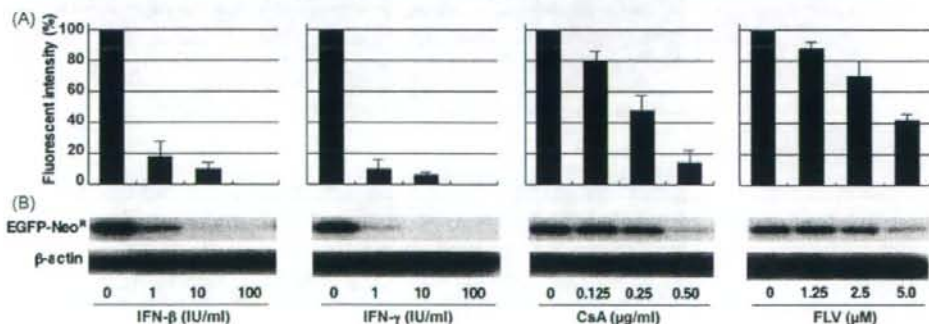


**Fig. 5.** Effect of IFN- $\alpha$  on genome-length HCV RNA replication in OGF7 and OR6 cells. OGF7 and OR6 cells were treated with IFN- $\alpha$  (0, 1, 10, and 100 IU/ml) for 72 h. After the measurements of the fluorescent intensity of OGF7 cells, the cells were subjected to quantitative RT-PCR analysis 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- $\alpha$ , 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.

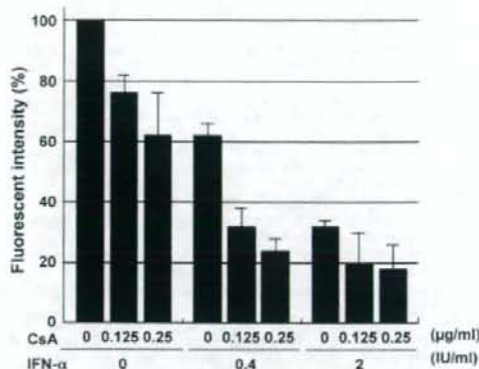


Fig. 7. Effect of IFN- $\alpha$  in combination with CsA on genome-length HCV RNA replication in OGF7 cells. OGF7 cells were co-treated with IFN- $\alpha$  (0, 0.4, and 2.0 IU/ml) and CsA (0, 0.125, and 0.25  $\mu$ g/ml), and at 72 h after treatment, the fluorescent intensity of OGF7 cells was measured 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>a</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 AH3553R, 5'-CACACGCCGTGATCC AGGTCG-3' was used for RT. Primers 21 [11] and AH3519R, 5'-TGCCTGGCCG

<sup>a</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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TGGAAACCACCTG-3' were employed in the first round of PCR (35 cycles). An internal primer pair (21X [11] and AH3466RX: 5'-ATTATCTAGAGGCTGTGAGACTG 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'-ATTATCTAGACTGACATGGGACCAAGATCATCAC-3'; containing a XbaI site (underlined) and 9357RX: 5'-ATTATCTAGACCCGTTCCAGGTTGGGGAGCAG-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'-ATTATCTAGCTCACAGG CCGGACAGAAGACC-3'; containing a SpeI site (underlined) and 9162RB: 5'-ATTATCTAGCGCCAGTTGAAGAGGTACTTGGCC-3'; containing a BsiWI site (underlined). The amplified fragment was digested with SpeI and BsiWI, and ligated into the replicon cassette plasmid pNS5RZ2RU [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)<sub>2</sub> 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)<sub>2</sub> 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].

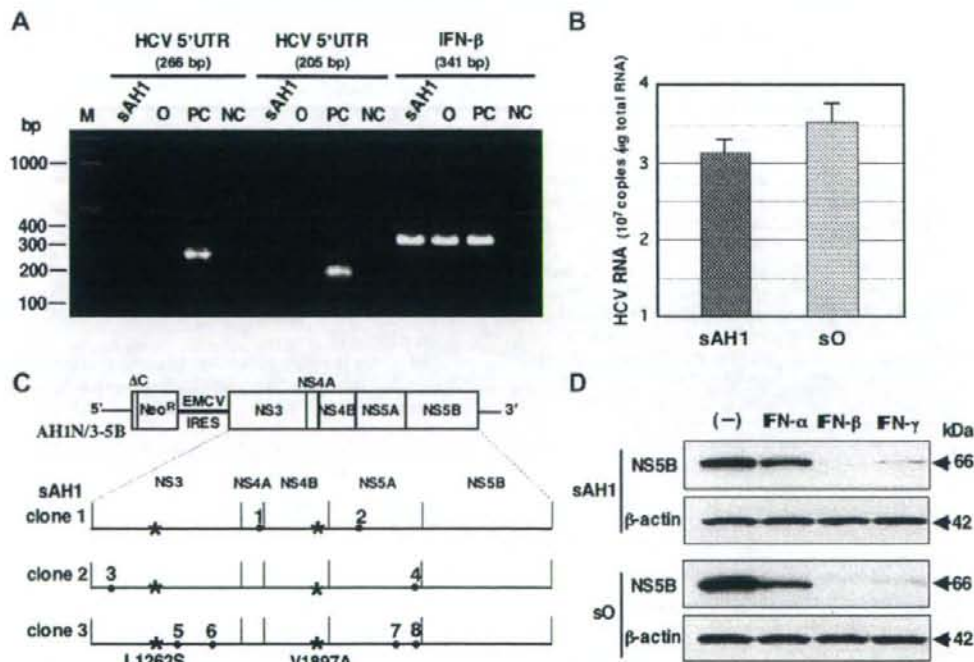
Western blot analysis. The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as previously described [11]. The antibodies used in this study were those against Core, E2, NS3, NS4A, NS5A, and NS5B [10].  $\beta$ -Actin antibody (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.

## Results

### Establishment of a G418-resistant cell line (sAH1) harboring HCV replicon RNA

An HCV replicon RNA library prepared from the AH1 strain was first transfected into sOc cells (cured sO cells) [11], and the G418-resistant cells were selected as described previously [11]. Although several G418-resistant colonies were obtained, production of these colonies was due to integration of the HCV RNA sequence into the chromosomal DNA (PC in Fig. 1A). Therefore, we further cleaned up the replicon RNA library with additional DNase treatment, and it was then transfected into OR6c cells (cured OR6 cells) [10]. Consequently, a G418-resistant colony was obtained and successfully proliferated; this colony was referred to as sAH1. To exclude the possibility of integration of a replicon RNA sequence into the genomic DNA, we examined the presence of the HCV 5'UTR sequence in the genomic DNA isolated from sAH1 cells by a PCR method described previously [11]. Genome-length HCV RNA-replicating O cells were also examined as a negative control. The results revealed that the HCV RNA sequence was not integrated into the genomic DNA in either sAH1 cells or O cells (Fig. 1A).

Regarding the level of replicon RNA in sAH1 cells, RT-qPCR analysis revealed that the titer of replicon RNA was approximately  $3 \times 10^7$  copies/ $\mu$ g total RNA, and its level was equivalent to that in sO cells (Fig. 1B), suggesting that the efficiency of RNA replication in sAH1 cells is similar to that in sO cells.

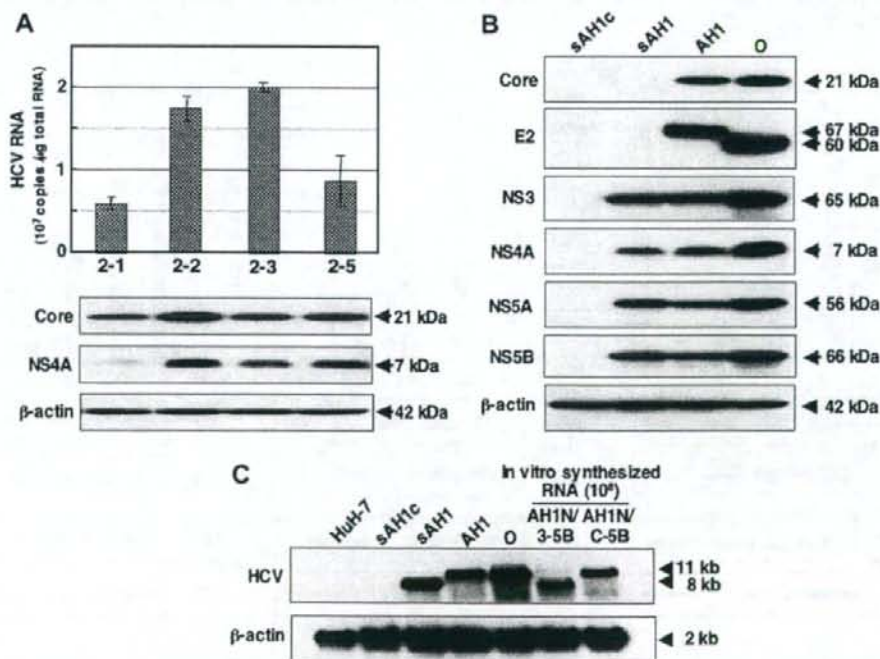
To exclude the possibility that sAH1 cells were derived from a small number of OR6 cells remaining after IFN treatment, and to determine whether replicon RNA in sAH1 cells possesses cell culture-adaptive mutations [5], which enhance the efficiency of RNA replication, we performed genetic analysis of the intracellular

AH1 replicon. The sequences of three independent clones were determined and compared with each other to avoid PCR error. The obtained consensus nucleotide and aa sequences of NS3–NS5B regions of the AH1 replicon showed 7.3% and 3.7% differences, respectively, from those of the HCV-O replicon [11], indicating that sAH1 cells were not contaminated by the OR6 cells. In contrast, to find conserved mutations in the AH1 replicon, we determined the consensus nucleotide sequences of AH1 serum-derived HCV RNA by comparison of the nucleotide sequences of three independently isolated cDNA clones (Accession No. AB429050). The K1609E (NS3) and S2200R (NS5A) adaptive mutations found in O and OR6 cells were not detected in the AH1 replicon. However, instead of these mutations, L1262S (NS3) and V1897A (NS4B) conserved mutations were detected (Fig. 1C). Although V1897A has been detected as an adaptive mutation in Con-1 replicon [15], L1262S has until now remained undetected. In clone 2, the P1115L mutation (number 3 in Fig. 1C), which has been reported as an adaptive mutation [15,16], was detected.

To further characterize the sAH1 replicon, we compared the sensitivities of sAH1 and sO replicons against anti-HCV reagents (IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$ ) [5,6,11]. Western blot analysis of NS5B revealed that the IFN sensitivity of the sAH1 replicon was equivalent to that of the sO replicon (Fig. 1D).

### Establishment of a genome-length HCV-AH1 RNA-replicating cell line, AH1

To develop a genome-length HCV RNA replication system, we first constructed a pAH1N/C-5B/PL, LS, (VA)<sub>2</sub> by the replacement with sAH1 replicon clone 2 (Fig. 1C) into pAH1N/C-5B, AH1N/C-5B/PL, LS, (VA)<sub>2</sub> RNA was transfected into sAH1c cells, cured sAH1 cells. Following 3 weeks of culturing in the presence of



**Fig. 2.** Characterization of AH1 cells harboring genome-length HCV RNA. (A) Selection of G418-resistant cell lines. The levels of HCV RNA in G418-resistant cells were quantified by LightCycler PCR (upper panel). Core and NS4A were detected by Western blot analysis (lower panel). (B) Western blot analysis. AH1, O, sAH1, and sAH1c cells were used for the comparison. Core, E2, NS3, NS4A, NS5A, and NS5B were detected by Western blot analysis. (C) Northern blot analysis. AH1, O, sAH1, sAH1c, and HuH-7 cells were used for the comparison. In vitro-synthesized AH1N/3-5B and AH1N/C-5B RNAs were also used for the comparison.



G418, several colonies were obtained, and 4 colonies (2-1, 2-2, 2-3, and 2-5) then successfully proliferated. We selected colony 2-2 among them because it showed high levels of HCV RNA and proteins (core and NS4A) (Fig. 2A); this cell line was referred to as AH1. To compare the expression levels of HCV proteins in AH1 cells with those in O cells, Western blot analysis was further performed. Although the levels of HCV proteins in AH1 cells were slightly lower than those in O cells, the expression levels of NS proteins in AH1 cells were equivalent to those in sAH1 cells (Fig. 2B). In this analysis, we noticed that the size of the E2 protein in AH1 cells was 7 kDa larger than that in O cells (Fig. 2B). This difference may be due to the different numbers of N-glycosylation sites in E2 protein, since 11 and 9 N-glycosylation sites in E2 proteins are estimated in AH1 and HCV-O strains, respectively. Northern blot analysis also showed the presence of HCV-specific RNA with a length of approximately 11 kb in the extracts of total RNA prepared from AH1 cells, similar to that in the O cells (Fig. 2C). We confirmed the presence of replicon RNA (approximately 8 kb) in sAH1 cells (Fig. 2C). To check the additional adaptive mutations in the genome-length AH1 RNA, we performed sequence analysis of HCV RNA in AH1 cells. The results (Supplementary Fig. 2) revealed no additional mutations detected commonly among the three independent clones sequenced, suggesting that additional adaptive mutations are not required for genome-length HCV RNA replication. We therefore conclude that the AH1 cell line can be used as a genome-length HCV RNA replication system with acute hepatitis C-derived HCV strain.

#### Diverse effects of anti-HCV reagents on HCV RNA replication in AH1 and O cells

To compare the effects of anti-HCV reagents on RNA replication systems with different HCV strains, we examined the anti-HCV profiles of IFN- $\alpha$ , IFN- $\gamma$ , and cyclosporine A (CsA) [17] using AH1 and O

cells. Regarding IFN- $\alpha$ , the anti-HCV effect in AH1 cells was similar to that in O cells (Fig. 3A). Although RT-qPCR analysis showed a statistically significant difference in both cell systems when 1 IU/ml of IFN- $\alpha$  was used, such a difference was not observed in the Western blot analysis (Fig. 3A). In contrast, a significant different effect of IFN- $\gamma$  was observed in both cell systems. RT-qPCR and Western blot analyses revealed that RNA replication of the AH1 strain was less sensitive than that of the HCV-O strain when 1 or 10 IU/ml of IFN- $\gamma$  was used (Fig. 3B). Conversely, we observed that RNA replication of the AH1 strain was more sensitive to CsA than that of the HCV-O strain (Fig. 3C). These results suggest that anti-HCV profiles of IFN- $\gamma$  and CsA are rather different between AH1 and O cell systems.

Different anti-HCV profile of IFN- $\gamma$  is not correlated with the cellular potentials of the IFN- $\gamma$  signaling pathway

To clarify whether the different effects of IFN- $\gamma$  observed between AH1 and O cells are dependent on the cellular potentials of the IFN- $\gamma$  signaling pathway, we performed a dual-luciferase reporter assay using an IFN- $\gamma$ -inducible intrinsic GBP-1 gene promoter. As a control, IFN- $\alpha$ -inducible intrinsic 2'-5'-OAS gene promoter was also used for the analysis of the IFN- $\alpha$  signaling pathway. The results revealed that a good response of both AH1 and O cells to IFN- $\alpha$  and IFN- $\gamma$  stimulation, with their potentials for both signaling pathways being almost the same (Fig. 4). These results suggest that the diverse anti-HCV effects of IFN- $\gamma$  are dependent on the HCV strain, but not on the cellular potentials of the IFN- $\gamma$  signaling pathway.

#### Discussion

In the present study, we established for the first time an HCV RNA replication system with AH1 strain derived from a patient

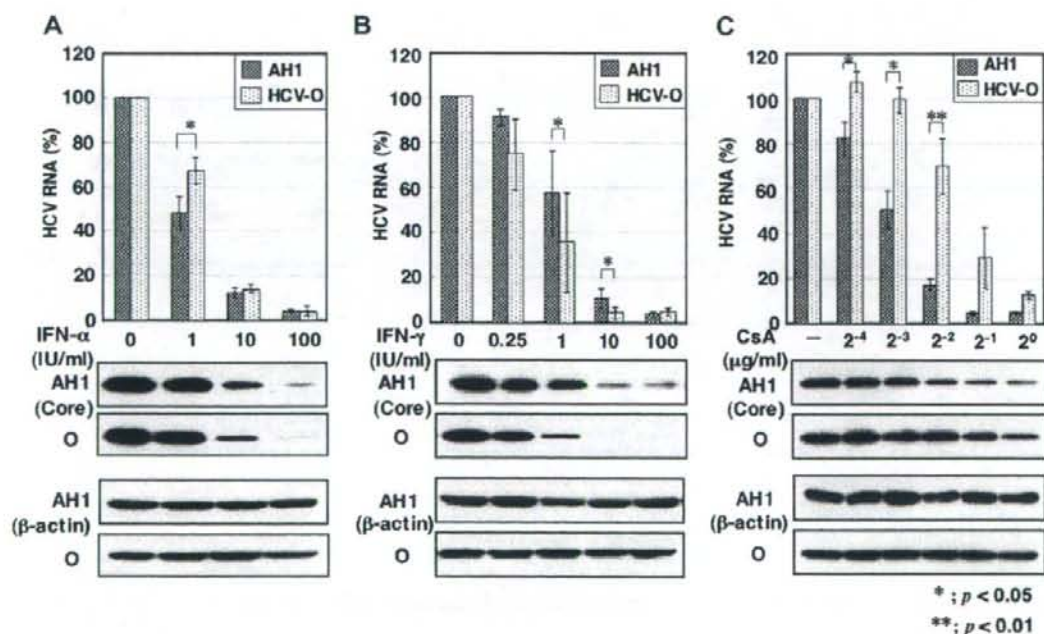


Fig. 3. The diverse effects of anti-HCV reagents on AH1 and HCV-O RNA replications. AH1 and O cells were treated with anti-HCV reagents for 72 h, and then extracted total RNAs and cell lysates were subjected to RT-qPCR for HCV 5' UTR (each upper panel) and Western blot analysis for the core protein (each lower panel), respectively. (A) Effect of IFN- $\alpha$ . (B) Effect of IFN- $\gamma$ . (C) Effect of CsA (Sigma).

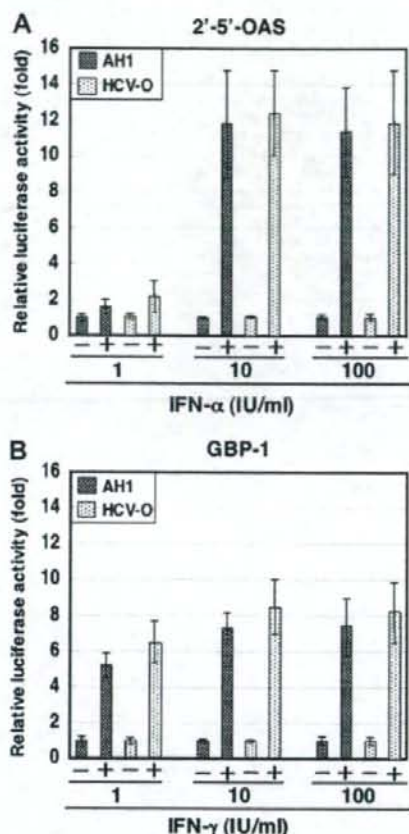


Fig. 4. Dual-luciferase reporter assay of IFN- $\alpha$  or IFN- $\gamma$ -inducible gene promoter. AH1 and O cells were treated for 6 h with IFN- $\alpha$  or IFN- $\gamma$  before the reporter assay. (A) 2'-5'-OAS gene promoter. (B) GBP-1 gene promoter.

with acute hepatitis C, and found diverse anti-HCV effects of IFN- $\gamma$  and CsA between AH1 and HCV-O strains.

The levels of HCV replicon RNA and genome-length HCV RNA in sAH1 and AH1 cells were assigned to  $3 \times 10^7$  and  $2 \times 10^7$  copies/ $\mu$ g total RNA, respectively. These values are similar to those obtained from previously established HCV RNA replication systems [5]. Since known adaptive mutations (P1115L and V1897A) and additional conserved mutations (L1262S) were detected in the developed sAH1 replicon, these mutations may contribute to enhanced levels of RNA replication. The expression levels of genome-length HCV RNA and proteins observed in the present study suggest that genome-length HCV RNA replication efficiently occurs in AH1 cells, and that this RNA replication system is useful for comparison with already developed genome-length HCV RNA replication systems with HCV-N [7], Con-1 [8,9], or HCV-O [10] strains.

In the comparative analysis of genome-length HCV RNA replication systems with AH1 and HCV-O strains, we found that IFN- $\gamma$  and CsA showed different anti-HCV profiles between AH1 and HCV-O strains. Regarding IFN- $\gamma$ , RNA replication of the AH1 strain ( $EC_{50} = 1.9$  IU/ml) was less sensitive than that of the HCV-O strain ( $EC_{50} = 0.3$  IU/ml). Windisch et al. [18] have previously reported that RNA replication in an HCV replicon system using HuH-6 hepatoma cells is highly resistant ( $EC_{50}$  was more than 100 IU/ml) to IFN- $\gamma$ , and that its resistant phenotype is not due to a general

defect in the IFN- $\gamma$  signaling pathway. In that study, they speculated that some mutations within a critical effector gene in HuH-6 cells might account for the inability of the cells to reduce the number of replicon RNAs in response to IFN- $\gamma$ . Although such a possibility is not completely excluded, the diverse effects of IFN- $\gamma$  observed in the present study were likely due to the difference in viral strains because RNA replication of the AH1 strain is still sensitive to IFN- $\gamma$ . To clarify this point, development of an additional HCV RNA replication system such as an OR6 assay system with more quantitative reporter genes [10] is needed.

Regarding CsA, RNA replication of the AH1 strain ( $EC_{50} = 0.13$   $\mu$ g/ml) showed more sensitivity than that of the HCV-O strain ( $EC_{50} = 0.35$   $\mu$ g/ml). Ishii et al. [17] have previously reported that RNA replication of the JFH1 strain (genotype 2a) is less sensitive to CsA than genotype 1b strains, including the HCV-O strain. In that study, they concluded that the difference in sensitivity of JFH1 and genotype 1b strains to CsA could be attributed to characteristic differences in the HCV strains, not to the parent cell strain. In addition, sensitivity to CsA was almost the same among genotype 1b strains in that study. Therefore, we estimate that the AH1 strain is more sensitive to CsA than these genotype 1b strains examined to date. Further analysis will be necessary to clarify the mechanism underlying differences in sensitivity to CsA among genotype 1b strains.

In conclusion, an HCV RNA replication system with the AH1 strain would be useful for comparison with other strain-derived systems in various HCV studies, including analysis of the effects of anti-HCV reagents.

#### Acknowledgments

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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.bbrc.2008.04.005.

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## Differential Effects of Calcineurin Inhibitors, Tacrolimus and Cyclosporin A, on Interferon-Induced Antiviral Protein in Human Hepatocyte Cells

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The premise of our study is that selective inhibition of interferon (IFN) by calcineurin inhibitors contribute to the increased severity of hepatitis C virus (HCV) posttransplantation. Therefore, we examined the influence of calcineurin inhibitors in the human hepatocyte cell line on IFN- $\alpha$ -induced phosphorylation of Janus kinase (Jak) and signal transducers and activators of transcription (STAT), nuclear translocation of IFN-stimulated gene factor 3 (ISGF-3), IFN-stimulated regulatory element (ISRE)-contained promoter activity, and the expressions of antiviral proteins. Tacrolimus (Tac), but not cyclosporin A (CyA), had an inhibitory effect on IFN- $\alpha$ -induced double-stranded ribonucleic acid (RNA)-dependent protein kinase (PKR) in a dose-dependent manner. STAT-1 also acted in a similar fashion to PKR. IFN- $\alpha$  combined with Tac attenuated the ISRE-containing promoter gene activity as compared with IFN- $\alpha$  alone. In contrast, its expression in pretreated CyA was slightly attenuated. In pretreated Tac, but not CyA, the levels of IFN- $\alpha$ -induced tyrosine phosphorylated STAT-1 and -2 were clearly lower than those induced by IFN- $\alpha$  alone. Tac and CyA did not decrease the IFN- $\alpha$ -induced JAK-1 phosphorylation. The nuclear translocation rate of tyrosine phosphorylated STAT-1 was inhibited by pretreatment of both Tac and CyA by western blotting and immunohistochemistry. In an HCV replicon system, pretreated Tac diminished the replication inhibitory effect of IFN- $\alpha$ . In this study, we show that calcineurin inhibitors, especially Tac, are the negative regulators of IFN signaling in the hepatocyte; the greatest cause of such inhibition is the phosphorylation disturbance of STAT-1, next to inhibition of the nuclear translocation of STAT-1. In conclusion, disturbance of tyrosine phosphorylation of STAT-1 resulted in diminished ISRE-containing promoter activity and a decline in antiviral protein expression. Moreover, the replication of HCV was activated. This phenomenon is detrimental to IFN therapy after liver transplantation, and the selection of calcineurin inhibitors may warrant further discussion depending on the transplant situation. *Liver Transpl* 14:292-298, 2008. © 2008 AASLD.

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Hepatitis C virus (HCV) infection is widespread worldwide. A major problem of chronic HCV infection is hep-

atocellular carcinoma. Currently, liver transplantation for HCV-related liver disease is an option worldwide.<sup>1</sup> Recently, it has been demonstrated that the prognosis for liver transplantation patients with HCV-related disease deteriorates with time,<sup>2</sup> resulting in a poorer out-

**Abbreviations:** CyA, cyclosporin A; HCV, hepatitis C virus; IFN, interferon; ISGF-3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated regulatory element; Jak, Janus kinase; NF-AT, nuclear factor of activated T cells; PKR, double-stranded RNA-dependent protein kinase; RNA, ribonucleic acid; STAT, signal transducers and activators of transcription; Tac, tacrolimus.

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come than in the non-HCV course.<sup>3,4</sup> The transplanted liver in HCV-related disease undergoes a rapid progression of fibrosis and worsens to cirrhosis and graft failure.<sup>5</sup> The factors for a worsening outcome were speculated to be increased donor age,<sup>3-5</sup> stronger immunosuppression,<sup>3</sup> and high levels of HCV-ribonucleic acid (RNA) at transplantation.<sup>4</sup> These factors have no small effect on the reinfection and reactivation of HCV in the graft liver.

Reinfection of HCV in the graft liver is rapid after transplantation, and the virus immediately proliferates in the graft. In the natural course of reinfection, approximately 10 to 25% of recipients will develop cirrhosis, and a strategy for the prevention of reinfection has not been developed.<sup>6</sup> At present, treatment of HCV after transplantation is inadequate, and does not result in a cure.<sup>7</sup> Recently, pegylated interferon (IFN) and ribavirin combination therapy has been effective in the treatment of HCV genotype 1a chronic hepatitis, with a sustained viral response rate of 45%.<sup>8</sup> However, reinfection after transplantation is the norm despite combined therapy.<sup>9,10</sup> Meanwhile, the patients with a sustained viral response after transplantation show no progression or reversal of liver fibrosis.<sup>11,12</sup> The refractory nature of pegylated IFN and ribavirin combination therapy for liver transplantation patients contributes to a worsening outcome in HCV-related transplantation.

We speculated that posttransplantation immunosuppression is part of the reason for IFN resistance to HCV reinfection of the graft liver. Methylprednisolone pulse therapy is a risk factor for severe outcome after transplantation, and the treatment of acute cellular rejection using heavy immunosuppressive agents is also a risk factor.<sup>3,4,6</sup> Previous reports described the fact that glucocorticoid inhibits the expression of signal transducers and activators of transcription (STAT)-1, as a signal transduction factor of IFN, and diminishes the signaling of IFN.<sup>13</sup> However, the effects on HCV reinfection and IFN therapy by calcineurin inhibitors, the most frequently used immunosuppressants, have not been fully evaluated, until now. Therefore, we have attempted to evaluate the influences of calcineurin inhibitors on IFN signaling in the hepatocytes.

IFN- $\alpha$  and  $\beta$ , after binding to their receptors, stimulate the intracellular IFN-signaling cascade including the Janus kinase (Jak)-STAT tyrosine kinases, the phosphorylation of STAT-1 and -2, and the formation of IFN-stimulated gene factor 3 (ISGF-3), which consists of STAT-1, STAT-2, and p48.<sup>14</sup> ISGF-3 translocates into the nucleus and binds to the IFN-stimulated regulatory element (ISRE) in the promoter sequences of a variety of IFN-inducible genes, including antiviral proteins such as double-stranded RNA-dependent protein kinase (PKR).<sup>15</sup> Several negative regulation systems of Jak-STAT signaling, including the suppressor of cytokines signaling family, the protein inhibitor of activated STAT family, and the SH2-containing protein tyrosine phosphatase family, are notorious contributors to a state of inflammation and carcinogenesis in the hepatocyte.<sup>16,17</sup> In addition, the nucleus-cytoplasm transport of ISGF-3 was regulated by translocated specific pro-

teins along with the phosphorylation of STAT.<sup>18</sup> We examined the influence of calcineurin inhibitors on IFN-induced phosphorylation of Jak and STAT, nuclear translocation of ISGF-3, ISRE contained promoter activity, and the expressions of antiviral proteins.

## MATERIALS AND METHODS

### Reagents and Cell Culture

Recombinant human IFN- $\alpha$ 2b, tacrolimus (Tac), and cyclosporine A (CyA) were generous gifts from Schering-Plough KK (Tokyo, Japan), Astellas Co. (Tokyo, Japan), and Novartis Pharma Co. (Basel, Switzerland), respectively. Hc human hepatocyte cells (Applied Cell Biology Research Institute, Kirkland, WA) and HuH-7 human hepatoma cells (American Type Culture Collection, Rockville, MD) were maintained in a chemically-defined medium, CS-C completed (Cell Systems, Kirkland, WA) and RPMI (Invitrogen, Grand Island, NY), respectively, supplemented with 5% fetal bovine serum. In the pretreatment of calcineurin inhibitors, the cells were cultured in 5% RPMI containing varying concentrations of Tac and CyA, and then the medium was exchanged and the cells were treated with IFN 100 IU/mL at the indicated time.

### HCV Replicon System

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE<sup>19</sup> were used to examine the influence on the anti-HCV effect of IFN of calcineurin inhibitors. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL; Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin; Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After the treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to luciferase assay according to the manufacturer's protocol.

### Western Blotting and Antibody

Western blotting with anti-PKR, anti-STAT-1, anti-STAT-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-tyrosine-701 phosphorylated STAT-1, anti-tyrosine-689 STAT-2, anti-JAK-1 or anti-tyrosine 1022/1023 JAK-1 (New England Biolabs, Beverly, MA) was performed as described previously.<sup>20</sup> Briefly, Hc cells were lysed by the addition of lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% sodium dodecyl sulfate buffer, 150 mmol/L NaCl, 1 mmol/L ethylene diamine tetraacetic acid, 1 mmol/L phenylmethanesulfonyl fluoride, 1  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin, 1 mmol/L sodium *o*-vanadate, and 1 mmol/L NaF). Extraction of nucleus and cytoplasm were performed using the NE-PER Nuclear and Cyto-



plasmic Extraction kit (Pierce, France). Samples were analyzed by electrophoresis on 8 to 12% sodium dodecyl sulfate buffer polyacrylamide gel and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G, and the immunoreactive bands were visualized by the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England). The density of each band was quantified using the National Institutes of Health image analysis software program.

### Reporter Gene Assay

pISRE-Luc containing 5 copies of the ISRE sequence and firefly luciferase gene and pRL-SV40 containing SV40 early enhancer/promoter and *Renilla* luciferase gene were obtained from Clontech (San Diego, CA) and Promega, respectively. The HuH-7 cells were grown in 24-well multiplates and transfected with 1  $\mu$ g of pISRE-Luc and 10 ng of pRL-SV40 as a standard by the lipofection method. One day later, the cells were incubated in the absence or presence of varying concentrations of Tac, CyA, and IFN- $\alpha$ , and the luciferase activities in the cells were determined using a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative ISRE-luciferase activity.

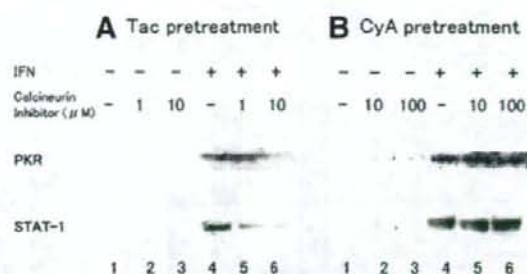
### Fluorescence Immunohistochemistry

The Hc cells were seeded onto 11-mm glass coverslips in 24-well plates at 240,000 cells/well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 10  $\mu$ mol/L of Tac, 100  $\mu$ mol/L of CyA, or vehicle, for 16 hours and then stimulated with 100 IU/mL of IFN- $\alpha$  for 10 minutes. Fluorescence immunohistochemistry was performed as described previously.<sup>21</sup> The cells were incubated with anti-tyrosine-701 phosphorylated STAT-1 antibody for 1 hour at room temperature, washed 3 times in phosphate buffered saline, incubated with rhodamine-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour, washed in phosphate buffered saline, and mounted in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Nuclear staining was performed using Hoechst 33258 (Invitrogen Japan K.K., Tokyo, Japan). Immunofluorescence analysis was done by an Olympus BX50 microscope (Tokyo, Japan) and the image was captured by a Nikon DXM 1200 digital camera (Tokyo, Japan).

## RESULTS

### Differential Effects of Tac and CyA on IFN- $\alpha$ -Induced Antiviral Protein Expression

To elucidate how calcineurin inhibitors exert influence on IFN-induced antiviral protein, the Hc cells were incubated in the absence or presence of IFN- $\alpha$  after the



**Figure 1.** Effect of (A) Tac and (B) CyA on IFN- $\alpha$ -induced PKR and STAT-1. Hc cells were treated with 100 IU/mL of IFN- $\alpha$  in the absence (lane 4) or presence of pretreatment (lanes 5 and 6). Indicated concentration of calcineurin inhibitor alone was lanes 2 and 3, lane 1 was not treated with IFN- $\alpha$  and calcineurin inhibitors. One day later, PKR and STAT-1 were determined by western blotting.

presence or absence of pretreatment of Tac (Fig. 1A) or CyA (Fig. 1B) for 16 hours, and then were harvested for the western blot analysis. Pretreated Tac had an inhibitory effect on IFN- $\alpha$ -induced PKR expression, antiviral protein as messenger RNA translation inhibitor activated by double-stranded RNA dependent, in a dose-dependent manner, but no inhibitory effect of pretreatment CyA for PKR expression was recognized in our experiment. STAT-1 is an essential signal transmitter substance of IFN and IFN-inducible proteins.

The expression of IFN-inducible STAT-1 also decreased in a dose-dependent manner after the administration of Tac, but not after the administration of CyA.

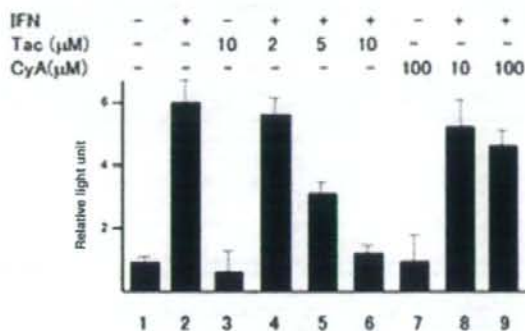
### Alterations of IFN- $\alpha$ -Stimulated Reporter Gene Expression by Tac and CyA

Because the formation of IFN stimulating gene factor (ISGF) 3 by IFN- $\alpha$  leads to transactivation of the ISRE in the promoter regions of the IFN- $\alpha$ -inducible genes, we performed the reporter gene transfection assay using plasmids containing ISRE in their promoter sequence. Because there were not enough Hc cells for reporter gene transfection, we used HuH-7 cells in the transfection assay. HuH-7 cells were transfected with pISRE-Luc containing 5 repeats of ISRE sequence and pRV-SV40 as a standard and then were treated with IFN- $\alpha$  after 16 hours in the presence or absence of pretreated Tac or CyA (Fig. 2). Tac and CyA alone did not influence the ISRE-luciferase activities. IFN- $\alpha$  combined with Tac and attenuated its expression compared with IFN- $\alpha$  alone. In contrast, there was a slight attenuation effect of its expression in 100  $\mu$ mol/L of pretreated CyA.

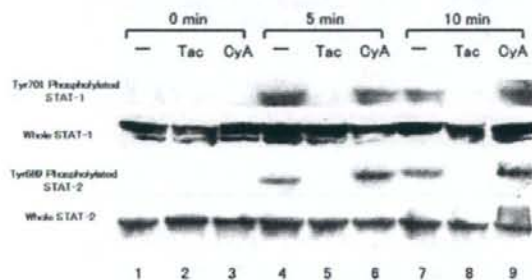
### Inhibitory Effect of Tac on IFN- $\alpha$ -Induced Tyrosine Phosphorylation of STATs

The activation of STAT-1 and -2 by phosphorylation of tyrosine-701 and 689 residues, respectively, is essential for the relay of IFN- $\alpha$  signal with the formation of ISGF-3. Therefore, we examined the effect of Tac and CyA on the IFN- $\alpha$ -induced tyrosine phosphorylation of



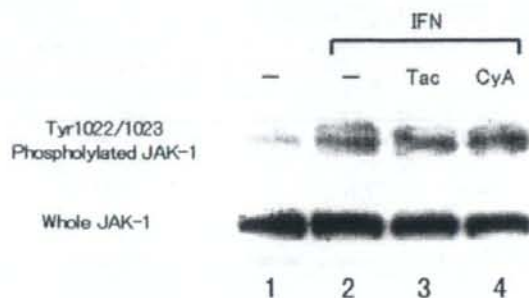


**Figure 2.** Suppression effect of calcineurin inhibitors on IFN- $\alpha$ -induced reporter gene assay. HuH-7 cells transfected with reporter gene (pISRE-Luc and pRL-SV40) were either untreated (lanes 1, 2) or pretreated with Tac (lanes 3-6) or CyA (lanes 7-9) for 16 hours, followed by IFN- $\alpha$  100 IU/mL (lanes 2, 4-6, 8, and 9) or absence (lanes 3 and 7). Six hours later, the relative ISRE-luciferase activity ( $n = 4$ ) was determined as described in Materials and Methods. The data are expressed as the mean  $\pm$  SD and are representative examples of four similar experiments.



**Figure 3.** Effect of calcineurin inhibitors on STAT-1 and STAT-2. After pretreatment with 10  $\mu$ M Tac (lanes 2, 5, and 8) and 100  $\mu$ M CyA (lanes 3, 6, and 9) for 16 hours, Hc cells were untreated (lane 1) or treated with 100 IU/mL IFN- $\alpha$  (lanes 4-9) for the indicated periods and phosphorylated STAT-1 at tyrosine-701 residue (first panel), expression of STAT-1 (second panel), phosphorylated STAT-2 at tyrosine-689 residue (third panel), and expression of STAT-2 (fourth panel) were analyzed by western blotting. The density of each band was quantified and the nuclear translocation rate was calculated by the following: nuclear intensity (N)/nuclear intensity (N) + cytoplasmic intensity (C).

STAT-1 and -2 (Fig. 3). IFN- $\alpha$  clearly induced the tyrosine phosphorylation of STAT-1 and -2, but Tac and CyA could not. However, when the Hc cells were pretreated with Tac, but not CyA, before IFN- $\alpha$  stimulation, the levels of tyrosine phosphorylated STAT-1 and -2 were clearly lower than those induced by IFN- $\alpha$  alone. In the case of pretreatment with CyA, the IFN- $\alpha$ -induced tyrosine phosphorylation levels were similar to IFN- $\alpha$  alone. Then, the cells were changed from Hc cells to HuH-7 cells and a similar experiment was done. The inhibitory effect of Tac to IFN- $\alpha$ -induced STAT-1 and -2 tyrosine phosphorylation was the same (data not shown).



**Figure 4.** Evaluation of IFN- $\alpha$ -induced phosphorylated JAK-1 by calcineurin inhibitors. After pretreatment of 10  $\mu$ M Tac (lane 3) and 100  $\mu$ M CyA (lane 4) for 16 hours, Hc cells were untreated (lane 1) or treated with 100 IU/mL IFN- $\alpha$  (lanes 2-4) for 3 minutes, then phosphorylated JAK-1 at tyrosine-1022/1023 residue (first panel) and expression of JAK-1 (second panel) were analyzed by western blotting.

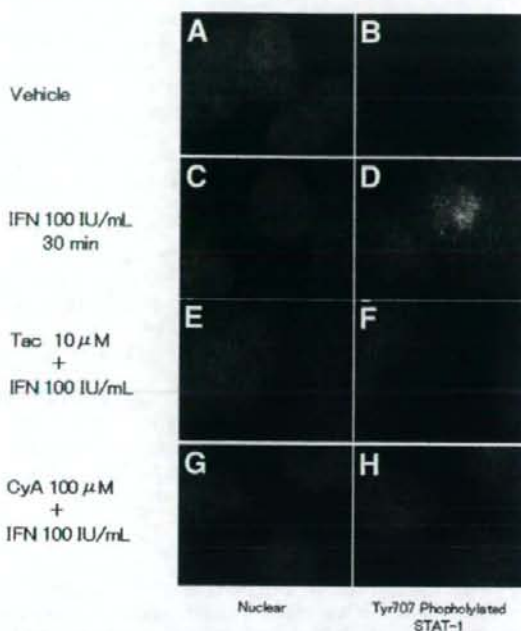


**Figure 5.** Alteration of distribution of IFN- $\alpha$ -induced phosphorylated STAT-1 and STAT-2 by calcineurin inhibitors. Hc cells were pretreated with absence (lanes 1-4) or presence of 10  $\mu$ M Tac (lanes 5 and 6) or 100  $\mu$ M CyA (lanes 7 and 8). And then, Hc cells were stimulated by 500 IU/mL IFN- $\alpha$  (lanes 3-8) for 10 minutes. Hc cells harvested by extraction kit of nucleus (lanes 1, 3, 5, and 7) and cytoplasm (lanes 2, 4, 6, and 8). Phosphorylated STAT-1 at tyrosine-701 residue (upper panel) and phosphorylated STAT-2 at tyrosine-689 residue (lower panel) were analyzed by western blotting.

When we performed western blotting of phosphorylated JAK-1 under the same conditions, Tac and CyA did not decrease the IFN- $\alpha$ -induced JAK-1 phosphorylation (Fig. 4).

### Influence of Calcineurin Inhibitors on IFN- $\alpha$ -Induced Nuclear Translocation of Tyrosine Phosphorylated STATs

For transcription of the IFN- $\alpha$ -induced antiviral gene, the ISGF-3 complex, including activated STAT-1, STAT-2, and p48, could be translocated to the nucleus. Initially, we detected tyrosine phosphorylated STAT-1 and -2 extracted it from the nucleus and cytoplasm by western blotting. In this experiment, detectable band intensities were quantified by National Institutes of Health image software and we evaluated the nuclear translocation rate of activated STAT-1 and -2 (Fig. 5).

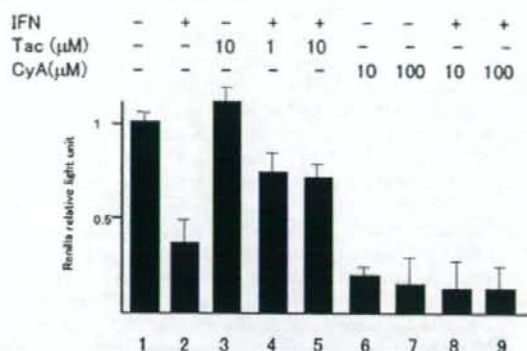


**Figure 6.** Inhibition of IFN- $\alpha$ -induced nuclear translocation of phosphorylated STAT-1 by calcineurin inhibitor. Hc cells were pretreated in the absence (A-D) or presence of 10  $\mu\text{mol/L}$  Tac (E,F) or 100  $\mu\text{mol/L}$  CyA (G,H). After pretreatment, Hc cells were stimulated by 100 IU/L IFN- $\alpha$  (C-H) for 30 minutes. Thereafter, the cells were fixed, permeabilized, processed for immunofluorescence (B,D,F,H) and Hoechst staining (A,C,E,G), and visualized with fluorescence microscopy. The results shown are from one representative experiment from a total of three performed.

The total IFN- $\alpha$ -stimulated tyrosine phosphorylated STAT-1 was decreased by pretreatment with Tac; furthermore, the nuclear translocation rate of activated STAT-1 was inhibited both by pretreatment with Tac and CyA. However, in the case of pretreatment with Tac and CyA, there was no effect on the nuclear translocation of tyrosine phosphorylated STAT-2. Secondly, we evaluated the location of tyrosine phosphorylated STAT-1 by fluorescence immunohistochemistry of cultured Hc cells (Fig. 6). The IFN- $\alpha$ -induced nuclear translocation of tyrosine phosphorylated STAT-1 was observed, but its translocation was inhibited by pretreatment with Tac. Along with the nuclear translocation rate of activated STAT-1 by western blotting (Fig. 5), pretreatment with Tac also attenuated the nuclear staining of activated STAT-1 compared to IFN- $\alpha$  alone, but did not attenuate the expression of activated STAT-1 by immunohistochemistry.

#### Inhibitory Effect of Tac on IFN- $\alpha$ -Induced Anti-HCV Efficiency

To examine the effect of calcineurin inhibitors on IFN- $\alpha$ , we used the full-length HCV replication system, OR6 cells. The cells were treated with IFN- $\alpha$  after 16 hours in



**Figure 7.** Alteration of IFN- $\alpha$ -suppressed HCV replication by Tac. OR6 cells, full-length replicon system, were treated with 100 IU/mL of IFN- $\alpha$  in the absence (lane 2) or presence of pretreatment (lanes 4, 5, 8, and 9). Indicated concentration of calcineurin inhibitor alone was lanes 3, 6, and 7, lane 1 was not treated with IFN- $\alpha$  and calcineurin inhibitors. One day later, *Renilla* luciferase activity was determined by luminometer.

the presence or absence of pretreated Tac or CyA (Fig. 7). IFN- $\alpha$  or CyA alone repressed the *Renilla* luciferase activity, which is well correlated with HCV-RNA concentration in OR6 cells.<sup>19</sup> In contrast, Tac alone had little effect on *Renilla* luciferase activity. However, pretreatment with Tac attenuated the IFN- $\alpha$ -induced repression of *Renilla* luciferase activity (Fig. 7; lane 2 versus lanes 4 and 5), but pretreatment with CyA did not (Fig. 7; lanes 8 and 9).

#### DISCUSSION

We herein show that calcineurin inhibitors, especially Tac, are negative regulators of IFN signaling in the hepatocyte, and the greatest cause of this phenomenon is phosphorylation of STAT-1, next to inhibition of nuclear translocation of STAT-1. Disturbance of STAT-1 phosphorylation caused diminished ISRE-containing promoter activity, for example PKR and STAT-1, and antiviral protein expression declined. Pretreatment with Tac diminished the replication inhibitory effect of IFN- $\alpha$ . This phenomenon has a detrimental effect on IFN therapy after HCV-related liver transplantation. In our experiments, we speculated that Tac is not better suited for posttransplantation IFN therapy than CyA, but it did not report that IFN- $\alpha$  response is different between Tac and CyA in human study in previous time. When the alternative of potent immunosuppressant for prevention of rejection, or antiviral-activity for HCV reactivation is weighed, we might need to consider other factors in choosing between Tac and CyA. We had compared high concentration CyA with low concentration Tac, since rejection was controlled by serum trough values of tacrolimus of 5 ng/mL and of cyclosporin of 100 ng/mL in our hospital in the period of stability after liver transplantation.

Recently, the difference between Tac and CyA has been regarded in another function than immunosuppression, and we presume that this discrepancy de-



pendent on differences of "immunophilins." Immunophilins are a ubiquitous family of proteins. All cells contain several members of this family, which bind specific calcineurin inhibitors and participate in many cellular functions.<sup>22</sup> Tac has been reported to have neuroprotection,<sup>23</sup> but CyA did not, whereas CyA had anti-HCV action,<sup>24-26</sup> but Tac did not. Tac binds specific FK506 binding protein members of the immunophilin family, whereas cyclosporin binds a different subset of immunophilins (cyclophilins). FK506 binding protein and CyP have the same function as peptidyl prolyl *cis-trans* isomerase and they inhibited the nuclear translocation of nuclear factor of activated T cells (NF-AT). Despite this common pathway, the cell protection activity has been reported to require the induction of heat shock protein 70 by Tac but not CyA,<sup>27</sup> and the anti-HCV activity contributed to a specific blockade of CyP B by CyA.<sup>25</sup> The differences in the medical effects for immunosuppression between Tac and CyA require attention, when these immunosuppressants are used in posttransplantation-related HCV infection.

In our study, the IFN-induced tyrosine phosphorylated STAT-1 and -2 both decreased after the administration of Tac, but Tac is known essentially for the inhibition of serine/threonine protein phosphatase. Calcineurin, regardless of independent Jak-1 tyrosine phosphorylation, and CyA did not have such a tyrosine phosphatase action against STAT-1 and -2. We could not resolve this phosphatase mechanism, but we speculated that Tac induced the tyrosine phosphatase kinase and inhibited tyrosine phosphorylation of STAT-1 and -2. Tac did not induce suppression of cytokines signaling-1 and 3, Jak inhibitors, by western blotting in our study (data not shown); however, we could not rule out the induction of other types of tyrosine phosphatase. Previous studies described that suppressor of cytokines signaling-1, 3 and SH2-containing protein tyrosine phosphatase inhibited NF-AT activation,<sup>28-30</sup> and therefore the relationship between Tac and tyrosine phosphatase might be reconsidered. Barat and Tremblay<sup>31</sup> and Zhu and McKeon<sup>32</sup> previously described the protein-tyrosine phosphatase inhibitor bisperoxovanadium as a potent activator of T cell receptor signaling, and SH2-containing protein tyrosine phosphatase-1, T cell protein-tyrosine phosphatase, Tac, and CyA are inhibitors of such activation. We were interested in the inhibition of protein-tyrosine phosphatase inhibitor by Tac and CyA, because Tac and CyA possessed the same action as SH2-containing protein tyrosine phosphatase-1 and protein-tyrosine phosphatase.<sup>32</sup> Furthermore, this action of Tac was stronger than CyA.<sup>31</sup> From these studies, we assume that Tac has tyrosine phosphatase action in the hepatocyte and inhibits tyrosine phosphorylation of STAT-1 and -2.

The inhibition of IFN-induced antiviral proteins by Tac, and the inhibition of nuclear trafficking of tyrosine phosphorylated STAT-1, is the common phenomenon between Tac and CyA in this study. This phenomenon was observed in the western blotting findings (Fig. 3) and immunohistochemistry of the cultured cells (Fig. 6).

NF-AT activation requires the suppression of Crm1-

dependent export from nucleus to cytoplasm by calcineurin,<sup>33</sup> and the presence of importin, bounded to calcineurin, in the nucleus.<sup>34</sup> In IFN-induced Jak-STAT signaling, nuclear trafficking of ISGF-3 requires suppression of Crm1 and binding importin<sup>16</sup> in the same fashion as NF-AT. Calcineurin inhibitors bind to immunophilin and inhibit dephosphorylation of NF-AT, then they inhibit the transcription activity of NF-AT. In addition to such action, it might be considered that the nuclear trafficking of NF-AT is regulated by the calcineurin inhibitor and immunophilin complex. We speculated that the decrease of the nuclear import of tyrosine phosphorylated STAT-1 is the function, the calcineurin inhibitor and immunophilin complex modified Crm1 and importin in the same fashion as NF-AT. Then, we recognized that the mechanisms of diminished tyrosine phosphorylation STATs and nuclear translocation STAT-1 were different.

Presently, there is no definite opinion regarding the selection of calcineurin inhibitors for liver transplantation.<sup>6</sup> However, reports of inhibition of HCV replication by CyA *in vitro* were noted recently<sup>24-26</sup> and the result were same in our full-length replicon system (Fig. 7). In our data, we consider that CyA has the effect of, not only the previously reported anti-HCV replication action itself, but it creates much less interference with IFN treatment for HCV reactivated after liver transplantation than does Tac. It has been reported that CyA increased the chance of a sustained viral response after liver transplantation.<sup>35</sup> However, we used care with our data, because both Tac and CyA inhibit the nuclear translocation of tyrosine phosphorylated STAT-1. Our data revealed that when an excess of CyA was used after liver transplantation, it resulted in a decrease in the amount of IFN-induced antiviral protein, because of inhibition of nuclear transportation of tyrosine phosphorylated STAT-1 (Figs. 5 and 6). The immunosuppression levels of Tac and CyA have already been reported to decrease significantly in patients responding favorably to anti-HCV therapy post-liver transplantation.<sup>36</sup> In this study, we therefore considered it necessary to pay attention to an excess dose of CyA, when IFN treatment for reactivation of HCV is required.

In conclusion, Tac has been shown to influence the tyrosine phosphorylation of STAT-1, and the result was a decline in antiviral protein PKR. In addition, Tac and CyA have been shown to interfere with the translocation of STAT-1. We speculated that posttransplantation immunosuppression is part of the reason for IFN resistance to HCV reinfection of the graft liver. As the course, calcineurin inhibitors, especially Tac, were pointed out in this study, and we clarified a part of the IFN resistance. Although the mechanism of inhibition of IFN signaling has not yet been fully investigated, it is necessary to compare the antirejection action of Tac to the anti-HCV action of CyA when selecting calcineurin inhibitors.

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## Possible Molecular Mechanism of the Relationship Between NS5B Polymorphisms and Early Clearance of Hepatitis C Virus During Interferon Plus Ribavirin Treatment

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We previously reported the relationship between viral polymerase polymorphisms and the initial decline in viral load induced by interferon- $\alpha$  and ribavirin therapy in genotype 1b-related chronic hepatitis C patients. The presence of E124K and I85V of NS5B was closely associated with viral clearance at 8 weeks of treatment. The aim of this study was to investigate the mechanisms by which this polymorphism of NS5B protein affects early viral clearance. We used a replicon system derived from strain O, genotype 1b virus. Three mutants (V85I), (K124E), and (V85I/K124E) were introduced to the replicon. OR6c, a derivative of HuH7 cells, was transfected with the replicon including a luciferase reporter gene. Luciferase activities were measured 72 hr post-transfection. All three mutants showed higher luciferase activity than that of the wild type, and the V85I mutant showed the highest activity. This result was also confirmed by neomycin gene-containing replicons with same mutations. All replicons were down-regulated by ribavirin, but the level of reduction in the V85I mutant was the lowest. Our results suggested that this mutation at least partly contributes to resistance to early viral clearance during interferon and ribavirin combination therapy. *J. Med. Virol.* 80:632–639, 2008. © 2008 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis C virus; NS5B polymorphism; replicon; interferon and ribavirin combination therapy; viral proliferation

### INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public

health. An estimated 65–80% of the individuals infected with HCV develop persistent infection while 20–50% develop cirrhosis and 5% develop hepatocellular carcinoma (HCC) [Liang et al., 2000; Gao et al., 2004]. Until recently, interferon (IFN)- $\alpha$  and IFN- $\beta$  were the only available treatments for HCV infection, although only 10–15% of treated subjects achieved sustained viral eradication with IFN monotherapy, and early viral clearance after initiation of IFN monotherapy was correlated with sustained viral clearance [Saito et al., 2000].

The current approved treatment for HCV infection is pegylated IFN- $\alpha$  (peg-IFN) in combination with ribavirin (RBV). This combination therapy leads to viral clearance in 50–80% of cases, depending on the infecting HCV genotype, and 50% of patients with HCV genotype 1b and high baseline levels of viral RNA do not achieve a sustained virological response with the combination therapy after 48 weeks [Manns et al., 2001; Fried et al., 2002; Feld and Hoofnagle, 2005]. Several prior studies have attempted to predict the efficacy of IFN plus RBV combination therapy. A quantitative measurement of HCV viremia or the initial decline in viral load is a reliable marker for early prediction of the therapeutic response to IFN and RBV combination therapy [Zeuzem et al., 1998; Bouvier-Alias et al., 2002; Takahashi et al., 2005; Lukasiewicz et al., 2007].

RBV is a broad-spectrum nucleoside analogue antiviral drug which is especially noted for its actions

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against RNA viruses and exhibits *in vitro* activity against some DNA and RNA viruses, including certain members of *Flaviviridae* [Sidwell et al., 1972]. It has recently been demonstrated that the antiviral activity of RBV can result from the ability of a viral RNA-dependent RNA polymerase (RdRP) to utilize RBV triphosphate and to incorporate this nucleotide into the viral genome with reduced specificity, thereby mutagenizing the genome and decreasing the yield of infectious virus [Crotty et al., 2000; Lanford et al., 2003]. Moreover, RBV exhibits an antiviral effect through a mechanism of error-prone replication in the HCV subgenomic replication system [Contreras et al., 2002]. Although RBV by itself cannot decrease serum HCV RNA levels in patients, it has been demonstrated that combination therapy with RBV and either IFN- $\alpha$  or peg-IFN yields a higher sustained response rate than is achieved with IFN- $\alpha$  monotherapy [Pol et al., 2000; Poynard et al., 2000; Saracco et al., 2001].

We previously reported the relationship between viral RdRP polymorphisms and the initial decline in viral load induced by IFN- $\alpha$  and RBV therapy in genotype 1b-related chronic hepatitis C patients [Kumagai et al., 2004]. Substitution of glutamic acid to lysine at the 124th position (E124K) and of isoleucine to valine at the 85th position (I85V) of NS5B was closely associated with viral clearance at 8 weeks of treatment.

In this study, we used the genotype 1b HCV replicon system [Ikeda et al., 2005] to generate NS5B mutants (E124K, I85V, and both) and we compared the replication activity with that of the wild-type replicon and to analyze how this polymorphism of NS5B protein affects early viral clearance during combination therapy with IFN and RBV. We also examined the significance of NS5B polymorphisms in the RBV-induced decrease in viral replication. We concluded that the identified polymorphism of NS5B partly affects viral replication.

## MATERIALS AND METHODS

### Cell Culture System

OR6 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM), in addition to G418 (300  $\mu$ g/ml; Geneticin, Invitrogen), and were then passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN- $\alpha$  treatment (500 IU/ml for 2 weeks) without G418, as previously described [Ikeda et al., 2005].

### Plasmids

The plasmids pON/C-5B/KE (Fig. 1a) and pHCV-O were described previously [Ikeda et al., 2005]. This plasmid includes the adaptive mutation of K1609E of NS3 to enhance the efficiency of replication, this adaptive mutation was reported by Lohman et al. [22]. The plasmid pON/C-5B/KE contains neomycin phosphotransferase (Neo) downstream of HCV IRES and the full length HCV-O polyprotein coding sequence downstream of encephalomyocarditis virus (EMCV) IRES. To introduce a pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/KE/(V85I&K124E), we first made PCR fragments including the partial NS5B region with the primers 5'-ggatcccgatctcagcgacgg-3' and 5'-tctagaggtccattcgcattac-3'. This 2.4-kb fragment was subcloned into pSTBlue1 Blunt vector (Novagen, Madison, WI) to generate pSTBlue-1MN002. Each vector expressing the V85I mutant, K124E mutant, and V85I&K124E double mutant of HCV-O was generated by Quick Change mutagenesis (Stratagene, La Jolla, CA) to generate pSTBlueMN002(V85I), pSTBlueMN002(K124E) pSTBlueMN002(V85I&K124E). Next, pON/C-5B was

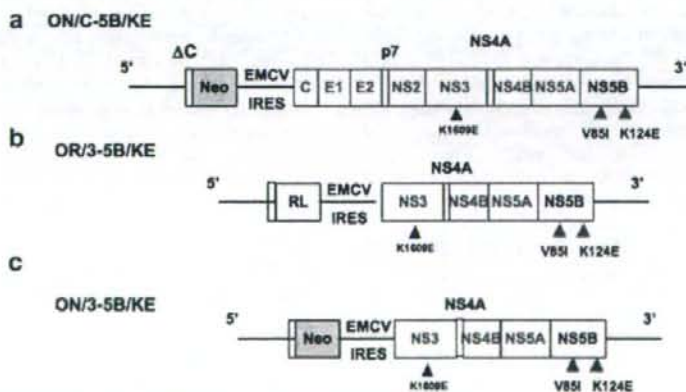


Fig. 1. a: Organization of genome-length HCV RNA derived from HCV-O. Open reading frames, untranslated regions, EMCV IRES, and Neo genes are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively.  $\Delta$ C indicates the 12 N-terminal amino acid residues of the core as a part of IRES. This construct also contains adaptive mutation K1609E which is indicated by a black triangle. We use this

construct as a wild type. Grey triangle is the position of 85 and 124 in NS5B which we generated mutation to the replicon for this experiment. b: The construct of the reporter subgenomic HCV replicon carries the renilla luciferase gene (RL). c: The construct of the reporter subgenomic HCV replicon carries the Neo gene.



digested with *Sna*1 and *Xba*1 and subcloned into pSTBlue-1 to create pSTBlueMN001. All of the pSTBlueMN002 mutants were digested with *Bam*H1 and *Xba*1, which were subcloned into pSTBlueMN001 to create pSTBlueMN001 mutant. The pSTBlueMN001 mutants were digested with *Sna*1 and *Xba*1 and re-ligated in pON/C-5B/KE to introduce pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/(V85I&K124E). The plasmids pOR/3-5B/KE/(V85I), pOR/3-5B/KE/(K124E) and pOR/3-5B/KE/(V85I&K124E), were constructed from pOR/3-5B/KE (Fig. 1b) by swapping for fragments of pSTBlueMN001 mutants digested with *Sna*1 and *Xba*1. DNA sequencing of the manipulated regions of the plasmids verified all mutations.

#### RNA Transfection and Selection of G418-Resistant Cells

For electroporation, OR6c cells were washed twice with ice-cold phosphate buffered saline (PBS) and resuspended at  $10^7$  cells/ml in PBS. Twenty microgram of ON/C-5B/KE or its mutant derived RNA was mixed with 500  $\mu$ l of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25  $\mu$ F, and maximum resistance. Following 10 min of incubation at room temperature, cells were seeded into 10-cm dishes. Cells were selected in complete DMEM with 300  $\mu$ g/ml G418. About 3 weeks after transfection and G418 selection, cells were fixed and stained with Coomassie brilliant blue (0.6 g/l in 50% methanol–10% acetic acid) and the number of colonies was counted.

#### Transient-Replication Assays With Luciferase Replicons

OR6c cells were transfected by electroporation as the same protocol described above using 20  $\mu$ g of OR/3-5B/KE or its mutants derived RNAs carrying the renilla luciferase (RL) gene. After addition of 2 ml of complete DMEM,  $2 \times 10^4$  of aliquot OR6c cells were plated in 24-well plates at least in triplicate for each assay and harvested at various time points with renilla lysis reagent (Promega KK, Tokyo, Japan) and subjected to the RL assay according to the manufacturer's protocol (Promega). Values obtained with cells harvested 6 hr after electroporation were used to correct for the transfection efficiency.

#### IFN and Ribavirin Treatment

To monitor the anti-HCV effect of IFN and RBV on replication, OR6c cells were transfected by electroporation using 10  $\mu$ g of OR/3-5B/KE derived RNAs as described elsewhere [Crotty et al., 2000]. OR6c cells ( $2 \times 10^4$ /well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 hr. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, and 20 units/ml or RBV at a final concentration of 50, 100, and 200  $\mu$ M for 72 hr, harvested with renilla

lysis reagent (Promega), and assayed for luciferase activity according to the manufacturer's protocol. We also studied about the additional effect of RBV (100  $\mu$ M) on IFN (1 u/ml).

#### Cell Viability

We checked toxic effect of IFN and RBV. Effect of IFN (1 and 4 units/ml), and RBV (50 and 100  $\mu$ M) on cell viability was investigated. To examine the cytotoxic effect of IFN and RBV on OR6c cells with OR/3-5B/KE replicon RNA, the cells were seeded at a density of  $2 \times 10^5$  cells per dish onto 6-well plates. After 24-hr culture, the cells were treated with IFN or RBV at final concentrations of 2 and 4 units/ml or 50 and 100  $\mu$ M, respectively, in the absence of G418. After incubation for 72 hr, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen) treatment.

#### Indirect Immunofluorescence

Cells were grown on four-well chamber slides until 70–80% confluent, washed three times with PBS, and fixed in methanol–acetone (1:1, v/v) for 10 min at room temperature. Dilutions of primary murine monoclonal antibody to residues 21–40 of the core protein (2Zcp11; Tokushu Men-eki Institute, Tokyo) (1:1,000), were prepared in PBS containing 3% bovine serum albumin and incubated with fixed cells for 2 hr at room temperature. After additional washes with PBS, specific antibody binding was detected with a goat anti-mouse immunoglobulin G-fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Cells were washed with PBS, and mounted in DAKO Fluorescent mounting medium (DAKO Japan, Tokyo, Japan) prior to examination using a Zeiss AxioPlan2 fluorescence microscope.

#### Statistical Analysis

Difference in relative luciferase activity among mutant replicons and differences in anti-HCV activity of RBV among mutant replicons were tested using Student's *t*-test and Mann–Whitney *U*-test as appropriate. *P*-values <0.05 were considered statistically significant.

## RESULTS

#### Mutation in NS5B Enhances Levels of Replication on Transient Assay

To investigate whether the mutations in NS5B of the HCV genome affect replication, we used subgenomic HCV replicons with the renilla luciferase gene for transient assay [Ikeda et al., 2005]. In a previous study [Kumagai et al., 2004], substitution of glutamic acid at the 124th position with lysine and substitution of isoleucine at the 85th position with valine in NS5B yielded a complete match with the population of good



responders (5 out of 5 patients). We introduced mutations to two different types of replicon to obtain ON/C-5B/KE(V85I), ON/C-5B/KE/(K124E), ON/C-5B/KE(V85I&K124E), OR/3-5B/KE/(V85I), OR/3-5B/KE(K124E), and OR/3-5B/KE(V85I&K124E) as described in Materials and Methods Section. The subgenomic replicons with V85I showed higher replication activity than the wild-type replicon in OR6c cells (Fig. 2). Also the replicon with K124E and the replicon with V85I&K124E showed slightly higher replication activity than the wild type, but the replicon with K124E single amino acid mutation did not show statistically higher replication activity than the wild type (Fig. 2). We initially expected that double mutations (V85I&K124E) would lead to better replication than either of the single mutations (V85I or K124E), but interestingly the V85I mutation on NS5B replicated best. This result indicated that the level of replication was affected by amino acid substitution at the 85th position.

#### Mutation in NS5B Enhances the Efficiency of Colony Formation in Cured Cells

In colony formation assay, we used cured subgenomic replicon cells (OR6c), since cured cells enhanced colony formation of the replicon more efficiently than did parental HuH-7 cells. We examined the effect of these mutations in full-length replicon, ON/C-5B/KE by a colony formation assay. In the initial experiment, we introduced each 20  $\mu$ g of RNA derived from the ON/C-5B/KE, ON/C-5B/KE/(V85I), ON/C-5B/KE/(K124E), and ON/C-5B/KE/(V85I&K124E) into OR6c cells. After 3 weeks of G418 selection at a concentration of 300  $\mu$ g/ml, only one colony was obtained and the same result was obtained with ON/C-5B/KE(K124E) and ON/C-5B/KE/(V85I&K124E) transcripts. In repeated experiments, the number of G418-resistant colonies was reproducibly one or zero, but when ON/C-5B/KE/

(V85I) transcripts was electroporated, G418 resistant 4–6 colonies was obtained in repeated experiments. These results also confirm that the replication level of ON/C-5B/KE/(V85I) is higher than that of ON/C-5B/KE, ON/C-5B/KE/(K124E) and ON/C-5B/KE/(V85I&K124E).

As the efficiency of colony formation with full-length replicon (ON/C-5B/KE; Fig. 1a) was quite low, we investigated colony formation with subgenomic replicon, ON/3-5B/KE (Fig. 1c). Figure 3 shows the representative result of three independent colony formation assays. The efficiency of colony formation of ON/3-5B/KE was better than that of full-length replicon and the colony formation of *in vitro* transcript of ON/3-5B/KE, ON/3-5B/KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 39 (61), 157 (132), 44 (54), and 134 (107), respectively (the numbers in parentheses show another set of result). The efficiency of colony formation of ON/3-5B/KE/(V85I) was greater than that of ON/C-5B/KE and it showed a similar result with that obtained from genome-length replicon.

#### Inhibition of HCV RNA Replication by IFN and RBV

We examined the inhibitory effect of IFN and RBV on the replication of OR/3-5B/KE. In this experiment, the subgenomic replicon system was used. OR6c cells were treated with IFN at concentrations of 1–20  $\mu$ M (Fig. 4) and RBV at concentrations of 50, 100, and 200  $\mu$ M (Fig. 5) after transfection of OR/3-5B/KE derived RNA. Since it is important to know how IFN and RBV treatment is toxic to the cells, we examined cell viability after treatment with 50 and 100  $\mu$ M of RBV or 2 and 4 units/ml of IFN. The cell viability of OR6c was not

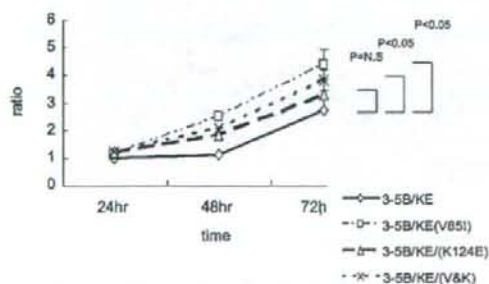


Fig. 2. Effect of amino acid substitutions in NS5B on transient replication activity of replicon. The replication activity of reporter subgenomic HCV replicon with mutation of V85I, K124E, or V85I and K124E (V&K) was compared with that of wild-type in OR6c cells (transient transfection). After 72 hr of transfection, the Renilla luciferase (RL) assay was performed as described in the Materials and Methods section. The relative RL activity (ratio) of mutants was calculated in comparison to that of subgenomic replicon of wild-type (assigned as 1). The data indicate means  $\pm$  SD of triplicates from three independent experiments. 3-5B/KE: OR/3-5B/KE, 3-5B/KE(V85I): OR/3-5B/KE(K124E), 3-5B/KE/(K124E): OR/3-5B/KE/(K124E), 3-5B/KE/(V85I&K124E): OR/3-5B/KE/(V85I&K124E).

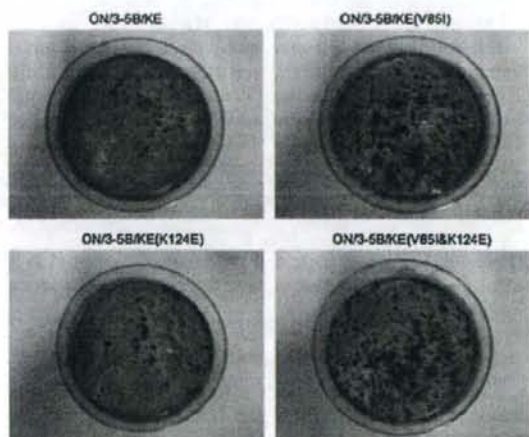


Fig. 3. Colony formation assay of OR6c cells transfected with wild-type and three different mutant replicons. A representative result of colony formation assay using subgenomic replicon RNA (ON/3-5B/KE) system. The efficacy of colony formation was much higher than that of full-length replicon RNA (ON/C-5B/KE). In this series of photographs, colony forming unit of ON/3-5B/KE, ON/3-5B/KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 2.7/ $\mu$ g, 6.7/ $\mu$ g, 3.0/ $\mu$ g and 5.4/ $\mu$ g, respectively.