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

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### ABSTRACT

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

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Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV infection has now become a serious health problem because at least 170 million people worldwide are currently infected with HCV [1]. HCV is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues [2,3]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [3].

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

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

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

### Materials and methods

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

**Reverse transcription (RT)-nested PCR.** RNA from a serum of patient AH1 [13] with acute hepatitis C was prepared using the ISOGEN-LS extraction kit (Nippon Gene Co., Japan). This RNA sample was used as a template for RT-nested PCR to amplify the HCV RNA. RT-nested PCR was performed separately in two parts; one part (3.5 kb) covered from HCV 5'UTR to NS3, and the other part (6 kb) covered from NS2 to NS5B. For the first part, the antisense primer AH3553R, 5'-CACACCGCTTGATCC AGGTGC-3' was used for RT. Primers 21 [11] and AH3519R, 5'-TGCGTGGCGG

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

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TGGAAACCACCTG-3' were employed in the first round of PCR (35 cycles). An internal primer pair (21X [11] and AH3466RX: 5'-ATTATCTAGAGGCCTGTGAGACTGTGATGATGC-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'-ATTATCTAGACTGACATGGAACCAAGATCATCAC-3'; containing a XbaI site (underlined) and 9357RX: 5'-ATTATCTAGACCCGTTACCGGTTGGGAGCAG-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'-ATTATCTAGCTCACAGCCGGGACAAGAACC-3'; containing a SpeI site (underlined) and 9162RB: 5'-ATTATCTAGCGCCAGTGAAGAGGACTTGTCC-3'; containing a BsiWI site (underlined). The amplified fragment was digested with SpeI and BsiWI, and ligated into the replicon cassette plasmid pNS1R22RU [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 Pml and ClaI, and then replaced with the Pml–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].

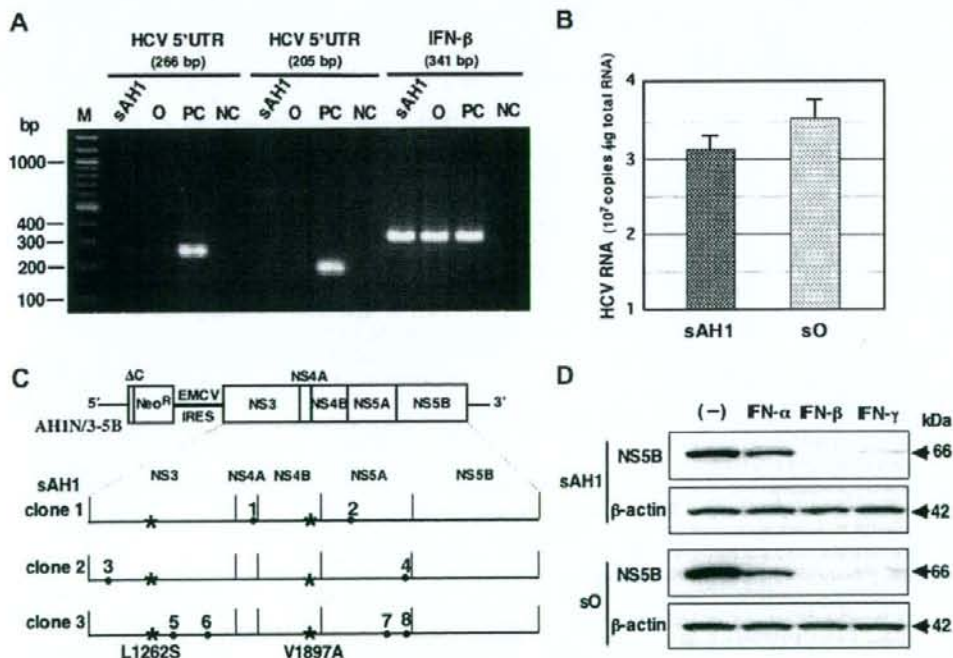
**Western blot analysis.** The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as previously described [11]. The antibodies used in this study were those against Core, E2, NS3, NS4A, NS5A, and NS5B [10].  $\beta$ -Actin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

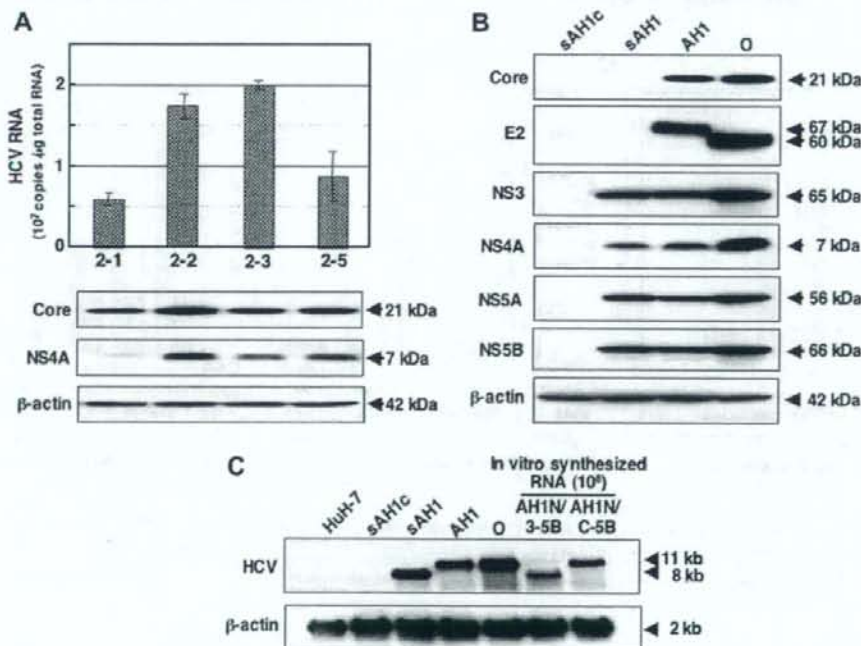
To exclude the possibility that sAH1 cells were derived from a small number of OR6 cells remaining after IFN treatment, and to determine whether replicon RNA in sAH1 cells possesses cell culture-adaptive mutations [5], which enhance the efficiency of RNA replication, we performed genetic analysis of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

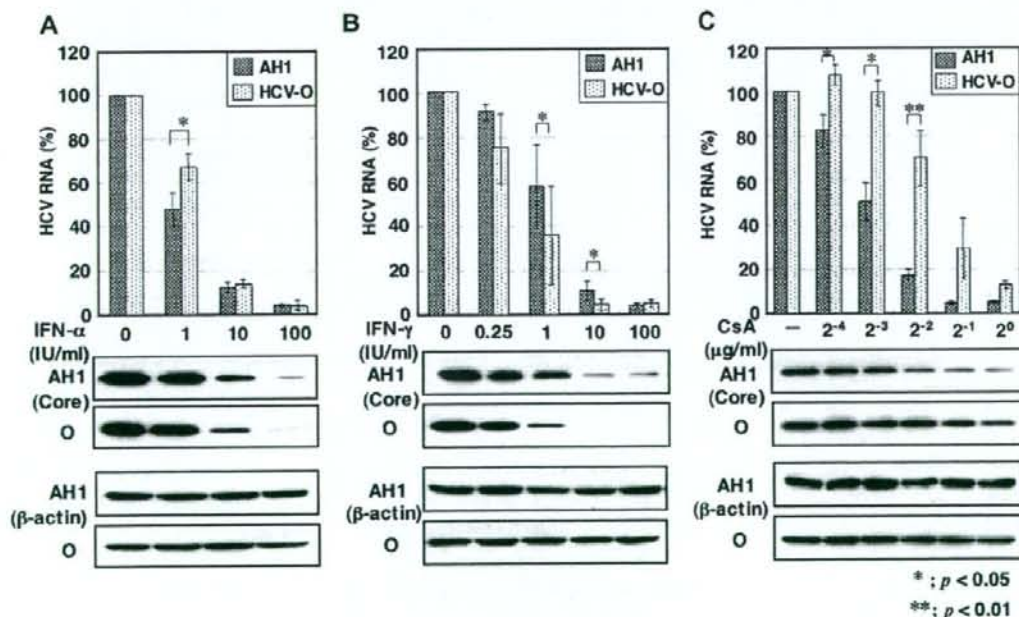
cells. Regarding IFN- $\alpha$ , the anti-HCV effect in AH1 cells was similar to that in O cells (Fig. 3A). Although RT-qPCR analysis showed a statistically significant difference in both cell systems when 1 IU/ml of IFN- $\alpha$  was used, such a difference was not observed in the Western blot analysis (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).

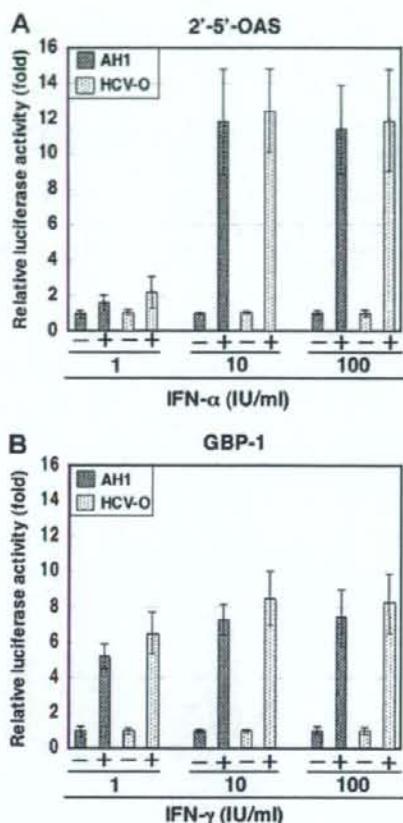


Fig. 4. Dual-luciferase reporter assay of IFN- $\alpha$  or IFN- $\gamma$ -inducible gene promoter. AH1 and O cells were treated for 6 h with IFN- $\alpha$  or IFN- $\gamma$  before the reporter assay. (A) 2'-5'-OAS gene promoter. (B) GBP-1 gene promoter.

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.

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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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## BASIC STUDIES

**Mitochondrial electron transport inhibition in full genomic hepatitis C virus replicon cells is restored by reducing viral replication**Mie Ando<sup>1</sup>, Masaaki Korenaga<sup>2</sup>, Keisuke Hino<sup>1</sup>, Masanori Ikeda<sup>3</sup>, Nobuyuki Kato<sup>3</sup>, Sohji Nishina<sup>2</sup>, Isao Hidaka<sup>2</sup> and Isao Sakaida<sup>2</sup><sup>1</sup> Department of Basic Laboratory Sciences, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan<sup>2</sup> Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan<sup>3</sup> Department of Molecular Biology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan**Keywords**

fluvastatin – interferon – oxidative stress – reactive oxygen species

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**Abstract**

**Background/Aim:** Hepatitis C virus (HCV) core protein has been shown to inhibit mitochondrial electron transport and to increase reactive oxygen species (ROS) *in vitro* and *in vivo*. The aim of this study was to investigate whether inhibiting HCV replication could restore the mitochondrial redox state and electron transport activity. **Methods:** We measured ROS, mitochondrial reduced glutathione content, and mitochondrial complex I, II, III and IV activities and protein expression in full genomic HCV replicon cells and cured cells that had been prepared by eliminating HCV RNA from replicon cells by interferon (IFN)- $\alpha$  treatment. **Results:** Cured cells had significantly lower ROS production and greater mitochondrial glutathione content than replicon cells. Complete inhibition of HCV replication by IFN- $\alpha$  restored complex I and IV activities by 20–30% ( $P < 0.01$ ) and complex I expression ( $P < 0.05$ ). Treatment with fluvastatin, one of the 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, which is known to have anti-HCV activity, partially inhibited core protein expression and restored complex I activity in full genomic HCV replicon cells to a lesser degree ( $P < 0.05$ ). **Conclusions:** Our results show that the mitochondrial redox state and electron transport activity can be restored by reducing HCV replication.

Hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). Because current antiviral treatment can only eliminate the virus in about 50% of patients (2, 3), therapies to reduce disease progression in chronically infected individuals would be of great benefit. In this respect, it is still a matter of debate whether reduction of HCV replication, even if not eliminating HCV, is beneficial to the outcome of disease. Although the mechanisms of its pathogenesis are incompletely understood, there have been several lines of evidence suggesting that oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases and is closely related to disease progression (4, 5). We and others have shown that HCV core protein induces the production of reactive oxygen species (ROS) (6–8) and that mitochondrial electron transport inhibition by HCV is associated with ROS production (9). Therefore, whether reduc-

tion of HCV replication restores mitochondrial electron transport activity is of interest in exploring treatments to reduce disease progression in HCV-associated chronic liver disease.

Establishment of the HCV subgenomic replicon has made it possible to assess the antiviral activities of interferon (IFN) and other reagents *in vitro* (10). We also developed a genome-length HCV RNA replication reporter system (11) and found that different statins, which are 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, have different anti-HCV profiles while using this reporter system (12). In the present study, we chose to use fluvastatin, which exhibited the strongest inhibition of HCV replication among the statins (12), to reduce HCV replication in full genomic HCV replicon cells without complete inhibition. The aim of this study was to examine whether mitochondrial electron transport activity was restored by reduction of HCV replication.



## Materials and methods

### Cell cultures

Full genomic HCV replicon cells were described in detail by Ikeda *et al.* (11). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin and G418 (300 µg/ml; Calbiochem, Darmstadt, Germany) and passaged twice a week at a 5:1 split ratio. Cured cells were established by eliminating genome-length HCV RNA from replicon cells by IFN- $\alpha$  treatment (500 IU/ml for 2 weeks; Sigma-Aldrich, St Louis, MO, USA) without G418, as described (11). In some experiments, full genomic HCV replicon cells were incubated in the presence of 10 µmol/L fluvastatin (Novartis Pharmaceutical, Tokyo, Japan) for 96 h.

### Measurement of reactive oxygen species

The cellular ROS level was measured by oxidation of the cell-permeable, oxidation-sensitive fluorogenic precursor dihydrodichlorocarboxyfluorescein diacetate (DCFDA; Molecular Probes Inc., Eugene, OR, USA). Cells in six-well plates were treated with tertiary butyl hydroperoxide (t-BOOH) for 5 h or not, followed by a 30-min incubation with DCFDA (500 nmol/L final concentration) at 37 °C. Fluorescence was measured with a CytoFluorII fluorescence plate reader (PerSeptive Biosystems, Framingham, MA, USA) at an excitation wavelength of 486 nm and an emission wavelength of 530 nm as described (7).

Localization of ROS production on the subcellular level was observed with a Zeiss (Oberkochen, Germany) LSM5 Pascal inverted laser scanning confocal microscope. Cells were pre-incubated with 5 µmol of hydroxyphenyl fluorescein (HPF; Alexis Corporation, Lausen, Switzerland) (13) for 5 min at 37 °C. They were then imaged at 30-s intervals after treatment with 10 nmol/L t-BOOH. The green fluorescence of HPF (excitation, 488 nm; emission, 505–530 nm) was observed after excitation with an argon-krypton laser.

### Isolation of mitochondria

Mitochondrial pellets were obtained as described previously with some modification (7, 9). Briefly, harvested cells were centrifuged at 500g for 5 min. The pellets were homogenized with 25 strokes using a Dounce homogenizer (Wheaton Science Products, Millville, NJ, USA) and a tight-fitting pestle with isolation buffer [70 mM sucrose, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM HEPES, 220 mM mannitol, 5 mM sodium succinate and 0.1% bovine serum albumin (BSA), pH 7.4]. The homogenate was centrifuged at 1330g for 5 min at 4 °C. The super-

natant fraction was retained, whereas the pellet was washed with isolation buffer and centrifuged again. The combined supernatant fractions were centrifuged at 1000g for 15 min at 4 °C to obtain a crude mitochondrial pellet. Purified mitochondria were prepared by sucrose gradient (1.5 M sucrose and 1 M sucrose) centrifugation as described (14) with some modification. An aliquot was removed for determination of the protein concentration with the Bio-Rad protein DC assay kit (Bio-Rad, Hercules, CA, USA), using BSA as the standard.

### Measurement of reduced glutathione content

Crude mitochondrial samples (3–4 mg of mitochondrial protein) were sonicated using a Sonifier cell disruptor 200 (VWR Scientific, Danbury, CT, USA) for 15 s at power setting 3 in ice-cold 5% metaphosphoric acid and centrifuged at 3000g at 4 °C for 10 min. The concentration of reduced glutathione was measured by the thioester method using a GSH-400 kit (Oxis International Inc., Portland, OR, USA).

### Immunoblotting

Crude mitochondrial pellets were suspended in lysis buffer (T-PER Tissue Extraction Reagent; Pierce, Rockford, IL, USA) and centrifuged at 10 000g for 15 min at 4 °C. The supernatant (20 µg of protein) was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 16% gel. The proteins were electrophoretically blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), blocked overnight at 4 °C with 5% skim milk and 0.1% Tween 20 in Tris-buffered saline, and subsequently incubated for 1 h at room temperature with an anti-hepatitis C core protein antibody (1:1000, Affinity Bio Reagents, Golden, CO, USA), anti-OxPhos complex I antibody (1:1000), anti-OxPhos complex II antibody (1:2000), anti-OxPhos complex III antibody (1:2500) or anti-OxPhos complex IV antibody (1:1000, Molecular Probes Inc). The membranes were washed, incubated with appropriate secondary antibodies and detected with ECL<sup>TM</sup> Western blot detection reagents (Amersham Biosciences, Piscataway, NJ, USA). The degree of protein expression was expressed as the normalized quotient, which was derived by dividing the intensity of the blot density of each protein by that of  $\beta$ -actin protein.

### Measurement of complex I, II, III and IV activities

Submitochondrial particles were prepared from mitochondria by incubation for 3 min at 37 °C followed by sonication in a microcentrifuge tube immersed in ice water. Forty micrograms of submitochondrial



particles was pelleted at 15 000g for 10 min. Enzyme activity assays were performed at 25 °C by a previously established method (15). Complex I [nicotinamide adenine dinucleotide (NADH)-decylubiquinone oxidoreductase] activity was measured as the initial (5 min) rate of decrease of  $A_{340}$  using the acceptor 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB 80  $\mu$ M) and 200  $\mu$ M NADH as the donor in 10 mM Tris (pH 8.0) buffer containing 1 mg/ml BSA, 0.24 mM KCN and 0.4  $\mu$ M antimycin A. Complex II (succinate decylubiquinone 2,6-dichlorophenolindophenol reductase) activity was measured at 600 nm using 80  $\mu$ M DCPIP as the acceptor and 10 mM succinate as the donor in 10 mM  $\text{KH}_2\text{PO}_4$  (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4  $\mu$ M rotenone, 0.2 mM ATP and 0.4  $\mu$ M antimycin A. Complex III (ubiquinol cytochrome *c* reductase) activity was measured at 550 nm using 40  $\mu$ M oxidized cytochrome *c* as the acceptor and 80  $\mu$ M decylubiquinol as the donor in 10 mM  $\text{KH}_2\text{PO}_4$  (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4  $\mu$ M rotenone and 0.2 mM ATP for 2 min. Complex IV (cytochrome *c* oxidase) activity was measured using a cytochrome *c* oxidase assay kit (Sigma-Aldrich), following the manufacturer's instructions.

#### Statistical analysis

Quantitative values are expressed as mean  $\pm$  standard deviation. Two groups were compared by the Student *t*-test. A *P* value of  $< 0.05$  was considered to be significant. Two groups among multiple groups were compared by the rank-based, Kruskal-Wallis analysis of variance test followed by Scheffe's test.

## Results

### Increased reactive oxygen species production and mitochondrial oxidant status in full genomic hepatitis C virus replicon cells

To assess the effect of HCV replication on ROS production, we used the ROS-sensitive fluorescent probe DCFDA. As compared with cured cells, HCV replication increased ROS 1.4-fold (Fig. 1A). Because HCV infection results in an inflammatory response and an increase in the basal oxidative stress, we next determined the effect of an exogenous oxidant, 500 nmol/L t-BOOH, on ROS production. This treatment had no effect on cured cells, but increased ROS production in full genomic HCV replicon cells to a level 2.5-fold greater than that of cured cells (Fig. 1A;  $P < 0.01$ ). Cells were then imaged by confocal micro-

scopy at 30-s intervals after exposure to HPF, which is more sensitive to ROS production than DCFDA. As shown in Figure 1B, treatment with t-BOOH significantly increased the oxidized fluorescent product as time passed in full genomic HCV replicon cells, but not in cured cells ( $P < 0.0005$ ). Thus, a small volume of exogenous oxidant (10 nmol/L) that did not induce ROS production in cured cells significantly increased ROS production in full genomic HCV replicon cells.

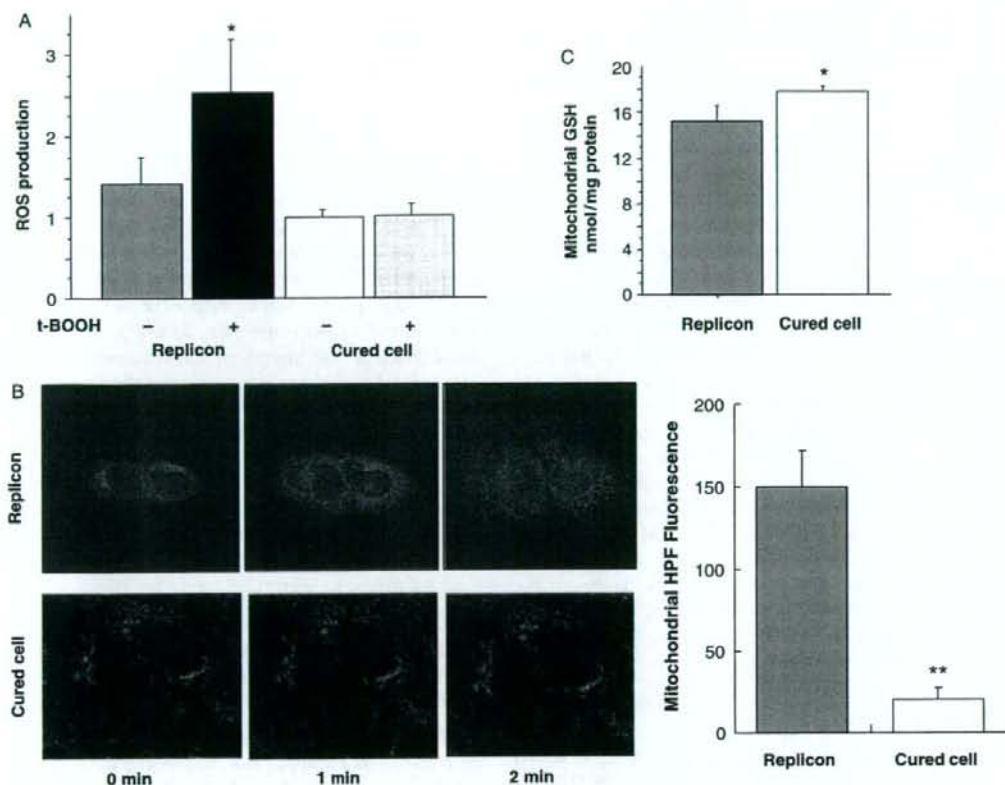
We previously demonstrated, by confocal microscopy, that the mitochondria are the primary site of initial ROS production in cells expressing HCV core protein and cytochrome P450 2E1 (7). Because confocal microscopic images of the oxidized fluorescent product in replicon cells were almost the same as those in our previous study, we measured mitochondrial reduced glutathione content to assess mitochondrial antioxidant capacity. The level of mitochondrial reduced glutathione was significantly lower in full genomic HCV replicon cells than in cured cells (Fig. 1C;  $P < 0.05$ ), suggesting that HCV replication was responsible for the mitochondrial oxidant status and sensitized to exogenous oxidative stress.

### Restoration of mitochondrial electron transport activity by complete inhibition of hepatitis C virus replication

Our previous study has demonstrated that core protein causes oxidation of the glutathione pool, increases ROS production and inhibits complex I activity (9). Because increased ROS production and mitochondrial oxidant status were found in full genomic HCV replicon cells as well, we next measured complex I, II, III and IV activities in submitochondrial particles to determine whether inhibiting HCV replication restored mitochondrial electron transport activity. Complete inhibition of HCV replication by IFN- $\alpha$  restored complex I and IV activities by 20–30% ( $P < 0.01$ ) (Fig. 2). However, complex II and III activities were not changed after treatment with IFN- $\alpha$  in these cells (Fig. 2).

We further assessed the expression levels of complexes I, II, III and IV in full genomic HCV replicon cells and cured cells. As shown in Figure 3, immunoblotting revealed that complete inhibition of HCV replication by IFN- $\alpha$  restored the complex I expression as well ( $P < 0.05$ ). Although the complex IV activity was restored by IFN- $\alpha$ , the expression of complex IV did not change after complete inhibition of HCV replication. Thus, it should be noted that both activity and expression of complex I were restored by completely inhibiting HCV replication in full genomic HCV





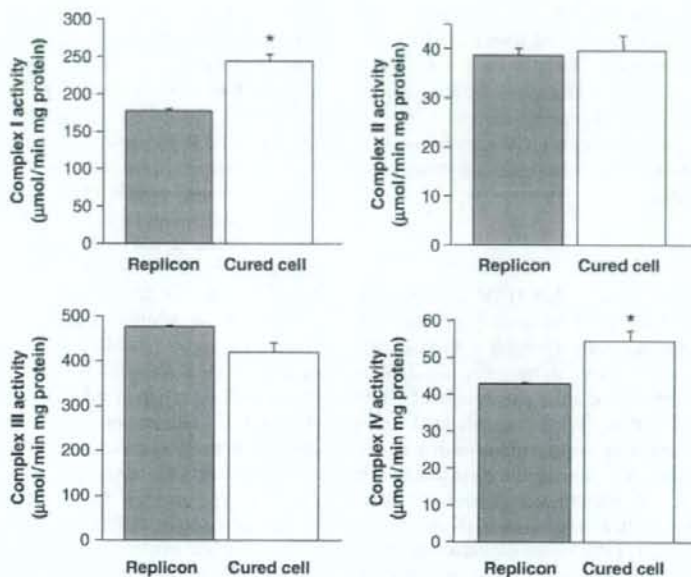
**Fig. 1.** Effects of HCV replication on ROS production and mitochondrial reduced glutathione level. (A) ROS production was measured by oxidation of DCFDA in HCV replicon cells and cured cells under control conditions or after 5-h incubation with t-BOOH (500 nmol/L). \* $P < 0.01$  compared with untreated cured cells. (B) Confocal images of ROS generation. HCV replicon cells and cured cells were pre-incubated with HPF, subsequently treated with t-BOOH (10 nmol/L) and imaged at 30-s intervals. The increase in HPF fluorescence intensity 2 min after treatment with t-BOOH was compared between HCV replicon cells and cured cells. \*\* $P < 0.0005$  compared with HCV replicon cells. (C) Reduced glutathione content was measured in crude mitochondrial fractions prepared from HCV replicon cells and cured cells. \* $P < 0.05$  compared with HCV replicon cells. DCFDA, dihydrodichlorocarbonyfluorescein diacetate; HCV, hepatitis C virus; HPF, hydroxyphenyl fluorescein; ROS, reactive oxygen species.

replicon cells, even though the significance of reduced complex IV activity remains elusive.

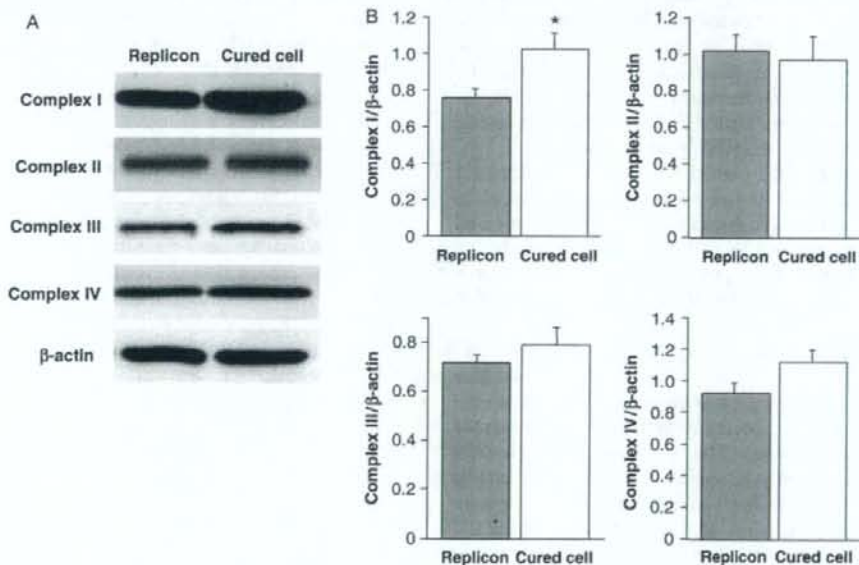
#### Incompletely inhibited hepatitis C virus replication partially restores mitochondrial electron transport activity

Fluvastatin, a 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitor, has been shown to have an inhibitory effect on HCV replication in the present full genomic HCV replicon cells (12). We used fluvastatin for partially inhibiting HCV replication in full genomic HCV replicon cells, because it has a lesser inhibi-

tory effect on HCV replication than IFN- $\alpha$  (12). In fact, expression of core protein was present in mitochondria, but was significantly lowered by treatment with fluvastatin in full genomic HCV replicon cells ( $P < 0.05$ ; Fig. 4A). Partial inhibition of HCV replication restored complex I activity by ~13% ( $P < 0.05$ ; Fig. 4B). Although statins including fluvastatin are known to have an anti-oxidative effect (16, 17), treatment with fluvastatin did not improve complex I activity in cured cells (Fig. 4B), suggesting that this activity was restored by its inhibitory effect on HCV replication rather than its anti-oxidative property. However, partial inhibition of HCV replication did



**Fig. 2.** Mitochondrial complex I, II, III and IV activities. Complex I (NADH-decylubiquinone oxidoreductase) activity, complex II (succinate decylubiquinone 2,6-dichlorophenolindophenol reductase) activity, complex III (ubiquinol cytochrome c reductase) activity and complex IV (cytochrome c oxidase) activity were measured in submitochondrial fractions prepared from HCV replicon cells and cured cells. \* $P < 0.01$  compared with HCV replicon cells. HCV, Hepatitis C virus; NADH, nicotinamide adenine dinucleotide.



**Fig. 3.** Expression of mitochondrial complex I, II, III and IV. (A) Immunoblots for complex I, II, III and IV were performed using crude mitochondrial fractions prepared from HCV replicon cells and cured cells. (B) The degree of protein expression was normalized with  $\beta$ -actin protein. \* $P < 0.05$  compared with HCV replicon cells. HCV, hepatitis C virus.



not lead to a significant reduction of ROS production (Fig. 4C), a significantly increased level of mitochondrial reduced glutathione (Fig. 4D) or complex I expression (Fig. 4A). Thus, incomplete inhibition of HCV replication restored mitochondrial electron transport activity in full genomic HCV replicon cells, even though it was not sufficient to reduce mitochondrial oxidative status.

## Discussion

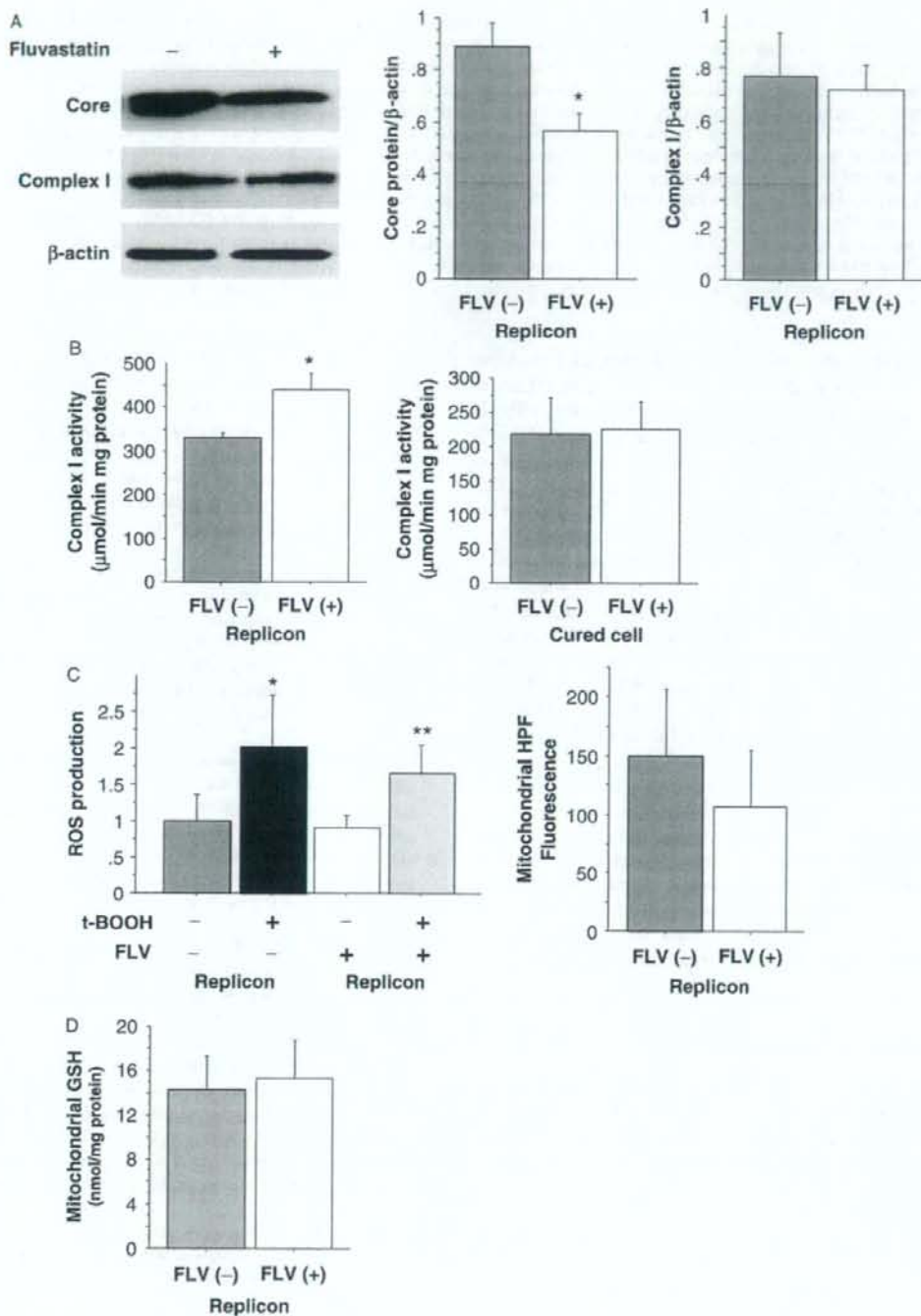
We have previously reported that HCV core protein inhibits mitochondrial electron transport and increases ROS production in the transgenic mouse liver (9). Our present results have shown that these phenomena can be reproduced in the presence of HCV replication. As replication of a full-length HCV genome rather than mere core protein expression is closer to the disease condition occurring in patients with chronic hepatitis C, the present results have strengthened the possibility that mitochondrial oxidation, ROS production and inhibition of mitochondrial electron transport are actually caused in chronic hepatitis C. Thanks to the establishment of HCV replicon cells, we could investigate the effect of inhibiting HCV replication on mitochondrial electron transport activity that was closely related to ROS production. In the present study, we focused on restoration of mitochondrial electron transport activity by inhibiting HCV replication, regarding complete inhibition of HCV replication *in vitro* as that of HCV eradicated with IFN therapy and partial inhibition of HCV replication *in vitro* as that in cells undergoing IFN therapy without HCV eradication.

Consistent with a previous observation, complex I activity, but not complex III activity, was reduced in full genomic HCV replicon cells. Complex I appeared to be the source of HCV-induced ROS, because mitochondrial ROS generation can occur at either complex I or complex III (18–20). We also found decreased expression of complex I in full genomic HCV replicon cells as compared with cured cells. Complex I is the site most sensitive to oxidative damage of the electron transport carriers, and inhibition of complex I occurs during the early stages of mitochondrial damage (21). Increased mitochondrial ROS production due to reduction of complex I activity amplifies mitochondrial oxidation, which in turn may inhibit the expression of complex I. Complex IV activity, i.e. that of cytochrome *c* oxidase, was reduced in full genomic HCV replicon cells as well. Complex IV localizes at the end of mitochondrial electron transport, accepts one electron at a time from cytochrome *c*

and passes them four at a time to oxygen. Therefore, decreased activity of complex IV may amplify mitochondrial ROS production, possibly by inhibiting electron flow in the respiratory chain.

Thus, it is likely that HCV replication increases mitochondrial ROS production through inhibition of electron transport, causing oxidative stress within the liver in patients with chronic hepatitis C. Several different experimental models of HCV protein expression reproduced this finding (6–8). However, whether reduction of HCV replication restores mitochondrial function remains unknown. In the present study full genomic HCV replicon cells had ~30% reduction of complex I activity ( $P=0.0001$ ) and ~20% reduction in complex IV activity ( $P < 0.01$ ) as compared with cured cells (Fig. 2). In other words, complete inhibition of HCV replication by IFN- $\alpha$  restored the activities of complex I and complex IV, leading to reduced ROS production in the presence of an exogenous oxidant and to an increase of mitochondrial reduced glutathione content (Fig. 1). There have been several lines of clinical evidence suggesting that HCV elimination by IFN treatment reverses the progression of liver fibrosis and significantly suppresses the development of HCC afterwards (22, 23). Restoration of mitochondrial electron transport activity by complete inhibition of HCV replication may well account for this clinical evidence, because the progression of liver fibrosis and development of HCC in chronic hepatitis C have been shown to be closely related to excess oxidative stress within the liver (24, 25).

In the clinical setting, however, HCV eradication by combination therapy with peginterferon- $\alpha$  and ribavirin has been successful in 50–60% of patients with refractory chronic hepatitis C at most (2, 3). Therefore, it is a critical issue for patients for whom this is unsuccessful if needed, whether prolonged reduction of HCV replication, not elimination of HCV, reduces or delays the progression of liver fibrosis and development of HCC. Although there have been several studies suggesting the inhibitory effect of IFN therapy on HCC development in patients with HCV-associated chronic liver diseases (26, 27), it is still controversial (28). Cured cells did not show core protein expression at all (data not shown), whereas fluvastatin-treated replicon cells had core protein expression that was significantly lower than in replicon cells without treatment. Thus, incomplete inhibition of HCV replication by fluvastatin was useful as a model for assessing if reduction of HCV replication, not elimination of HCV, could restore mitochondrial function. We found that incomplete inhibition of HCV replication restored complex I activity, but did





**Fig. 4.** Effect of fluvastatin on core protein expression, mitochondrial complex I activity and expression, ROS production and mitochondrial reduced glutathione level. (A) Immunoblots for core protein and complex I were performed using crude mitochondrial fractions prepared from HCV replicon cells treated with/without fluvastatin. The degree of protein expression was normalized with  $\beta$ -actin protein. (B) Complex I (NADH-decylubiquinone oxidoreductase) activity was measured in submitochondrial fractions prepared from HCV replicon cells and cured cells, both of which were treated with/without fluvastatin. (C) ROS production was measured by oxidation of DCFDA in HCV replicon cells treated with/without fluvastatin under control conditions or after 5-h incubation with t-BOOH (500 nmol/L). HCV replicon cells treated with/without fluvastatin also were pre-incubated with HPF and subsequently treated with t-BOOH (10 nmol/L). The increase in HPF fluorescence intensity 2 min after treatment with t-BOOH was compared between HCV replicon cells with fluvastatin and those without. \* $P < 0.05$  as compared with HCV replicon cells without both t-BOOH and fluvastatin treatment. \*\* $P < 0.005$  as compared with fluvastatin-treated HCV replicon cells without t-BOOH. (D) Reduced glutathione content was measured in crude mitochondrial fractions prepared from HCV replicon cells treated with/without fluvastatin. DCFDA, dihydrodichlorocarboxyfluorescein diacetate; FLV, fluvastatin; HCV, hepatitis C virus; HPF, hydroxyphenyl fluorescein; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; t-BOOH, tertiary butyl hydroperoxide.

not lead to the reduction of mitochondrial oxidative status. Thus it should be noted that restoration of complex I activity resulted from the inhibitory effect of HCV replication by fluvastatin rather than its antioxidant property. Even if incomplete inhibition of HCV replication fails to reduce mitochondrial oxidative status *in vitro*, restoration of complex I activity for a certain period *in vivo* may lead to a reduction of mitochondrial oxidative status. However, we need to recognize that inhibition of HCV replication *in vitro* by statins does not necessarily imply the same effect *in vivo*, because the absence of a clinical anti-HCV effect of statins has been reported (29). The present results showing that even incomplete inhibition of HCV replication can restore mitochondrial function to a lesser degree than completely inhibited HCV replication provides us with a rationale for suppressing HCV replication by anti-HCV agents in nonsustained responders to the current combination therapy.

In conclusion, our study shows that HCV replication causes oxidation of the mitochondrial glutathione pool, increases ROS production and inhibits mitochondrial electron transport activity, and that these changes in the mitochondrial redox state can be reversed by reducing HCV replication.

## Acknowledgements

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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 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.

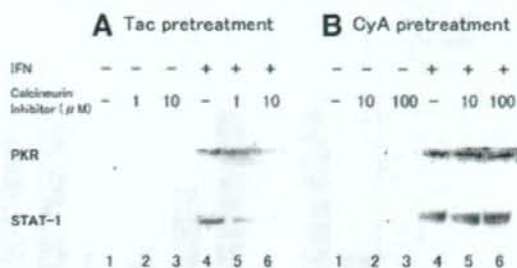
### Fluorescence Immunohistochemistry

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.

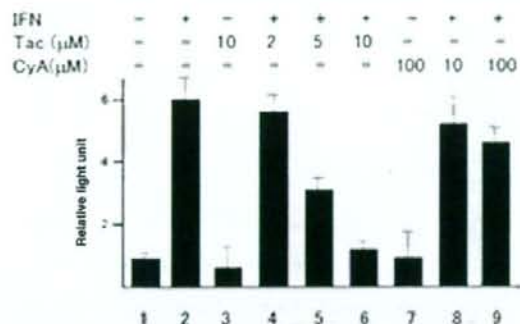
The expression of IFN-inducible STAT-1 also decreased in a dose-dependent manner after the administration of Tac, but not after the administration of CyA.

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

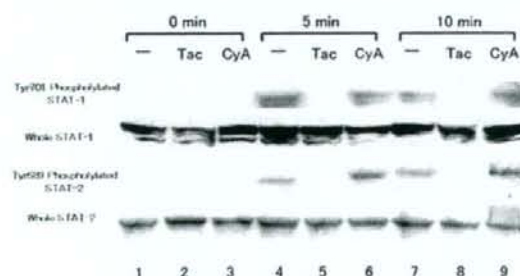
Because the formation of IFN stimulating gene factor (ISGF) 3 by IFN- $\alpha$  leads to transactivation of the ISRE in the promoter regions of the IFN- $\alpha$ -inducible genes, we performed the reporter gene transfection assay using plasmids containing ISRE in their promoter sequence. Because there 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 pRL-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

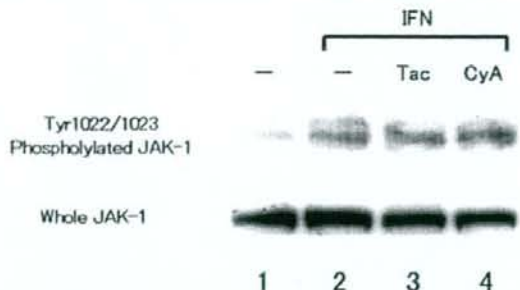


**Figure 2.** Suppression effect of calcineurin inhibitors on IFN- $\alpha$ -induced reporter gene assay. Hc cells transfected with reporter gene (pISRE-Luc and pRL-SV40) were either untreated (lanes 1, 2) or pretreated with Tac (lanes 3-6) or CyA (lanes 7-9) for 16 hours, followed by IFN- $\alpha$  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.

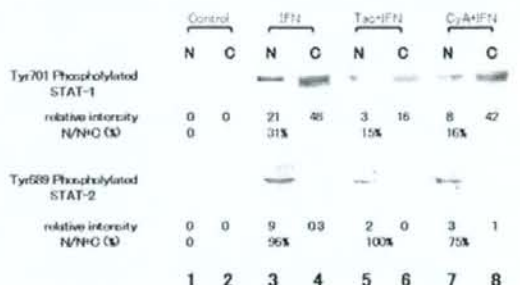


**Figure 3.** Effect of calcineurin inhibitors on STAT-1 and STAT-2. After pretreatment with 10  $\mu$ M Tac (lanes 2, 5, and 8) and 100  $\mu$ M CyA (lanes 3, 6, and 9) for 16 hours, Hc cells were untreated (lane 1) or treated with 100 IU/mL IFN- $\alpha$  (lanes 4-9) for the indicated periods and phosphorylated 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/L 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-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).