig. 1A). We introduced *in vitro*-synthesized HCV replicon RNAs (10 μg) into OR6c cells, in which HCV RNA was eliminated from OR6 cells by IFN-α treatment. After 3 weeks of G418 selection, we obtained HCV replicon-harboring cell colonies, i.e., more than 100 colonies from KAH5 and 20 colonies from 1B-4. However, no colony formation was observed among 1B-5 replicon-RNA-introduced cells. Therefore, we next attempted to perform the electroporation of a 1B-5 replicon with mutations derived from the HCV sequence in s1B-5 replicon-harboring cells, in which the replicating HCV replicon possessed only neomycin-resistant genes in the first cistron (data not shown). The mutations introduced into 1B-5 replicon were E1758D and I1851F in NS4B and R2192W and E2414Q in NS5A. Consequently, we established 9, 4, and 6 replicon-harboring cells from KAH5, 1B-4, and 1B-5, respectively, and confirmed the expression of HCV RNA and proteins. In addition to three replicon RNAs, the previously described ORN/3-5B/KE replicon RNA was also introduced into OR6c cells and selected as sOR in this study (Ikeda et al., 2005). The representative clonal cell lines, which grow healthy and stably expressed abundant HCV proteins, are used in the following experiments (Supplemental Fig. 1A, B, and C). These replicon-harboring cell lines were established from genotype 1b HCV strains: 1B-4, KAH5, O, and 1B-5 and were designated as s1B-4R, sKAH5R, sOR, and s1B-5R, respectively. We confirmed the expression of NS3, NS5A, and NS5B proteins in all replicon-harboring cells (Fig. 1B). The expression levels of HCV RNAs in the replicon-harboring cells were examined for the 5′-UTR by quantitative RT- PCR (RT-qPCR) (Fig. 1C). s1B-4R cells exhibited the highest levels of expression of HCV RNA (approximately 10^8 copies/μg total RNA), followed by sKAH5R, sOR, and s1B-5R cells (Fig. 1C). All of the replicon-harboring cells expressed HCV RNA at levels greater than at least 4×10^7 copies/μg total RNA. Northern blot analysis also demonstrated the presence of HCV-specific RNA with a length of approximately 9 kb in the total RNA extracts from four replicon-harboring cells (Fig. 1D). These four genotype 1b HCV replicon reporter systems were established and used for further analyses of sensitivity to anti-HCV reagents.

3.2. Diverse activity of various IFN types on HCV replicons

IFN-α belongs to the type I IFN group and is currently used as standard therapy for patients with CH C. Therefore, first we evaluated the activity of IFN-α using the four developed replicons and a reporter assay. The s1B-4R and sKAH5R replicons showed almost equal and moderate sensitivity to IFN-α (50% effective concentration (EC_{50}): 1.44 and 1.37 IU/ml, respectively) (Fig. 2). The s1B-5R and sOR replicons, respectively, exhibited the highest (EC_{50}: 1.10 IU/ml) and lowest (EC_{50}: 2.35 IU/ml) sensitivity to IFN-α among the replicons tested (Fig. 2). We also examined the activity of IFN-α on HCV protein expression levels in these four replicons. The findings from the Western blot analysis of the sensitivity to IFN-α

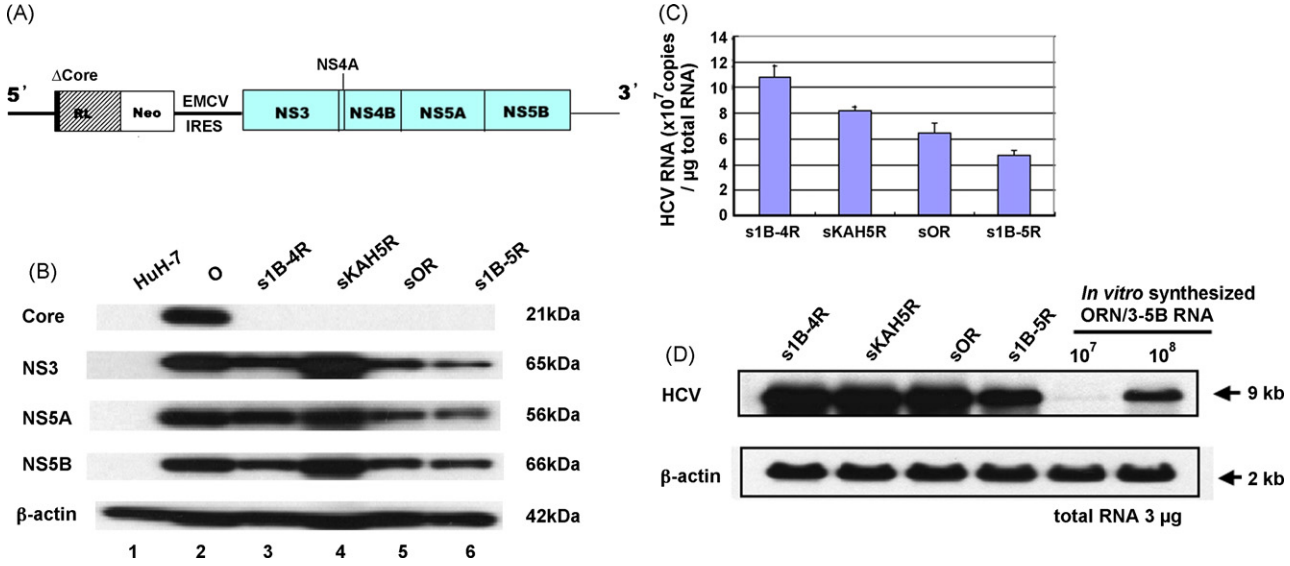

Fig. 1. The expression of HCV proteins and HCV RNAs in four replicon-harboring cell lines. (A) Schematic gene organization of subgenomic replicon RNA. The NS3 to NS5B region and 12 N-terminal amino acid residues of the Core (ΔC) are depicted in closed boxes. Untranslated regions, EMCV IRES, RL, and Neo genes are indicated by thin lines, thick line, shaded box, and open box. (B) Western blot analysis of HCV proteins. Production of Core, NS3, NS5A, and NS5B in HuH-7 cells (lane 1), O cells (lane 2), s1B-4R cells (lane 3), sKAH5R cells (lane 4), sOR cells (lane 5), and s1B-5R cells (lane 6) were analyzed by immunoblotting using anti-Core, anti-NS3, anti-NS5A, and anti-NS5B antibodies. (C) RT-qPCR analysis. The levels of HCV RNA in G418-resistant cells were quantified by LightCycler PCR. (D) Northern blot analysis. RNAs from s1B-4R, sKAH5R, sOR, and s1B-5R cells were used for comparison. *In vitro*-synthesized ORN/3-5B RNA was also used for comparative analyses.

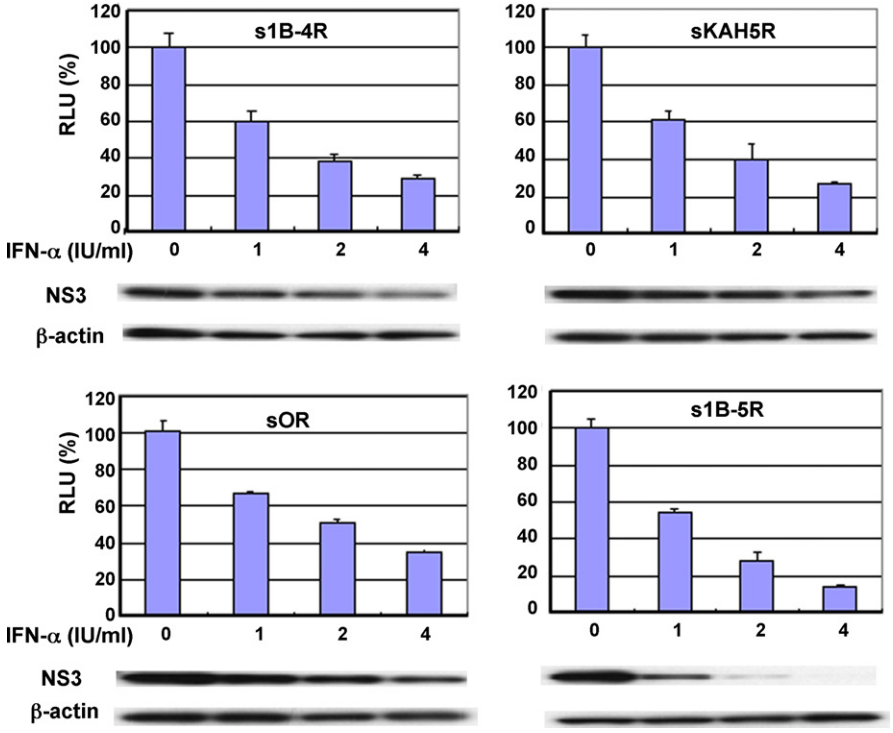

Fig. 2. The activity of IFN-α on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-α treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-α (0, 1, 2, and 4 IU/ml) for 72 h. Then, the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels). The percent relative luciferase unit (RLU (%)) was calculated with the RL activity of untreated cells assigned at a value of 100%. The data indicate means ± S.D.s of triplicate samples. All of the luciferase assays were repeated at least three times. β-Actin was used as a control for the amount of proteins loaded per lane.

coincided with the results of the reporter assay. Thus, these results indicated that genotype 1b replicons possess different sensitivities to IFN-α.

Next, we examined the sensitivity of four replicons to type II IFN, IFN-γ, because in our previous study, HCV (genotype 1b, AH1 strain) from a patient with AH C was found to be more resistant to IFN-γ than was HCV-O (Mori et al., 2008). In this study, sKAH5R was also derived from the serum of a patient with AH C. The reporter assay revealed that sKAH5R has the lowest sensitivity to IFN-γ (EC_{50}: 2.26 IU/ml) among the replicons tested (Fig. 3). To calculate the EC_{50} of IFN-γ to sKAH5R, we also treated sKAH5R with IFN-γ at 2 and 4 IU/ml for 72 h (data not shown). The EC_{50} of IFN-γ to s1B-4R, sOR,

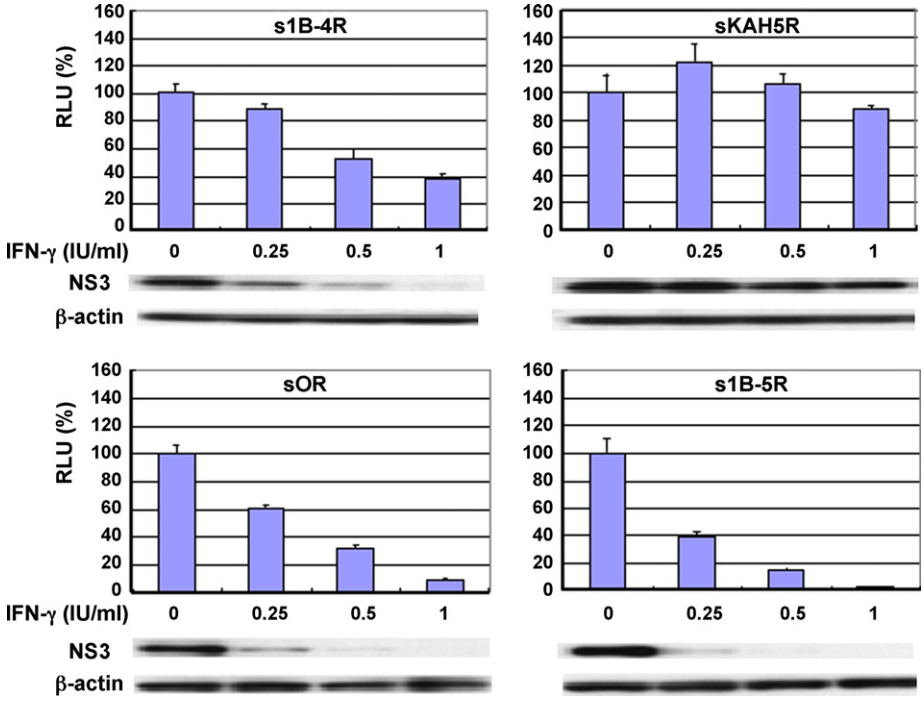

Fig. 3. The activity of IFN-γ on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-γ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-γ (0, 0.25, 0.5, and 1 IU/ml) for 72 h and then the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels). All of the luciferase assays were repeated at least three times.

and s1B-5R was 0.54, 0.33, and 0.21 IU/ml, respectively. The results of Western blot analyses of sensitivity to IFN-γ coincided with those of the reporter assay. Interestingly, again in this study, the HCV RNA derived from the patient with AH C was resistant to IFN-γ, as was the AH1 strain. These results may suggest that AH C in pathologic states of HCV infection may be involved in the IFN-γ resistance feature of the replicon. Further studies will be needed to clarify this issue.

We analyzed a recently identified type III IFN, IFN-λ, in terms of its anti-HCV activity against four HCV replicons. IFN-λ shares the same Jak/Stat signaling pathway with type I IFNs, which express a common set of IFN-stimulating genes (ISGs). However, IFN-λ uses distinct receptors composed of IFNLR1 and IL10R2. Here, sKAH5R and s1B-4R, respectively, exhibited the lowest and highest sensitivities to IFN-λ (EC_{50}: 8.25 and 1.50 ng/ml) (Fig. 4A). Additionally, sOR and s1B-5R exhibited moderate sensitivity to IFN-λ (EC_{50}: 4.48 and 4.82 ng/ml, respectively) (Fig. 4A). These diverse inhibitory activities of IFN-λ were also confirmed by Western blot analysis (Fig. 4B). Moreover, s1B-4R and sKAH5R showed similar sensitivities to IFN-α. However, it was of note that these replicons exhibited different degrees of sensitivity to IFN-λ, which uses a common Jak/Stat signaling pathway. These results suggest the presence of a complicated antiviral mechanism in type I and III IFNs. Recently, it was reported that IFN-λ in combination with IFN-α or IFN-γ enhanced anti-HCV activity (Pagliaccetti et al., 2008). Therefore, s1B-4R and sKAH5R are useful for the study in combination treatment of IFNs.

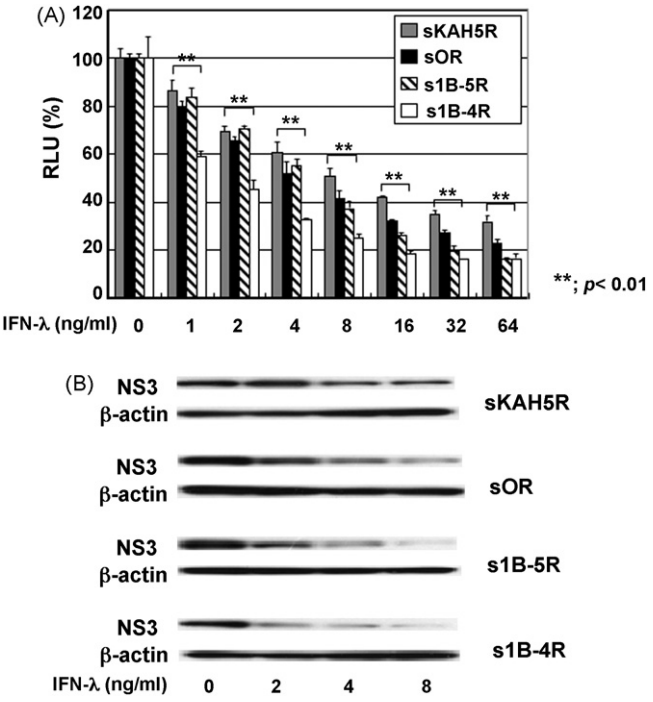

Fig. 4. Effects of IFN-λ on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in IFN-λ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-λ (0, 2, 4, 8, 16, 32, and 64 ng/ml) for 72 h, and then the cells were subjected to RL assay (B) and Western blot analysis. Four replicon-harboring cell types were treated with IFN-λ (0, 2, 4, and 8 ng/ml) for 72 h and were subjected to Western blot analysis of NS3. All of the luciferase assays were repeated at least three times.

3.3. Diverse effects of PTV but not CsA on HCV replicons

Anti-HCV reagents other than IFNs were examined in terms of their effectiveness in the presence of various replicons. As CsA is a well-characterized anti-HCV reagent, we examined the sensitivities of the replicons to CsA by reporter assay. There were no significant differences in sensitivity to CsA among the replicons (Fig. 5). The EC_{50} of CsA to s1B-4R, sKAH5R, sOR, and s1B-5R was 0.71, 0.96, 1.10, and 0.85 μg/ml, respectively. We also obtained similar results by Western blot analysis. In contrast to the findings of the IFN study, there were no significant differences in sensitivity to CsA among the genotype 1b replicons tested.

Statins, which are HMG-CoA reductase inhibitors, are yet another well-characterized anti-HCV reagent. Therefore, we

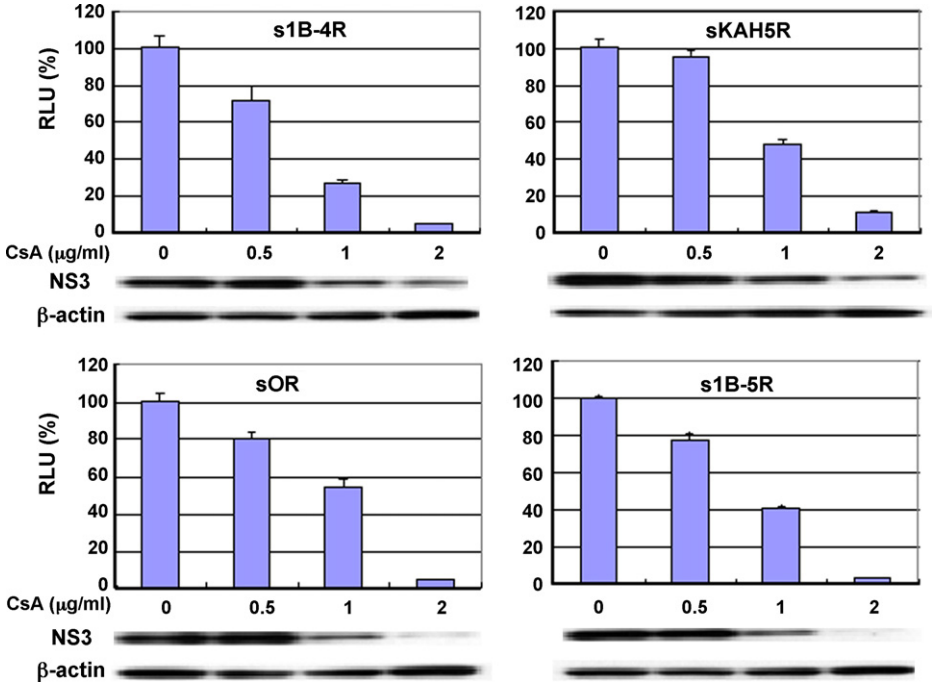

Fig. 5. The activity of CsA on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in CsA treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with CsA (0, 0.5, 1, and 2 μg/ml) for 72 h, and then the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels), as described in Fig. 2. All of the luciferase assays were repeated at least three times.

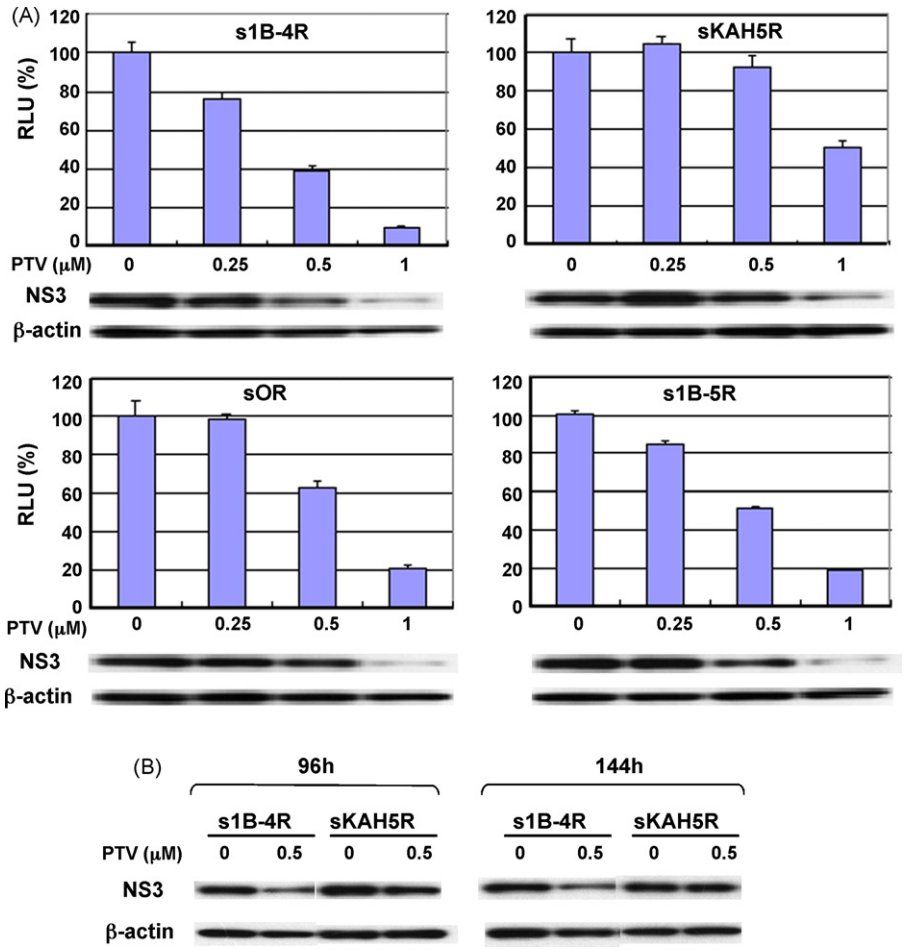

Fig. 6. The activity of PTV on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in PTV treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with PTV (0, 0.25, 0.5, and 1 μM) for 72 h, and then the cells were subjected to RL assay and Western blot analysis, as described in Fig. 2. All of the luciferase assays were repeated at least three times. (B) s1B-4R cells and sKAH5R cells were treated with PTV (0 and 0.5 μM) for 96 and 144 h and were subjected to Western blot analysis.

examined the sensitivity of the replicons to PTV. The reporter assay revealed that sKAH5R has the lowest sensitivity to PTV (EC_{50}: 1.00 μM) among the replicons tested (Fig. 6A). The sensitivities of the other replicons to PTV were almost identical, and the EC_{50} values of s1B-4R, sOR, and s1B-5R were 0.40, 0.64, and 0.51 μM, respectively (Fig. 6A). We also obtained similar results by Western blot analysis using cell lysates at 72 h after treatment. The inhibition of HCV protein in s1B-4R persisted until 96 and 144 h after treatment with PTV (0.5 μM) (Fig. 6B). FBL2 is identified as a geranylgeranylated cellular protein required for HCV RNA replication (Wang et al., 2005). Therefore, we examined the expression levels of FBL2 in s1B-4R and sKAH5R. The expression levels of FBL2 mRNA were almost equal between both cells (Supplemental Fig. 2). This result indicates that low sensitivity of sKAH5R to statins is not due to the low expression of FBL2. Previously, we used an OR6 assay system to demonstrate that PTV inhibited genome-length HCV RNA replication, and the EC_{50} of PTV was found to be 0.45 μM (Ikeda et al., 2006; Ikeda and Kato, 2007). The EC_{50} values of PTV in three replicons other than sKAH5R were almost equal to that of PTV in OR6. These results, taken together, suggest that sKAH5R is resistant to PTV as well as to IFN-γ and IFN-λ.

3.4. Resistance to statins in a replicon from a patient with AH C

To further confirm that sKAH5R is resistant to statins, we examined the sensitivity of the replicons to FLV and RSV using a reporter assay. Here, sKAH5R exhibited the lowest sensitivity to FLV and RSV (Fig. 7). In the case of sKAH5R, the EC_{50} of FLV was 7.87 μM, and the EC_{50} of RSV exceeded 20 μM, because RSV was toxic to cells at concentrations of more than 20 μM. Moreover, sOR and s1B-5R showed almost equal and moderate sensitivities to both FLV and RSV. It was of note that these results were in agreement with those regarding PTV sensitivity, i.e., s1B-4R exhibited the highest sensitivity to both FLV and RSV. The EC_{50} values of FLV and RSV to s1B-4R were 2.82 and 10.12 μM, respectively. These results suggest that sKAH5R exhibits some resistance, and s1B-4R some sensitivity, to statins. Therefore, these replicons may serve as useful tools for investigating the mechanism of the anti-HCV activity of statins.

3.5. Polyclonal KAH5 replicon with a statin-resistant phenotype

sKAH5R replicon cells were found to possess the least sensitivity to statins among the replicon-harboring cells tested. However, the statin-resistant phenotype may be due to cell clonality rather than HCV strain, because the sKAH5R replicon cells used here were a cloned cell line selected from numerous G418-resistant colonies. We thus examined the sensitivity of polyclonal sKAH5R cells to statins, and then compared the results with those obtained using polyclonal s1B-4R cells in order to rule out this possibility. In polyclonal sKAH5R, the EC_{50} values of PTV and FLV were 0.88 and 6.56 μM, respectively (Fig. 8), and the EC_{50} of RSV exceeded 20 μM (Fig. 8), because RSV is toxic to these cells at concentrations of more than 20 μM. In polyclonal s1B-4R, the EC_{50} values of PTV, FLV, and RSV were 0.47, 3.41, and 10.00 μM, respectively (Fig. 8).

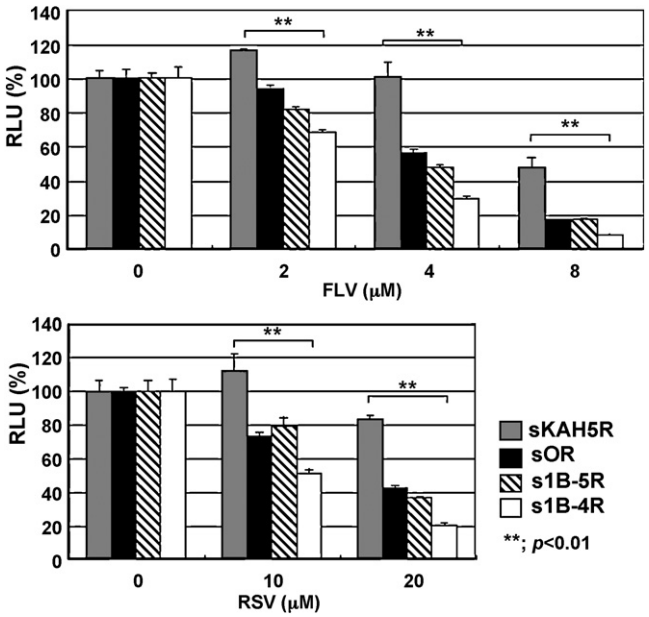

Fig. 7. HCV replicons exhibit diverse sensitivities to statins. Reporter assay of the sensitivity of HCV replicons to FLV. sKAH5R cells (light column), sOR cells (dark column) s1B-5R cells (shaded column), and s1B-4R cells (open column) were treated with FLV (0, 2, 4, and 8 μM) for 72 h (upper panel), and then the cells were subjected to an RL assay. A reporter assay of RSV sensitivity to HCV replicons was performed using RSV (0, 10, and 20 μM) (lower panel). All of the luciferase assays were repeated at least three times.

The polyclonal sKAH5R cells exhibited less sensitivity to PTV, FLV, and RSV than did polyclonal s1B-4R cells. These results suggest that the statin-resistant phenotype of sKAH5R is due to the KAH5 strain-specific viral factors rather than to the cell clonality of sKAH5R cells.

3.6. Second generation of sKAH5R possessed less sensitive phenotype to PTV than that of s1B-4R

To further demonstrate that the statin-resistant phenotype of sKAH5R is not due to the clonal specificity of the cells, we developed the second generation of sKAH5R and s1B-4R. Total RNAs from sKAH5R and s1B-4R were introduced into naïve OR6c cells. The second generation of sKAH5R and s1B-4R, designated as ssKAH5R and ss1B-4R, respectively, were selected as the polyclonal cells after 3 weeks G418 selection. ssKAH5R revealed less sensitive to PTV than ss1B-4R (EC_{50}: 0.76 μM vs. 0.43 μM) (Fig. 9A). These results further support that the viral factor plays the major role in the statin-resistant phenotype of sKAH5R.

On the contrary, there was no significant difference between ssKAH5R and ss1B-4R in the sensitivity to IFN-λ (EC_{50}: 4.1 ng/ml vs. 3.5 ng/ml) (Fig. 9B). These results suggest that cellular factors are dominant in the sensitivity to IFN-λ.

Table 1
EC_{50} of anti-HCV reagents to HCV replicons.

	s1B-4R	sKAH5R	sOR	s1B-5R
IFN-α (IU/ml)	1.44	1.37	2.35	1.10
IFN-γ (IU/ml)	0.54	2.26	0.33	0.21
IFN-λ (ng/ml)	1.50	8.25	4.48	4.82
CsA (μg/ml)	0.71	0.96	1.10	0.85
PTV (μM)	0.40	1.00	0.64	0.51
FLV (μM)	2.82	7.87	4.53	3.81
RSV (μM)	10.12	ND	17.52	17.10

ND: not determined.

4. Discussion

In the present study, we established an HCV replicon reporter assay system using four genotype 1b HCV strains (1B-4, KAH5, O, and 1B-5). Genotype 1 HCV infection accounts for most cases of resistance to current PEG-IFN-α and ribavirin therapy. However, in most previous reports, anti-HCV reagents have been assessed in terms of their effects using replicon(s) derived from only one or two HCV strain(s). Therefore, in order to further evaluate the anti-HCV activity of various reagents among the genotype 1b HCVs, we performed a comparative study using the present replicon reporter assay system, which was found to a precise, highly sensitive, and time-sparing assay compared to assays involving the quantification of HCV RNA. The EC_{50} values of anti-HCV reagents in four genotype 1b replicons are summarized in Table 1.

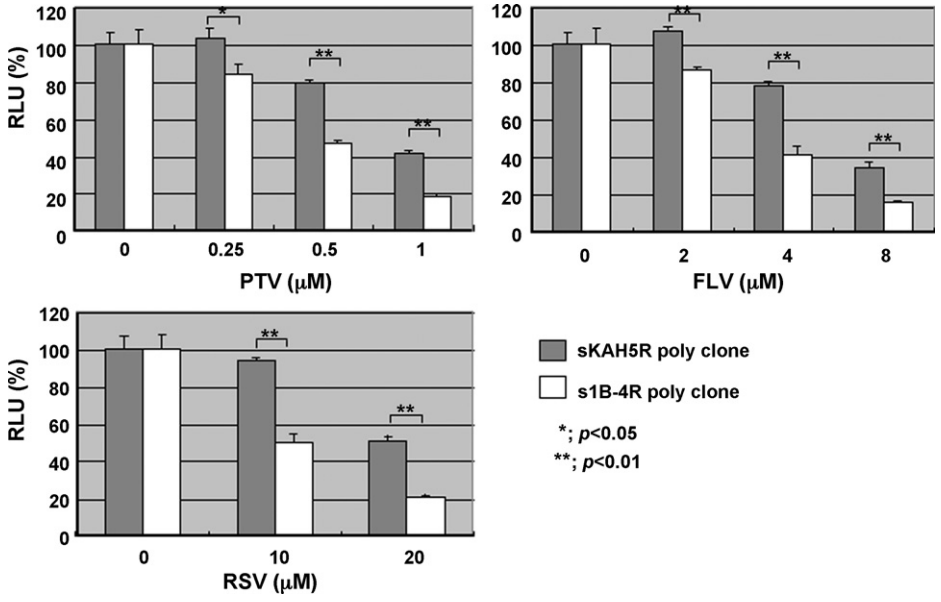

Fig. 8. Diverse sensitivities of polyclonal replicons to statins. Reporter assay of the sensitivity of polyclonal sKAH5R and s1B-4R replicons to PTV, FLV, and RSV. Polyclonal sKAH5R cells and polyclonal s1B-4R cells were treated with PTV (0, 0.25, 0.5, and 1 μM), FLV (0, 2, 4, and 8 μM), and RSV (0, 10, and 20 μM) for 72 h and then were subjected to RL assay. All the luciferase assays were repeated at least three times.

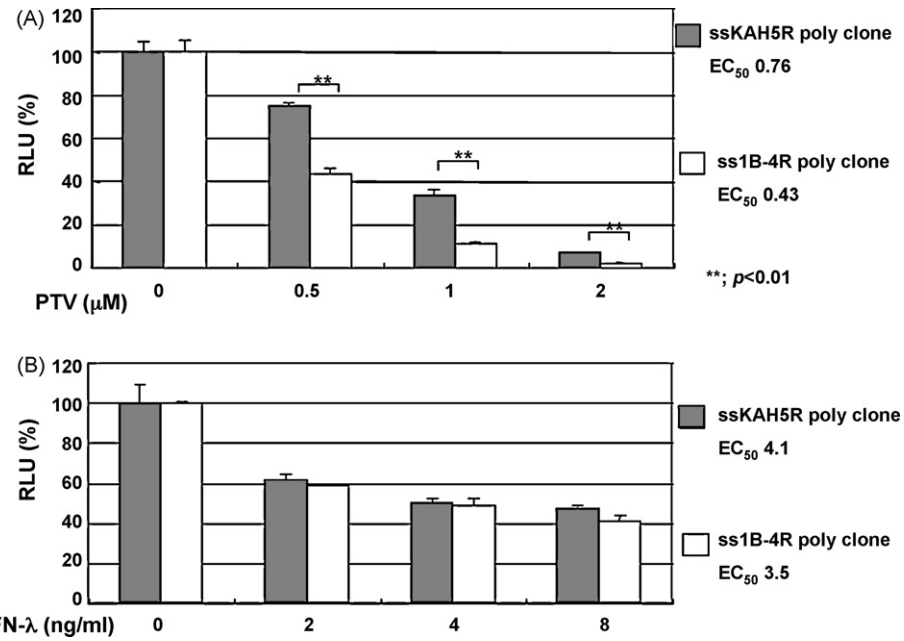

Fig. 9. The sensitivities of the second generation of sKAH5R and s1B-4R to PTV and IFN-λ. Polyclonal second generations of sKAH5R (ssKAH5R) and s1B-4R (ss1B-4R) were treated with PTV (0, 0.5, 1, and 2 μM) (A) and IFN-λ (0, 2, 4, and 8 ng/ml) (B) for 72 h and then were subjected to RL assay. All the luciferase assays were repeated at least three times.

Here, sOR exhibited the lowest level of sensitivity to IFN-α (EC_{50}: 2.35 IU/ml). In the clinical setting, high titers of HCV RNA are among the determining factors for IFN resistance. However, the sensitivity to IFN-α was found to be greater in the case of s1B-4R and sKAH5R than in sOR, although HCV RNA titers were higher than those of sOR. These results suggest that factor(s) other than the HCV RNA level may be involved in conferring sensitivity to IFN-α, and the genetic background of sOR may serve as a candidate for interpreting differences in IFN-α sensitivity among the genotype 1b HCVs tested. Previously the structural region of HCV was shown to be involved in viral resistance to type I IFN (Taylor et al., 1999). Therefore, the development of genome-length HCV RNA reporter systems from 1B-4, 1B-5, and KAH5R strains in addition to our developed OR6 cells will overcome the limitation of subgenomic replicon and will become powerful tool for the study of anti-HCV reagents including IFNs. Now we are planning to develop the genome-length HCV RNA reporter systems using these three HCV strains.

sKAH5R exhibited the lowest level of sensitivity to IFN-γ (EC_{50}: 2.26 IU/ml) among the replicons tested, as it is approximately 10 times more resistant to IFN-γ than s1B-5R (EC_{50}: 0.21 IU/ml). KAH5 was the only HCV strain derived from a patient with AH C in the present study. In our previous study, an AH1 strain derived from a patient with AH C also exhibited lower sensitivity to IFN-γ (EC_{50}: 1.9 IU/ml) than did O strain (EC_{50}: 0.3 IU/ml) in the genome-length HCV RNA replication system (Mori et al., 2008). In both subgenomic and genome-length HCV RNA replication systems, HCV strains from patients with AH C possess less sensitivity to IFN-γ than do HCV strains from healthy carriers. These results suggest that the NS region of HCV derived from AH C may be involved in IFN-γ resistance.

In 2003, IFN-λ was identified by two groups at the same time, and this novel IFN was classified as type III IFN. IFN-λ shares the Jak/Stat signaling pathway with the type I IFNs, although they bind to distinct membrane receptors, i.e., type I IFNs bind to the heterodimer of IFNAR1 and IFNAR2, whereas type III IFNs bind to the heterodimer of IFNLR1 and IL10R2 (Uze and Monneron, 2007). Therefore, we expected to obtain similar IFN-α sensitivity results in the four replicons tested here. However, unexpectedly, the profiles of replicon sensitivity to IFN-α and IFN-λ differed. The sensitivity of s1B-4R to IFN-λ was approximately five times greater than that of sKAH5R, although the sensitivities of these replicons to IFN-α were almost identical. There are several possible interpretations of these unexpected findings. First, an unidentified branched signaling pathway may account for variation in the anti-HCV activity of IFN-λ. Second, expression levels of the receptor for IFN-λ may vary. The second generation replicon assays suggest that the cellular factors may be dominant in the sensitivity to IFN-λ. Further study will be needed to clarify this issue. The anti-HCV activity of IFN-λ has already been reported by several groups using HCV RNA-harboring cells (Doyle et al., 2006; Marcello et al., 2006; Robek et al., 2005). The present study was the first to demonstrate the diverse anti-HCV activities of IFN-λ on HCV replicons.

In the case of CsA, we did not observe any significant differences among the genotype 1b replicons. Using a genome-length HCV RNA replication system, we recently demonstrated that an AH1 strain obtained from an AH C patient showed greater sensitivity than did an O strain (Mori et al., 2008). There are two possible explanations for this high sensitivity to CsA in the replicon derived from the AH C case: first, a high degree of sensitivity to CsA may not be a common feature in HCV from AH C and may instead be strain-dependent; second, a particular structural region of HCV from AH C may be responsible for this high level of sensitivity to CsA. However, further study will be needed to fully account for these findings.

Respectively, sKAH5R and s1B-4R exhibited the lowest (EC_{50}: 1.00 μM) and highest (EC_{50}: 0.40 μM) levels of sensitivity to PTV among the replicons tested. We also confirmed that sKAH5R and s1B-4R possessed the lowest and highest levels of sensitivity, respectively in both FLV and RSV treatment. Therefore, resistance to statins may be a unique feature of sKAH5R. It should be noted that we obtained these results using polyclonal sKAH5R and polyclonal s1B-4R cells. The polyclonal sKAH5R cells were less sensitive to PTV, FLV, and RSV than were the polyclonal s1B-4R cells. Therefore, the statin-resistant phenotype of sKAH5R cells is due to a KAH5 strain-specific characteristic, rather than to the clonality of the cells. The second generation of replicon harboring cells, ssKAH5R and ss1B-4R, further supported that the viral factor plays the major role in the statin-resistant phenotype of sKAH5R. These two cell lines with contrasting sensitivity to statins promise to be useful for

determining the statin resistance-responsible region of HCV and also for investigating the anti-HCV mechanism of statins in general.

In the present study, we demonstrated the diverse profiles of four HCV replicons to anti-HCV reagents. sKAH5R showed the lowest sensitivity to IFN-γ, IFN-λ, and statins (PTV, RSV, and FLV). In contrast, s1B-4R exhibited the highest level of sensitivity to IFN-λ and statins (PTV, RSV, and FLV). sKAH5R and s1B-4R possessed a sensitive and a resistant phenotype to various anti-HCV reagents. The nucleotide sequences in the NS3-NS5B regions of 1B-4, 1B-5, and KAH5 strains showed differences of 6.5%, 8.6%, and 6.1%, respectively, from those of the O strain. Similarly, the amino acid sequences in the NS3–NS5B regions of 1B-4, 1B-5, and KAH5 strains showed differences of 2.6%, 4.7%, and 2.5%, respectively, from those of the O strain. Phylogenetic analysis revealed that O, 1B-4, and KAH5 strains formed the cluster different from 1B-5 strain (Supplemental Fig. 3). These data indicate that sKAH5R and s1B-4R are at a similar genetic distance from the O strain. These two replicons were also found to possess similar features in terms of HCV RNA expression levels and sensitivity to IFN-α. Therefore, sKAH5R and s1B-4R are expected to be useful tools for comparative analyses of anti-HCV determining factors of HCV, especially as regards IFN-λ and statins.

In conclusion, we established an HCV replicon reporter assay system with four different genotype 1b HCV strains. This replicon system is a useful tool for investigating differences in sensitivity to anti-HCV reagents among genotype 1b HCV strains, and it is expected to increase the rate of resolution of HCV cases otherwise resistant to current IFN therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2009.01.007.

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