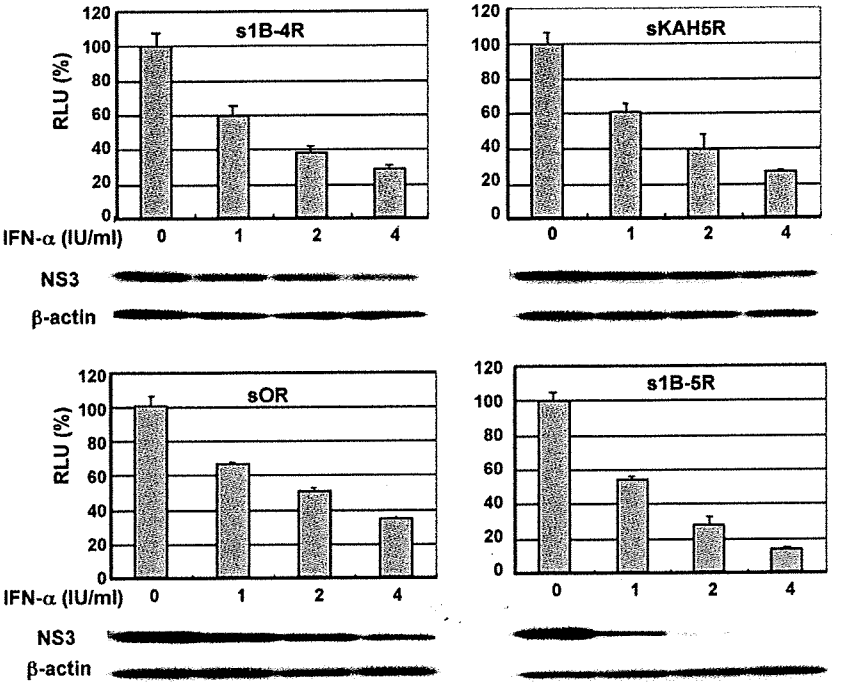

Fig. 2. The activity of IFN-α on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-α treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-α (0, 1, 2, and 4 IU/ml) for 72 h. Then, the cells were subjected to RL assay (upper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

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

Next, we examined the sensitivity of four replicons to type II IFN, IFN-γ, because in our previous study, HCV (genotype 1b, AH1 strain) from a patient with AH C was found to be more resistant to IFN-γ than was HCV-O (Mori et al., 2008). In this study, sKAH5R was also derived from the serum of a patient with AH C. The reporter assay revealed that sKAH5R has the lowest sensitivity to IFN-γ (EC_{50}: 2.26 IU/ml) among the replicons tested (Fig. 3). To calculate the EC_{50} of IFN-γ to sKAH5R, we also treated sKAH5R with IFN-γ at 2 and 4 IU/ml for 72 h (data not shown). The EC_{50} of IFN-γ to s1B-4R, sOR, and s1B-5R was 0.54, 0.33, and 0.21 IU/ml, respectively. The results

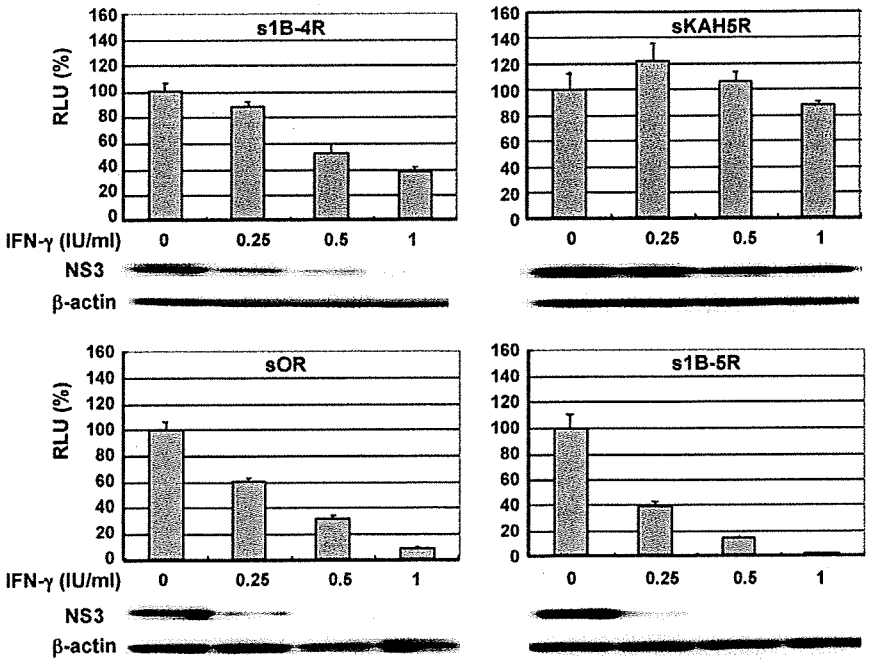

Fig. 3. The activity of IFN-γ on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-γ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-γ (0, 0.25, 0.5, and 1 IU/ml) for 72 h and then the cells were subjected to RL assay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

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

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

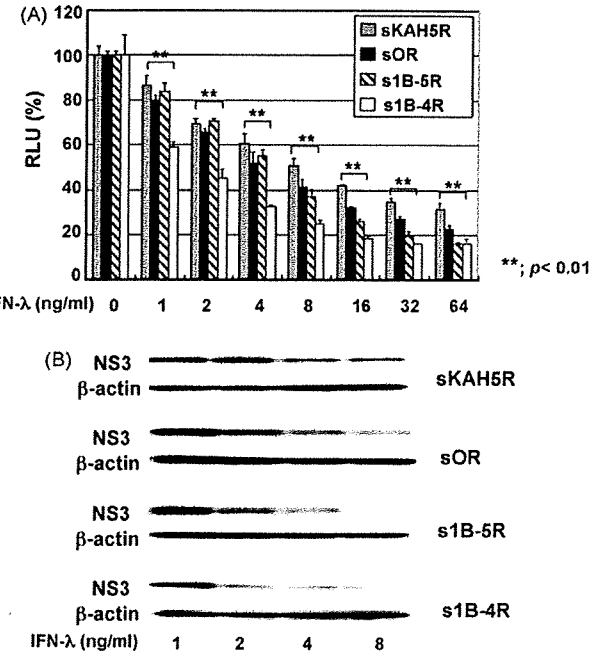

Fig. 4. Effects of IFN-λ on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in IFN-λ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-λ (0, 2, 4, 8, 16, 32, and 64 ng/ml) for 72 h, and then the cells were subjected to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

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

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

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

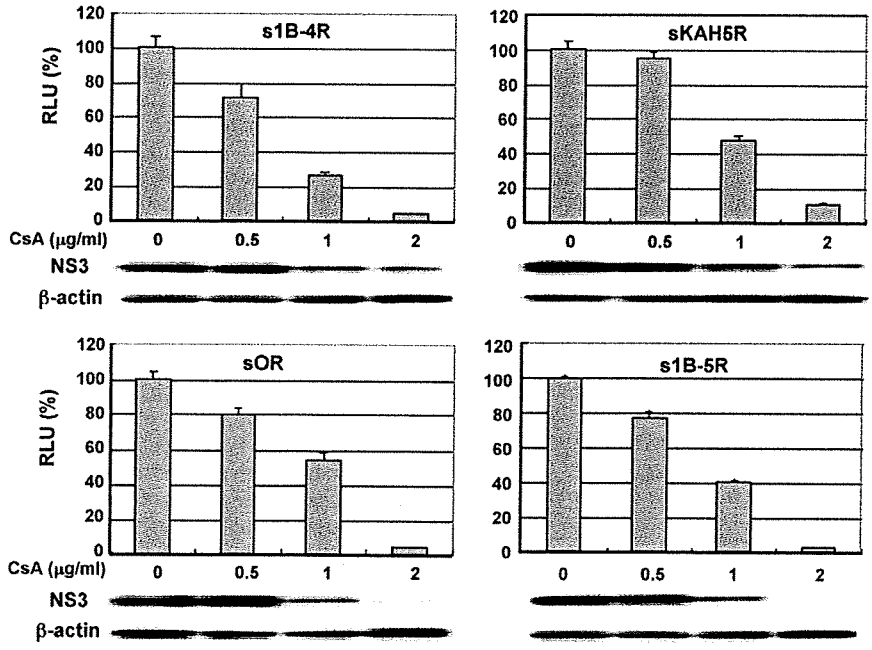

Fig. 5. The activity of CsA on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in CsA treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with CsA (0, 0.5, 1, and 2 μg/ml) for 72 h, and then the cells were subjected to RL assay (upper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

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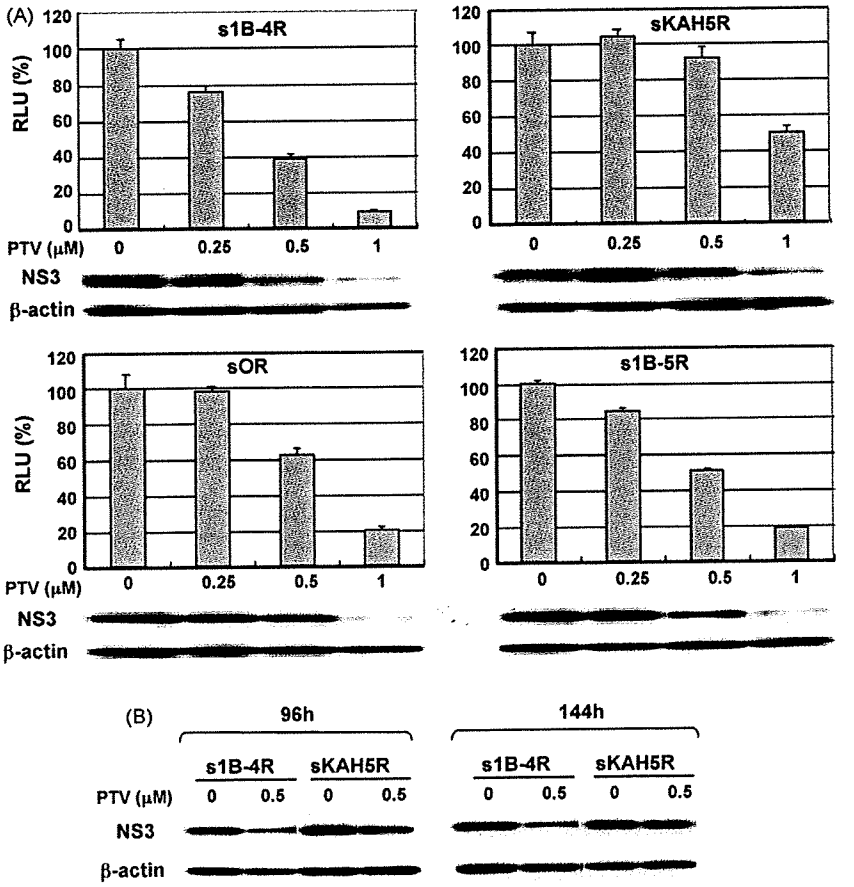

Fig. 6. The activity of PTV on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in PTV treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with PTV (0, 0.25, 0.5, and 1 μM) for 72 h, and then the cells were subjected to RL assay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

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

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

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

3.5. Polyclonal KAH5 replicon with a statin-resistant phenotype

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

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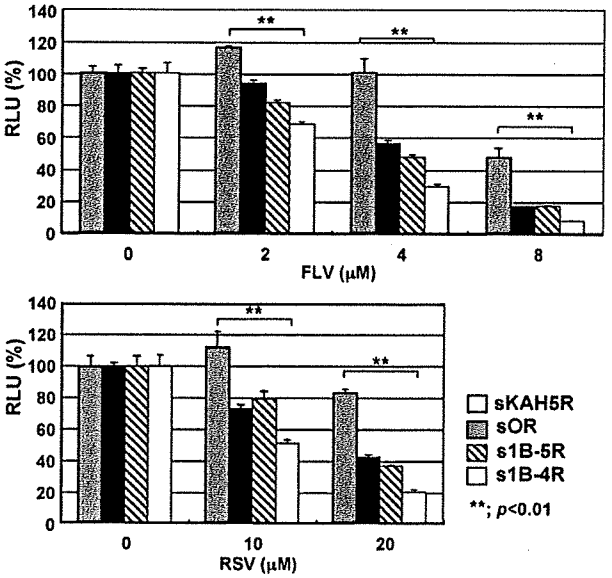

Fig. 7. HCV replicons exhibit diverse sensitivities to statins. Reporter assay of the sensitivity of HCV replicons to FLV. sKAH5R cells (light column), sOR cells (dark column) s1B-5R cells (shaded column), and s1B-4R cells (open column) were treated with FLV (0, 2, 4, and 8 μM) for 72 h (upper panel),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

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

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

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

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

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

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

ND: not determined.

4. Discussion

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

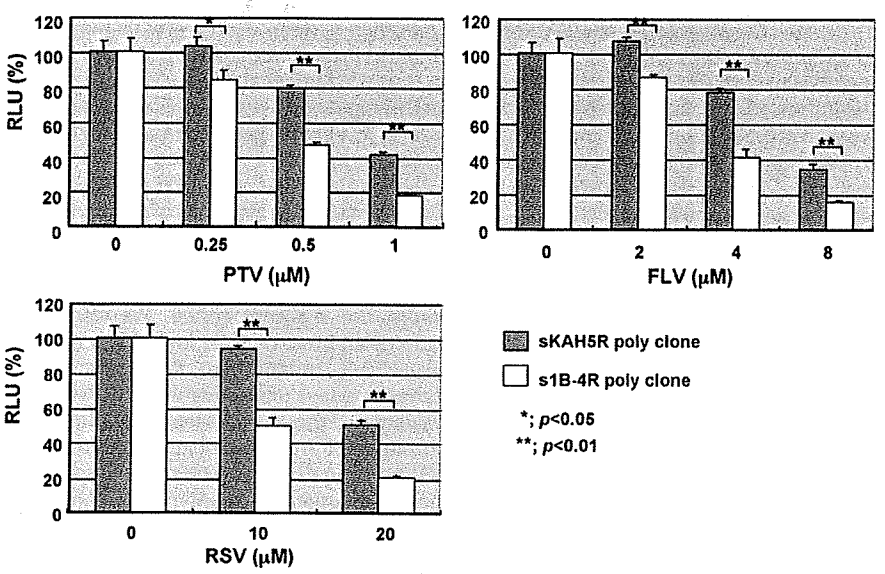

Fig. 8. Diverse sensitivities of polyclonal replicons to statins. Reporter assay of the sensitivity of polyclonal sKAH5R and s1B-4R replicons to PTV, FLV, and RSV. Polyclonal sKAH5R cells and polyclonal s1B-4R cells were treated with PTV (0, 0.25, 0.5, and 1 μM), FLV (0, 2, 4, and 8 μM), and RSV (0,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

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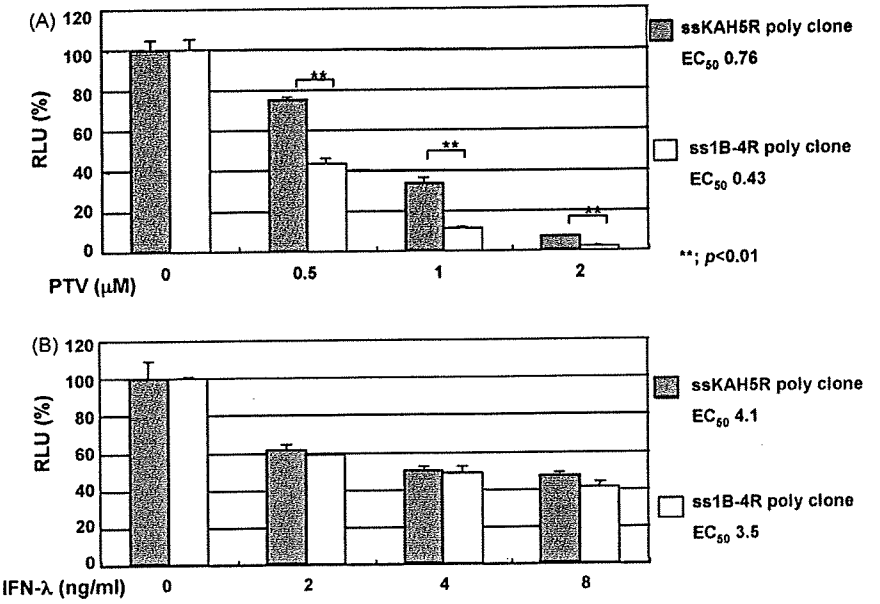

Fig. 9. The sensitivities of the second generation of sKAH5R and s1B-4R to PTV and IFN-λ. Polyclonal second generations of sKAH5R (ssKAH5R) and s1B-4R (ss1B-4R) were treated with PTV (0, 0.5, 1, and 2 μM) (A) and IFN-λ (0, 2, 4, and 8 ng/ml) (B) for 72 h and then were subjected to RL assay. All the luciferase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

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

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

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

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

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

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

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

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

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Appendix A. Supplementary data

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

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Arsenic Trioxide Inhibits Hepatitis C Virus RNA Replication through Modulation of the Glutathione Redox System and Oxidative Stress[▽]

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Arsenic trioxide (ATO), a therapeutic reagent used for the treatment of acute promyelocytic leukemia, has recently been reported to increase human immunodeficiency virus type 1 infectivity. However, in this study, we have demonstrated that replication of genome-length hepatitis C virus (HCV) RNA (O strain of genotype 1b) was notably inhibited by ATO at submicromolar concentrations without cell toxicity. RNA replication of HCV-JFH1 (genotype 2a) and the release of core protein into the culture supernatants were also inhibited by ATO after the HCV infection. To clarify the mechanism of the anti-HCV activity of ATO, we examined whether or not PML is associated with this anti-HCV activity, since PML is known to be a target of ATO. Interestingly, we observed the cytoplasmic translocation of PML after treatment with ATO. However, ATO still inhibited the HCV RNA replication even in the PML knockdown cells, suggesting that PML is dispensable for the anti-HCV activity of ATO. In contrast, we found that *N*-acetyl-cysteine, an antioxidant and glutathione precursor, completely and partially eliminated the anti-HCV activity of ATO after 24 h and 72 h of treatment, respectively. In this context, it is worth noting that we found an elevation of intracellular superoxide anion radical, but not hydrogen peroxide, and the depletion of intracellular glutathione in the ATO-treated cells. Taken together, these findings suggest that ATO inhibits the HCV RNA replication through modulation of the glutathione redox system and oxidative stress.

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6-kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (30).

Alpha interferon has been used as an effective anti-HCV reagent in clinical therapy for patients with chronic hepatitis C. The current combination treatment with pegylated alpha interferon and ribavirin, a nucleoside analogue, has been shown to improve the sustained virological response rate to more than 50% (15). However, the adverse effects of the combination therapy and the limited efficacy against genotype 1b warrant the development of new anti-HCV reagents.

Arsenic trioxide (ATO) (As_2O_3, arsenite) has been used as a therapeutic reagent in acute promyelocytic leukemia, which bears an oncogenic PML-retinoic acid receptor alpha fusion protein resulting from chromosomal translocation (51, 52, 68, 70). The ATO treatment induces complete remission through degradation of the aberrant PML-retinoic acid receptor α (70). The PML tumor suppressor protein is required for formation of the PML nuclear body (PML-NB), also known as nuclear dot 10 or the PML oncogenic domain, which is often disrupted by infection with DNA viruses, such as herpes simplex virus type 1, human cytomegalovirus, and Epstein-Barr virus (17). The treatment with ATO results in degradation of the PML protein and disruption of the PML-NB (70). Therefore, ATO has been become a useful probe for investigating the functions of the PML-NB, including cell growth, apoptosis, stress response, and viral infection. Indeed, ATO has been shown to increase retroviral infectivity, such as human immunodeficiency virus type 1 (HIV-1) and murine leukemia virus infectivity, but the mechanisms of this change are not well understood (5, 6, 32, 44, 47, 50, 57). In contrast, ATO was recently reported to inhibit the replication of HCV subgenomic replicon RNA (24). However, it also remains unclear how ATO inhibits the HCV RNA replication. In this study, using genome-length HCV RNA replication systems, we investigated the molecular mechanism(s) of the anti-HCV activity of ATO, and we provide evidence that ATO inhibits HCV RNA replication through modulation of the glutathione redox system and oxidative stress.

MATERIALS AND METHODS

Reagents. ATO, *N*-acetyl-cysteine (NAC), ascorbic acid (vitamin C), and L-buthionine sulfoximine (BSO) were purchased from Sigma (St. Louis, MO). Arsenic pentoxide (APO) (As_2O_5, arsenate) was purchased from Wako (Osaka, Japan). Both ATO and APO were dissolved in 1 N NaOH at 0.1 M as a stock solution. An inducible nitric oxide synthase (iNOS) inhibitor, 1400W, was purchased from Calbiochem (Merck Biosciences, Darmstadt, Germany).

Cell culture. 293FT cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. The following four HuH-7-derived cell lines or their parental HuH-7 cells

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were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum as described previously (25): O cells, harboring a replicative genome-length HCV-O RNA (O strain of genotype 1b) (25); OR6 cells, harboring the genome-length HCV-O RNA with luciferase as a reporter (25); sO cells, harboring the subgenomic replicon RNA of HCV-O (31); and RSc cured cells, which cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) (58) could infect and effectively replicate in (2, 3). The O, OR6, and sO cells were maintained in the presence of G418 (300 μg/ml Geneticin; Invitrogen).

RNA interference. Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences targeted to PML (56) in a lentiviral vector: 5′-GATCCCCAGATGC AGCTGTATCCAAGTTCAAGAGACTTGGATACAGCTGCATCTTTTTTG GAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAAGATGCAGCTGTATCCAA GTCTCTTGAACTTGGATACAGCTGCATCTGGG-3′ (antisense). These oligonucleotides were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (8), to generate pSUPER-PMLi. To construct pLV-PMLi, the BamHI-SalI fragments of pSUPER-PMLi were subcloned into the BamHI-SalI site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transduced cells (7). pLV-Chk2i was described previously (3).

Lentiviral vector production. The vesicular stomatitis virus (VSV) G-pseudotyped HIV-1-based vector system has been described previously (42). The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 (1, 71) and the VSV G envelope-expressing plasmid pMDG2 as well as pRDI292 into 293FT cells with FuGene6 (Roche Diagnostics, Mannheim, Germany).

HCV infection experiments. The supernatants was collected from cell culture-generated HCV-JFH1 (58)-infected RSc cells (2, 3) at 5 days postinfection and stored at −80°C after filtering through a 0.45-μm filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1 virus, RSc cells (1×10^5 cells/well) were plated onto six-well plates and cultured for 24 h. We then infected the cells with 50 μl (equivalent to a multiplicity of infection of 0.05 to 0.1) of inoculum. The culture supernatants were collected at 97 h postinfection, and the levels of the core protein were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using an RNeasy minikit (Qiagen, Hilden, Germany) for quantitative reverse transcription-PCR (RT-PCR) analysis of intracellular HCV RNA. The level of intracellular HCV RNA in the RSc cells was $>10^8$ copies/μg total RNA at 4 days postinfection.

Quantitative RT-PCR Analysis. The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously (25). We used the following forward and reverse primer sets for the real-time LightCycler PCR: PML, 5′-GAGGAGTTCCAGTTTCTGCG-3′ (forward), 5′-GCGCCTGGCAGATGGGGCAC-3′ (reverse); β-actin, 5′-TGACGG GGTCACCCACACTG-3′ (forward), 5′-AAGCTGTAGCCGCGCTCGGT-3′ (reverse); HCV-O, 5′-AGAGCCATAGTGGTCTGCGG-3′ (forward), 5′-CTT TCGCGACCCAACACTAC-3′ (reverse); and HCV-JFH1, 5′-5′-AGAGCCAT AGTGGTCTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse).

Western blot analysis. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-PML (A301-168A-1; Bethyl Laboratories, Montgomery, TX), anti-Chk2 (DCS-273; Medical & Biological Laboratories, MBL, Nagoya, Japan), anti-HCV core (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan), anti-HCV NS5A (no. 8926; a generous gift from A Takamizawa, The Research Foundation for Microbial Diseases of Osaka University, Japan), anti-signal transducer and activator of transcription 3 (anti-STAT3) (BD Bioscience, San Jose, CA), anti-phospho-STAT3 (Tyr705) (Cell Signaling Technology, Danvers, MA) anti-poly(ADP-ribose) polymerase 1 (anti-PARP-1) (C-2-10; Calbiochem), or anti-β-actin antibody (Sigma).

MTT assay. HuH-7 or O cells (5×10^3 cells/well) were plated onto 96-well plates and cultured for 24 h. The cells were treated with ATO, APO, or NaOH for 24, 48, or 72 h and then subjected to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (cell proliferation kit I; Roche). The absorbance was read using a microplate reader (model 2550; Bio-Rad Laboratories, Hercules, CA) at 550 nm with a reference wavelength of 690 nm.

RL assay. OR6 cells (1.5×10^4 cells/well) were plated onto 24-well plates and cultured for 24 h. The cells were treated with each reagent for 72 h and then subjected to the *Renilla* luciferase (RL) assay according to the manufacturer's instructions (Promega, Madison, WI). A Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

FL assay. Plasmids were transfected into O cells (2×10^4 cells/well in 24-well plates) using FuGene6 and cultured for 24 h. The cells were treated with or without 1 μM ATO for 24 h, and then firefly luciferase (FL) assays were performed according to the manufacturer's instructions (Promega).

Immunofluorescence and confocal microscopic analysis. Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% NP-40 in PBS at room temperature, and incubated with anti-PML antibody (PM001; MBL) at a 1:300 dilution in PBS containing 3% bovine serum albumin at 37°C for 30 min. They were then stained with fluorescein isothiocyanate-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) at a 1:300 dilution in PBS containing bovine serum albumin at 37°C for 30 min, followed by staining with 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 15 min. Following extensive washing in PBS, the cells were mounted on slides using a mounting medium of 90% glycerin–10% PBS with 0.01% *p*-phenylenediamine added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (LSM510; Zeiss, Jena, Germany).

Measurement of intracellular O_2^- and H_2O_2 production. The intracellular superoxide anion radical (O_2^-) levels were measured with an oxidation-sensitive fluorescent probe, dihydroethidium (DHE) (Invitrogen Molecular Probes), that is highly selective for detection of O_2^- among reactive oxygen species (ROS). DHE is cell permeative and reacts with O_2^- to form ethidium, which in turn intercalates in DNA, thereby exhibiting a red fluorescence. The intracellular hydrogen peroxide (H_2O_2) levels were measured with another oxidation-sensitive fluorescent probe dye, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (Invitrogen Molecular Probes). Carboxy-H_2DCFDA was intracellularly deacetylated with esterase and further oxidized with peroxidase to the fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). The ATO- or BSO-treated O cells were washed with PBS and incubated with 5 μM DHE and 20 μM carboxy-H_2DCFDA in PBS at 37°C for 30 min. Cells were then washed twice with PBS. The DHE or DCF fluorescence intensity was measured using a FACS-Calibur flow cytometer. For each sample, 10,000 events were collected. The O_2^- or H_2O_2 levels are indicated as mean fluorescence intensities, which were determined with the CellQuest software (BD Bioscience).

Detection of intracellular glutathione. Intracellular glutathione levels were analyzed using CellTracker Green (5-chloromethylfluorescein diacetate [CMFDA]; Molecular Probes, Invitrogen). CMFDA is a membrane-permeative dye used to determine intracellular glutathione levels. Cytoplasmic esterase converts the nonfluorescent CMFDA to the fluorescent 5-chloromethylfluorescein (CMF), which can then react with glutathione. The excitation peak is at 492 nm, and the fluorescence emission peak is at 517 nm. O cells treated with 1 μM ATO for 72 h were washed with PBS and incubated with 5 μM CMFDA at 37°C for 30 min. The CMF fluorescence intensity was measured using a FACSCalibur flow cytometer. For each sample, 10,000 events were collected. The glutathione levels are given as the relative mean fluorescence intensities, which were determined with CellQuest software.

RESULTS

ATO inhibits HCV RNA replication. First, we quantitatively examined the effect of ATO on the HCV RNA replication in HuH-7-derived O cells harboring a replicative genome-length HCV-O RNA (25). We found that submicromolar concentrations of ATO markedly inhibited genome-length HCV-O RNA replication in the O cells at 72 h after administration (Fig. 1A). The 50% effective concentration (EC_{50}) of ATO required for inhibition of genome-length HCV-O RNA replication was 0.19 μM (Fig. 1A). Consistent with this finding, the expression levels of the HCV core and NS5A proteins were also significantly decreased in the cell lysates of O cells treated with ATO for 72 h (Fig. 1B). In addition, ATO markedly inhibited the replication of the subgenomic replicon RNA (31), with an EC_{50} of 0.48 μM at 72 h after the treatment (Fig. 1C). We next examined the effect of ATO on HCV reproduction by HCV-JFH1 infection (58). The results revealed that ATO significantly inhibited the intracellular RNA replication of HCV-

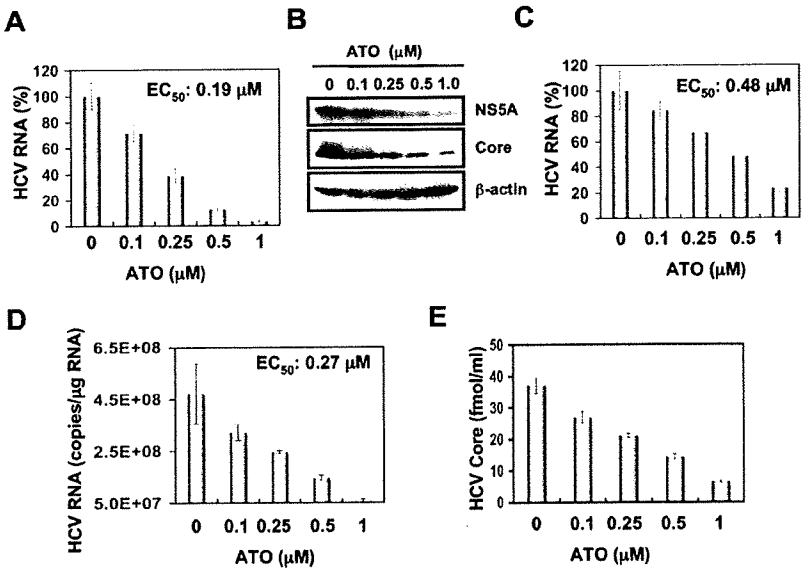

FIG. 1. Inhibition of HCV RNA replication by ATO. (A) The level of genome-length HCV RNA in O cells after the treatment with ATO was monitored by real-time LightCycler PCR. Experiments were done in triplicate and, bars represent the mean percentage of HCV RNA. Error bars indicate standard deviations. (B) HCV core and NS5A protein expression levels in O cells after treatment with ATO. The results of Western blot analysis of cellular lysates with anti-HCV core, anti-HCV NS5A, or anti-β-actin antibody in O cells at 72 h after treatment with ATO at the indicated concentration are shown. (C) The level of subgenomic replicon RNA was monitored by real-time LightCycler PCR. Results from three independent experiments conducted as described for panel A are shown. (D) The level of intracellular genome-length HCV-JFH1 RNA was monitored by real-time LightCycler PCR. RSc cells were pretreated with the indicated concentration of ATO for 13 h, followed by inoculation of the HCV-JFH1 virus, and then the infected cells were further incubated with ATO for 97 h. Results from three independent experiments conducted as described for panel A are shown. (E) The levels of the core protein in the culture supernatants treated as described for panel D were determined by enzyme-linked immunosorbent assay. Experiments were done in triplicate, and bars represent the mean core protein levels.

JFH1, with an EC_{50} of 0.27 μM, as well as the release of core protein into the culture supernatants in HuH-7-derived RSc cells at 97 h after inoculation of the HCV-JFH1 virus (Fig. 1D and E). Thus, we have demonstrated for the first time that ATO can inhibit the reproduction of HCV and particularly HCV RNA replication.

Effect of APO on HCV replication. Arsenic is known to exist in two oxidation states, As(III) in ATO and As(V) in APO. As ATO in the lower valence state has been reported to be more toxic than APO (48), we compared their anti-HCV activities using an OR6 assay system, which was recently developed as a luciferase reporter assay system for monitoring genome-length HCV RNA replication in HuH-7-derived OR6 cells (Fig. 2A) (25). The results showed that APO could not strongly suppress HCV replication at submicromolar concentrations, while ATO strongly inhibited it, with an EC_{50} of 0.33 μM (Fig. 2B and C), indicating that ATO has unique anti-HCV activity. In this context, it is relevant that the expression level of HCV core protein was also remarkably decreased in the cell lysates of O cells treated with ATO, but not those treated with APO, for 72 h (Fig. 2D). Thus, APO seems to be a useful negative probe to clarify the mechanism of the anti-HCV activity of ATO.

ATO does not affect cell growth at submicromolar concentrations. ATO has been reported to induce apoptosis (11, 14, 20, 21, 26–28, 33, 48, 66). Therefore, such an ATO-induced apoptosis may be involved in the anti-HCV activity. To test this possibility, we examined the effect of ATO or APO at various concentrations on cell proliferation by colorimetric MTT assay. In this context, we demonstrated that ATO did not affect the cell proliferation of O cells or the parental HCV-negative HuH-7 cells at submicromolar concentrations (Fig. 3A and E). In contrast, 4 or 8 μM ATO significantly inhibited cell proliferation (Fig. 3B and F). Similarly, APO did not affect the cell proliferation at less than 2 μM (Fig. 3C and D). Consistent with the above results, ATO-treated O cells exhibited normal growth rates and cell viabilities, at least at 1 μM for 72 h (Fig. 3G). Furthermore, we did not observe the cleavage of PARP-1, which is known to be an important substrate for activated caspase 3, in O cells treated with 1 μM ATO at least until 72 h (Fig. 3H), indicating that 1 μM ATO did not induce apoptosis in O cells. Thus, we concluded that the anti-HCV activity was independent of ATO-induced apoptosis or cell toxicity, at least at submicromolar concentrations.

PML and Chk2 are dispensable for the anti-HCV activity of ATO. Since PML is known to be a target of ATO (70), we first examined the subcellular localization of PML in O cells treated with either 1 μM ATO or 1 μM APO for 72 h by means of an anti-PML antibody (PM001; MBL) that can recognize most of the PML splicing variants and is useful for immunofluorescence analysis. The results showed that PML was localized predominantly in punctate nuclear speckles termed PML-NBs in control O cells (Fig. 4A). Interestingly, we noticed that some nuclear PML, but not all, disappeared and was translocated into discrete cytoplasmic bodies in the O cells treated with 1 μM ATO (Fig. 4A). We also observed cytoplasmic translocation of PML in the O cells treated with 1 μM APO for 72 h (Fig. 4A). Furthermore, we observed a similar cytoplasmic translocation of PML in the HCV-negative 293FT or HeLa

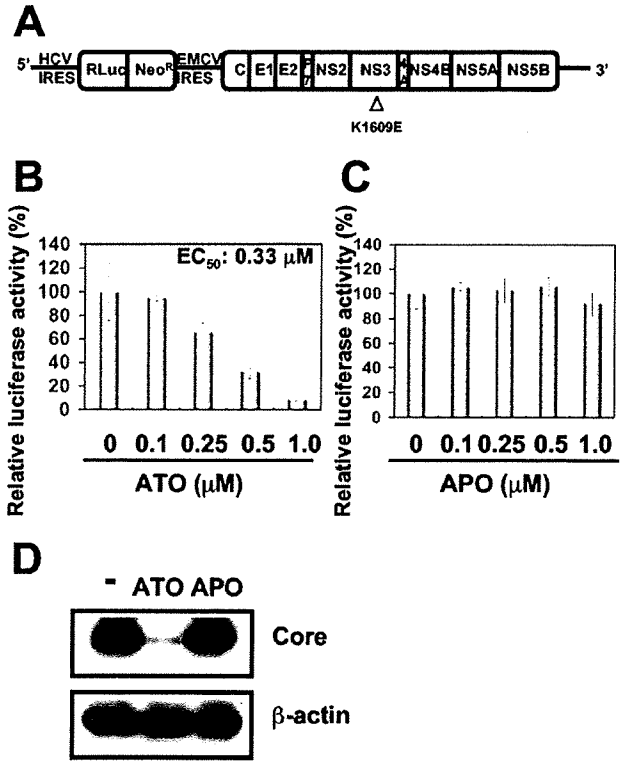

FIG. 2. Effect of APO on HCV replication. (A) Schematic representation of genome-length HCV RNA encoding the RL gene as a reporter (ORN/C-5B/KE RNA) replicated in OR6 cells. The RL is expressed as a fusion protein with neomycin phosphotransferase (Neo^R). The position of an adaptive mutation, K1609E in NS3, is indicated by an open triangle. (B) Effect of ATO on genome-length HCV RNA replication. At 72 h after treatment of OR6 cells with ATO at the indicated concentrations, the replication level of HCV RNA was monitored by the RL assay. The relative RL activity is shown. The results shown are means from three independent experiments. Error bars indicate standard deviations. (C) Effect of APO on genome-length HCV RNA replication. At 72 h after treatment of OR6 cells with APO at the indicated concentrations, the replication level of HCV RNA was monitored by the RL assay as described for panel B. (D) HCV core protein expression level in O cells after treatment with either ATO or APO. The results of Western blot analysis of cellular lysates with anti-HCV core or anti-β-actin antibody in O cells at 72 h after treatment with either 1 μM ATO or 1 μM APO are shown.

cells after the treatment with ATO (data not shown). Thus, we concluded that the cytoplasmic translocation of PML after the treatment with ATO was not associated with anti-HCV activity. Next, Western blot analysis to compare PML expression in the lysates of O cells treated with 1 μM ATO or 1 μM APO for 72 h was performed using another anti-PML antibody, A301-168A-1 (a gift from Bethyl Laboratories), which can recognize the longest isoform, PML I, but not shorter PML isoforms such as PML VI and which has been proven useful for Western blot analysis. Consistent with the previous finding that ATO promotes PML degradation (70), the expression level of the PML I protein was lower in the ATO-treated O cells than in the APO-treated O cells (Fig. 4B), suggesting that PML degradation by ATO is associated with anti-HCV activity. To further examine whether PML is directly involved in the anti-HCV activity of ATO, we used lentiviral vector-mediated RNA interference to stably knock down PML in the O cells. To express an shRNA targeted to all PML isoforms (56), we used the VSV G-pseudotyped HIV-1-based vector system (1, 42, 71). We used the puromycin-resistant pooled cells at 10 days after the lentiviral transduction in this experiment. Immunofluorescence and Western blot analysis demonstrated a very effective knockdown of PML in the O cells (Fig. 4C and D). We quantitatively examined the level of HCV RNA in the PML knockdown O cells treated with or without either 1 μM ATO (Fig. 4E) or 1 μM APO (Fig. 4F) for 72 h. The results showed that the replication level of genome-length HCV-O RNA in the untreated PML knockdown cells was similar to that in control cells (Fig. 4E), suggesting that PML is dispensable in HCV RNA replication. Importantly, ATO effectively inhibited the HCV RNA replication in both the PML knockdown cells and control cells compared with that of the APO-treated cells (Fig. 4E and F). Thus, we concluded that PML was dispensable for the anti-HCV activity of ATO. Since the Chk2 checkpoint kinase has recently been implicated in ATO-induced apoptosis and in association with PML (27, 63, 64, 66), we examined the anti-HCV activity in the ATO-treated Chk2 knockdown O cells (3). As we previously described, Western blot analysis demonstrated very effective knockdown of Chk2 in O cells (Fig. 4G). Accordingly, we examined the level of HCV RNA in Chk2 knockdown cells treated with or without either 1 μM ATO (Fig. 4H) or 1 μM APO (Fig. 4I) for 72 h. Consistent with our recent finding that Chk2 is required for HCV RNA replication, the replication of genome-length HCV RNA in the untreated Chk2 knockdown cells was remarkably suppressed (Fig. 4H). However, ATO strongly inhibited the HCV RNA replication in the Chk2 knockdown cells compared with that in the APO-treated Chk2 knockdown cells (Fig. 4H and I), suggesting that Chk2 is not implicated in the anti-HCV activity of ATO.

Effect of ATO on the stress-signaling pathways. To date, the focus has been on PML and PML-retinoic acid receptor α as major targets of ATO (70). On the other hand, arsenic has been reported to modulate other cell-signaling pathways, especially stress-responsive transcription factors, such as nuclear factor κB (NF-κB), activator protein 1 (AP-1), and STAT3 (12, 37, 38, 62). Therefore, we examined the involvement of several stress-responsive pathways in the anti-HCV activity of ATO by luciferase-based reporter assays or Western blot analysis using an antibody which specifically recognizes STAT3 phosphorylated at tyrosine 705. Although it has been reported that ATO inhibited the NF-κB signaling pathway through a direct interaction with IKKβ at a high concentration (more than 10 μM) (29), neither 1 μM ATO nor 1 μM APO affected the endogenous NF-κB transcriptional activity in the present study (Fig. 5A and B). Conversely, ATO at least slightly stimulated mitogen-activated protein kinase kinase kinase (MEKK)-mediated NF-κB activation (Fig. 5A and B). Since NF-κB activation has been shown to stimulate HCV replication (60), the NF-κB pathway would seem not to be essential for the anti-HCV activity of ATO. Next, regarding the AP-1 signaling pathway, both ATO and APO are known to activate c-Jun N-terminal kinase (JNK) (45). Importantly, there was no stimulation of JNK activity at a dose below 30 μM (45). In fact, 50 μM ATO but not 50 μM APO strongly stimulates AP-1 activity by in-

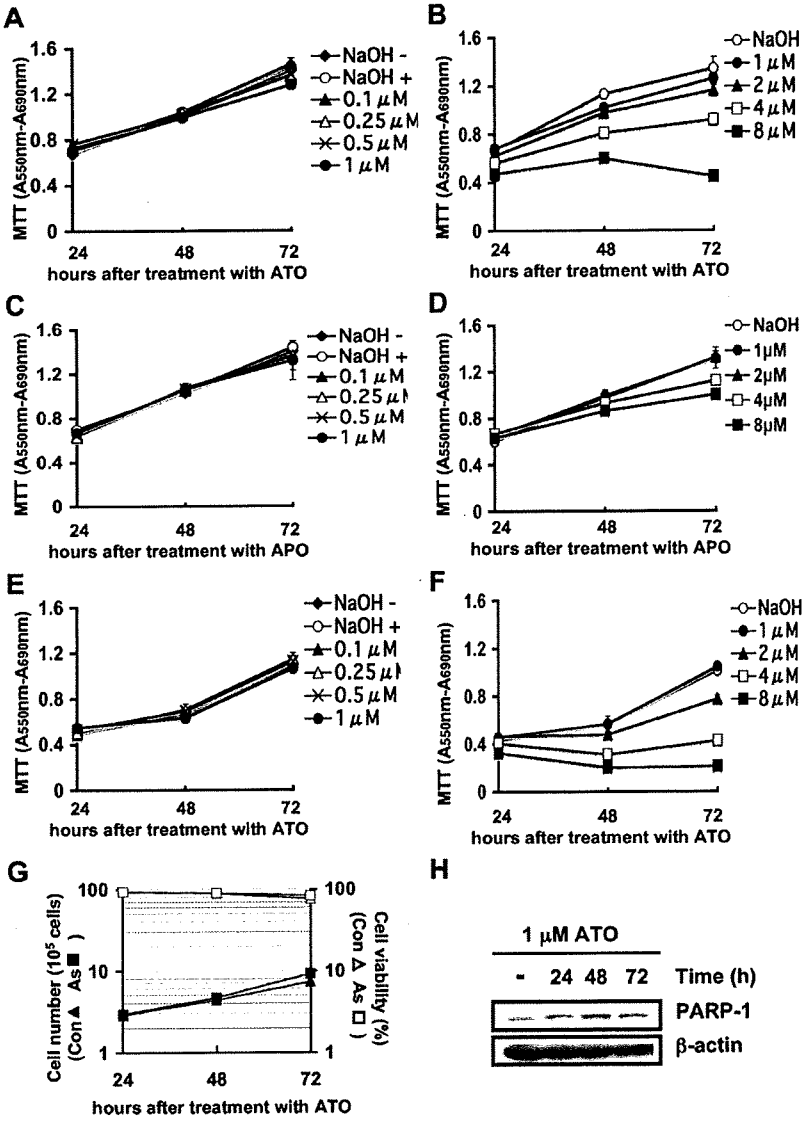

FIG. 3. Effect of ATO on cell growth and viability. (A and B) MTT assay of O cell lysates at the indicated times after treatment with ATO at various concentrations. NaOH (10 μM) was used as the solvent for ATO. The results shown are means from three independent experiments. Error bars indicate standard deviations. (C and D) MTT assay of O cell lysates at the indicated times after treatment with APO at various concentrations. (E and F) MTT assay of HuH-7 cell lysates at the indicated times after treatment with ATO at various concentrations. (G) Growth curve and viability of O cells after treatment with either 10 μM NaOH (Con) or 1 μM ATO (As). (H) Western blot analysis of cellular lysates with anti-PARP-1 or anti-β-actin antibody in O cells at the indicated times after treatment with 1 μM ATO.

hibiting a JNK phosphatase (10). Consistently, we found that both 1 μM ATO and 1 μM APO had a marginal effect on the AP-1 signaling pathway (Fig. 5C and D), suggesting that the AP-1 pathway is also not involved in the anti-HCV activity of ATO. Regarding the STAT3 signaling pathway, ATO has been reported to inhibit the phosphorylation of the STAT3 tyrosine at 705, leading to inactivation of the JAK-STAT signaling pathway (12, 62). In contrast, it has been reported that HCV constitutively phosphorylates and activates STAT3 (49, 59, 67). In this context, we observed constitutive tyrosine phosphorylation of STAT3 in untreated O cells (Fig. 5E). Furthermore, the marginal effect of 1 μM ATO on STAT3 phosphorylation and interleukin-6-mediated STAT3 activation was also observed (Fig. 5E and F). Taken together, these results at least suggest that the NF-κB, AP-1, and STAT3 pathways may not be associated with the anti-HCV activity of ATO at submicromolar concentrations.

The anti-HCV activity of ATO is associated with the glutathione redox system and oxidative stress. Finally, we focused on the involvement of the glutathione redox system and oxidative stress in the anti-HCV activity of ATO. For this, we analyzed the HCV replication level after combination treatment with ATO and antioxidants such as NAC and vitamin C using the OR6 assay system. When OR6 cells were treated with

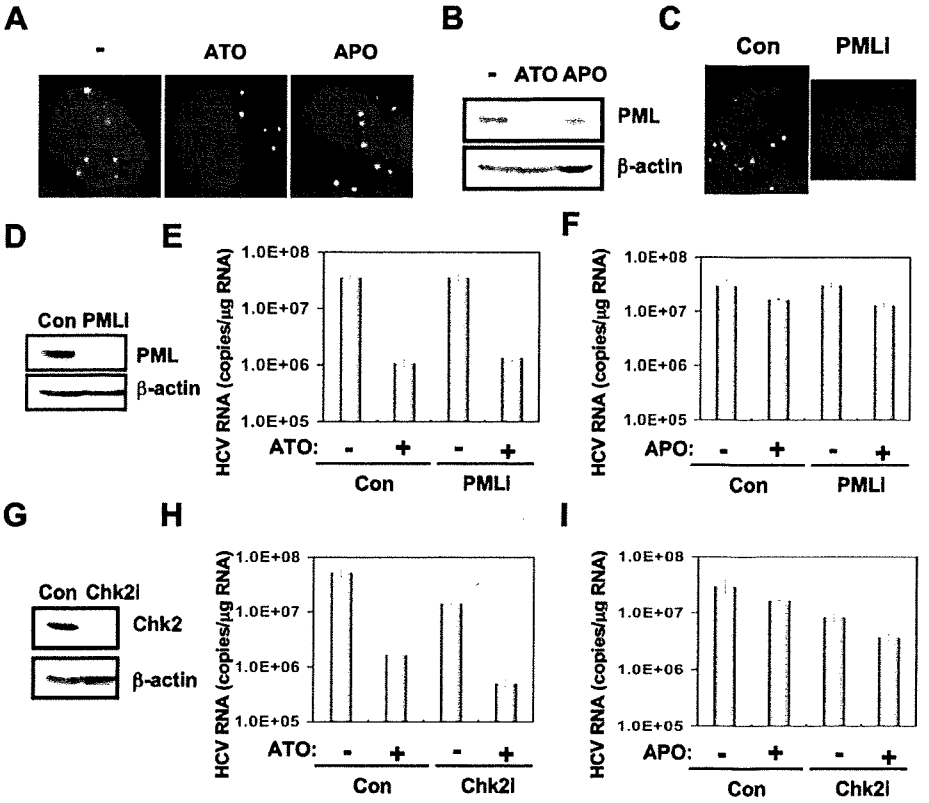

FIG. 4. PML and Chk2 are not required for the anti-HCV activity of ATO. (A) Subcellular localization of PML in O cells at 72 h after treatment with 10 μM NaOH (−), 1 μM ATO, or 1 μM APO. PML was detected by indirect immunofluorescence analysis with anti-PML antibody (PM001). DAPI staining of the nuclear DNA is also shown. (B) Induction of PML degradation by ATO but not by APO. The results of Western blot analysis of cellular lysates of O cells at 72 h after treatment with 10 μM NaOH (−), 1 μM ATO, or 1 μM APO with anti-PML (A301-168A-1) or anti-β-actin antibody are shown. (C) Stable knockdown of PML by shRNA-producing lentiviral vector in O cells. PML was detected by indirect immunofluorescence analysis with anti-PML antibody (PM001) in O cells expressing shRNA targeted to PML (PMLi) as well as in O cells transduced with a control lentiviral vector (Con). (D) Western blot analysis of cellular lysates with anti-PML (A301-168A-1) or anti-β-actin antibody in PML knockdown O cells (PMLi) as well as in control O cells (Con). (E and F) The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR in PML knockdown O cells (PMLi) as well as in control O cells (Con) after treatment with 10 μM NaOH (−), 1 μM ATO (+) (E), or 1 μM APO (+) (F) for 72 h. Results from three independent experiments conducted as described in the legend to Fig. 1A are shown. (G) Inhibition of Chk2 expression by shRNA-producing lentiviral vector. The results of Western blot analysis of cellular lysates with anti-Chk2 or anti-β-actin antibody in O cells expressing shRNA targeted to Chk2 (Chk2i) as well as in O cells transduced with a control lentiviral vector (Con) are shown. (H and I) The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR in Chk2 knockdown O cells (Chk2i) as well as in control O cells (Con) after treatment with 10 μM NaOH (−), 1 μM ATO (+) (H), or 1 μM APO (+) (I) for 72 h. Results from three independent experiments conducted as described in the legend to Fig. 1A are shown.

either 100 μM vitamin C or 10 mM NAC alone for 24 h or 72 h, the HCV replication was slightly enhanced (Fig. 6A and B), indicating that the antioxidant can activate HCV replication. Although the anti-HCV activity in the OR6 cells treated with 1 μM ATO and in combination with 100 μM vitamin C for 24 h was weakly reduced, 10 mM NAC completely and partially eliminated the anti-HCV activity of ATO after 24 h (Fig. 6A) and 72 h (Fig. 6B) of treatment, respectively, suggesting that oxidative stress and the glutathione redox system are associated with the anti-HCV activity of ATO. In contrast, the iNOS inhibitor 1400W did not suppress the HCV RNA replication or eliminate the anti-HCV activity of ATO, suggesting that NO is not involved in the anti-HCV activity of ATO (Fig. 6C). To further examine the involvement of oxidative stress in the anti-HCV activity of ATO, we examined ROS production in ATO-treated cells using two oxidative-sensitive fluorescent probes, DHE for detection of intracellular O_2^- and DCF for detection of intracellular H_2O_2. We found that 1 μM ATO could generate a significant level of intracellular O_2^- but not intracellular H_2O_2, while 2 μM BSO, an inhibitor of glutathione synthesis (14, 20, 33), could induce both O_2^- and H_2O_2 (Fig. 6D to H). Importantly, NAC diminished the ATO-dependent O_2^- induction (Fig. 6F). Since glutathione is a major antioxidant in cells and can clear away superoxide anion free radical, we also analyzed the changes of the intracellular glutathione level in ATO-treated O cells using CMF fluorescence, which can react with glutathione. As a result, we observed significant glutathione depletion in the cells treated with at least 1 μM ATO (Fig. 6I). To further confirm the involvement of glutathione in the anti-HCV activity of ATO, we examined the effect of cotreatment with ATO and BSO. When the OR6 cells were treated with 1 μM BSO alone, the HCV replication

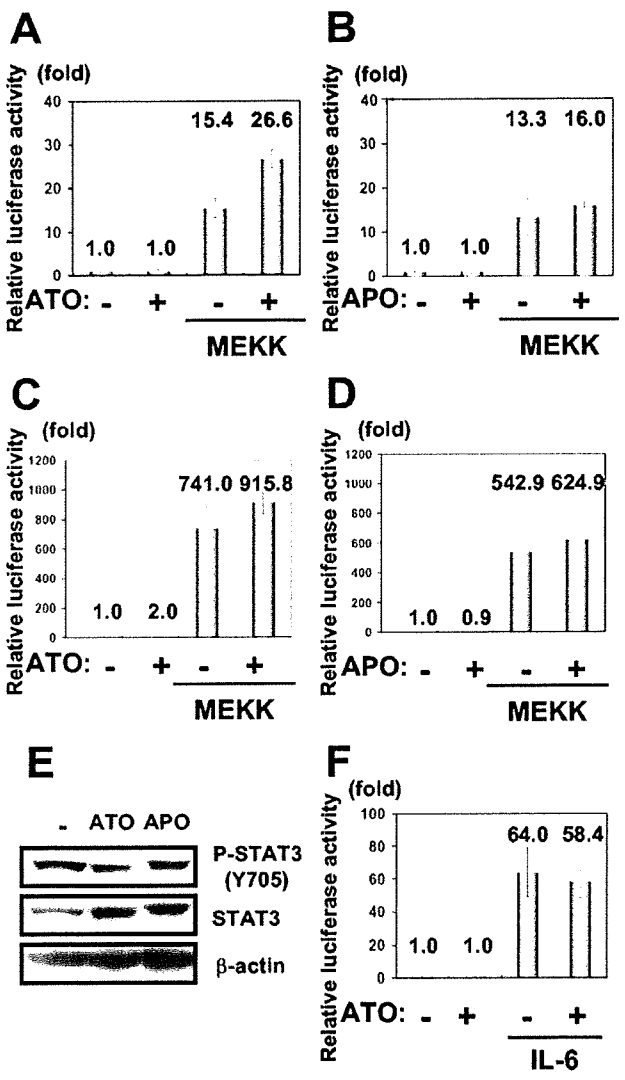

FIG. 5. Effect of ATO on the stress-signaling pathways. (A and B) Effect of ATO or APO on the NF-κB signaling pathway. O cells were transfected with 100 ng of reporter plasmid, pNF-κB-Luc, and/or 100 ng of pFC-MEKK (Stratagene, La Jolla, CA). Cells were treated with either 1 μM ATO (A) or 1 μM APO (B), and an FL assay was performed 24 h later. The results shown are means from three independent experiments. The relative FL activity is shown. (C and D) Effect of ATO or APO on the AP-1 signaling pathway. O cells were transfected with 100 ng of pAP-1-Luc and/or 100 ng of pFC-MEKK (Stratagene). Cells were treated with either 1 μM ATO (C) or 1 μM APO (D), and an FL assay was performed 24 h later as described for panels A and B. (E) Effect of ATO on the phosphorylation level of STAT3 at tyrosine 705. The results of Western blot analysis of cellular lysates with anti-phospho-STAT3 (Tyr705), anti-STAT3, or anti-β-actin antibody in O cells treated with either 1 μM ATO or 1 μM APO for 24 h are shown. (F) Effect of ATO on the STAT3 signaling pathway. O cells were transfected with 100 ng of STAT3 reporter APRE-Luc (41) (STAT3-Luc, a generous gift from T. Hirano, Osaka University, Japan). Cells were treated with 1 μM ATO for 19 h and then stimulated with 100 ng/ml of interleukin-6 for 5 h, and an FL assay was performed as described for panels A and B.

level was suppressed by about 30% compared with that of the control cells, and this occurred without cell toxicity (data not shown). However, consistent with previous reports in which ATO-induced apoptosis was enhanced by BSO (14, 20, 33), most of the cells died, possibly through apoptosis, when the OR6 cells were cotreated with 1 μM ATO and 1 μM BSO for 72 h (data not shown), suggesting that ATO and BSO synergistically generate ROS and deplete glutathione, resulting in induction of oxidative damage. Taken together, these results suggest that ATO may inhibit the HCV RNA replication by modulating the glutathione redox system and oxidative stress.

DISCUSSION

ATO has been reported to affect multiple biological functions, such as PML-NB formation, apoptosis, differentiation, stress response, and viral infection (38). Indeed, ATO has been shown to increase retroviral infectivity, including infectivity of HIV-1, HIV-2, feline immunodeficiency virus, simian immunodeficiency virus from rhesus macaques, and murine leukemia virus, although the mechanisms responsible for these changes are not well understood (5, 6, 32, 44, 47, 50, 57). PML, which is involved in host antiviral defenses, is required for the formation of the PML-NB, which is often disrupted or sequestered in the cytoplasm by infection with DNA or RNA viruses (17). The fact that ATO promotes the degradation of PML and alters the morphology or distribution of PML-NBs suggests that ATO enhances HIV-1 infection by antagonizing an antiviral activity associated with PML. In fact, HIV-1 infection has been reported to alter PML localization (57), although others have failed to confirm this finding (5). Furthermore, Berthoux et al. demonstrated that ATO stimulated retroviral reverse transcription (5). Moreover, ATO has been shown to have an inhibitory effect on host restriction factors, such as TRIM5a, Refl, and Lv1, in a cell type-dependent manner (5, 6, 32, 44, 47, 50). In contrast, we have demonstrated that ATO strongly inhibited genome-length HCV RNA replication without cell toxicity (Fig. 1A and 2A). In addition, we observed the cytoplasmic translocation of PML in the HCV RNA-replicating O cells after the treatment with ATO (Fig. 4A). However, PML was dispensable for the anti-HCV activity of ATO as well as HCV RNA replication (Fig. 4E). In this regard, it is worth noting the recent report by Herzer et al. that the HCV core protein interacts with PML isoform IV and abrogates the PML function (22). Thus, PML may be involved in the HCV life cycle. In any case, the sensitivity to ATO and the cellular target of ATO seem to be different between HCV and HIV-1.

HCV infection has been shown to cause a state of chronic oxidative stress like that seen in chronic hepatitis C, which may contribute to fibrosis and carcinogenesis in the liver (16, 18, 40). In particular, HCV replication has been associated with the endoplasmic reticulum (ER), where HCV causes ER stress. Indeed, HCV NS5A and core, the ER-associated proteins, have been reported to trigger ER stress (4, 55). Therefore, HCV infection causes production of ROS and lowering of mitochondrial transmembrane potential through calcium signaling (4, 36). Among the HCV proteins, core, E1, NS3, and NS5A have been shown to be potent ROS inducers, and these HCV proteins also alter intracellular calcium levels and induce oxidative stress, thereby inducing DNA damage, and constitu-

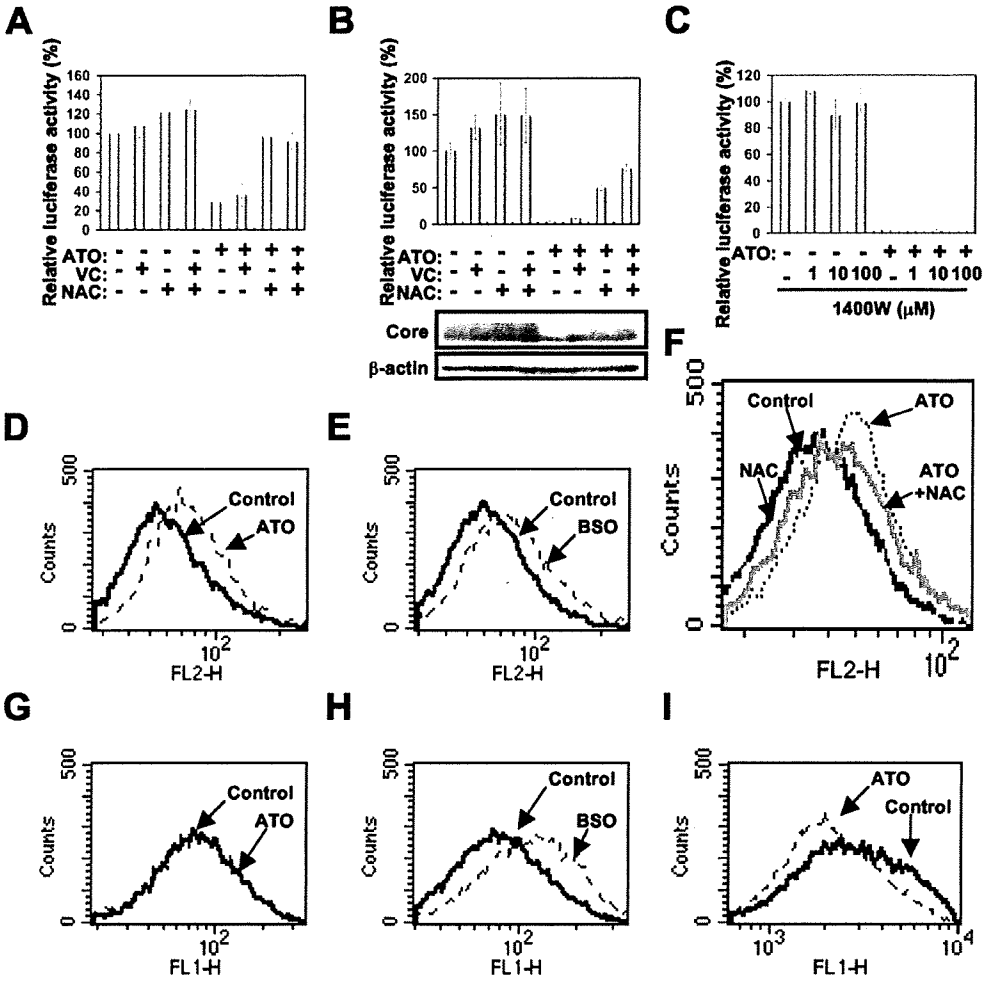

FIG. 6. The anti-HCV activity of ATO is associated with the glutathione redox system and oxidative stress. (A and B) The anti-HCV activity of ATO is eliminated by treatment with the antioxidant NAC. OR6 cells were treated with 1 μM ATO alone and in combination with 100 μM vitamin C (VC), with or without 10 mM NAC, for 24 h (A) or 72 h (B). The replication level of HCV RNA was monitored by the RL assay. The relative RL activity is shown. The results shown are means from three independent experiments; error bars indicate standard deviations. The results of Western blot analysis of cellular lysates with anti-HCV core or anti-β-actin antibody in OR6 cells at 72 h after the treatment with 1 μM ATO alone and in combination with 100 μM VC, with or without 10 mM NAC, are also shown. (C) Effect of combination treatment with ATO and the iNOS inhibitor 1400W on HCV RNA replication. OR6 cells were treated with 1 μM ATO alone and in combination with 1400W at the indicated concentrations for 72 h. The replication level of HCV RNA was monitored by the RL assay as described for panels A and B. (D and E) Effect of ATO on production of a ROS, O_2^-, in O cells. O cells were treated with 1 μM ATO (D) or 2 μM BSO (E) for 24 h. The intracellular O_2^- level was measured by flow cytometry using DHE as described in Materials and Methods. (F) Inhibition of ATO-dependent O_2^- induction by NAC. O cells were treated with either 1 μM ATO or 10 mM NAC alone and in combination with 10 mM NAC for 24 h. (G and H) Effect of ATO on production of a ROS, H_2O_2, in O cells. O cells were treated with 1 μM ATO (G) or 2 μM BSO (H) for 24 h. The intracellular H_2O_2 level was measured by flow cytometry using DCF as described in Materials and Methods. (I) Effect of ATO on the intracellular glutathione level in O cells. O cells were treated with 1 μM ATO for 72 h. The intracellular glutathione level was measured by flow cytometry using CellTracker Green CMFDA as described in Materials and Methods.

tively activate STAT3 and NF-κB, which are associated with HCV pathogenesis (19, 34, 36, 43, 49, 59, 60, 67). In fact, oxidative stress has been shown to trigger STAT3 tyrosine phosphorylation and nuclear translocation, which correlate with the activation of STAT3, leading to its DNA-binding activity (9). In contrast, ATO inhibited the STAT3 tyrosine phosphorylation through direct interaction with JAK kinase, thereby suppressing the transcriptional activity of STAT3 (12, 62). Importantly, STAT3 activation has been reported to be associated with HCV RNA replication (59, 69). The STAT3 Tyr705 dominant negative mutant has been shown to inhibit HCV RNA replication, suggesting that STAT3 positively regulates HCV replication (59). In contrast, others have reported that STAT3 induces anti-HCV activity (69). In this study, we analyzed the potential effect of ATO treatment on a set of stress-signaling events, including the NF-κB, AP-1, and STAT3 pathways, since ATO is known to modulate various signaling pathways. However, at 1 μM, which exerted an anti-HCV activity, the respective signaling pathways were not affected, arguing that the anti-HCV activity is independent of these

pathways (Fig. 5). In this regard, these stress-signaling pathways have been reported to be constitutively activated in HCV core- or NS5A-expressing cells (19, 36, 49, 59, 60, 67). In addition, previous studies demonstrated that ATO modulates the NF-κB, AP-1, and STAT3 pathways at higher concentrations (NF-κB, >10 μM; AP-1, >30 μM; STAT3, >4 μM). Therefore, we may have only observed the marginal effect of ATO in this study (Fig. 5). On the other hand, the HCV core or NS3 protein as well as HCV infection induces NO, leading to induction of double-stranded DNA breaks and accumulation of mutations of cellular genes (35). However, the iNOS inhibitor 1400W could not suppress HCV RNA replication and the anti-HCV activity of ATO, indicating that NO is not associated with the anti-HCV activity or with HCV replication (Fig. 6C).

It has been indicated that oxidative damage plays an important role in the effect of ATO (38). ROS generated in response to ATO exposure lead to accumulation of intracellular H_2O_2. Glutathione peroxidase and catalase are key enzymes regulating the levels of ROS and protecting cells from ATO-induced damage (26). However, the gastrointestinal glutathione peroxidase was drastically downregulated in cells harboring HCV replicons, which are rendered more susceptible to oxidative stress (39). The glutathione redox system has been implicated in the cellular defense system (14, 20). Glutathione, a major antioxidant in cells, is a tripeptide synthesized from cysteine, glutamic acid, and glycine, and it can scavenge superoxide anion free radicals. ATO has been shown to bind to the sulfhydryl group of glutathione and deplete the intracellular glutathione, resulting in enhancement of the sensitivity to oxidative damage (20, 33). Conversely, the antioxidant NAC is readily taken up by cells and serves as a precursor to elevate intracellular glutathione (53). In fact, ATO-induced apoptosis has been shown to be inhibited by NAC (11, 14, 21, 28). In this study, we have demonstrated that the anti-HCV activity of ATO was completely eliminated by treatment with NAC for 24 h (Fig. 6A). In addition, we found that ATO increased intracellular O_2^- but not H_2O_2 and depleted the intracellular glutathione in HCV RNA-replicating cells (Fig. 6D to I). Importantly, NAC diminished the ATO-dependent O_2^- induction (Fig. 6F). This finding could strengthen the link between ATO-dependent oxidative stress and anti-HCV activity. Similarly, Wen et al. reported an increase in ROS and enhanced susceptibility to glutathione depletion in the HCV core-expressing HepG2 cells (61). Accordingly, ROS have been shown to significantly suppress RNA replication in HCV replicon-harboring cells treated with H_2O_2 (13). In addition, HCV replication has been shown to be inhibited by lipid peroxidation of arachidonate, and this peroxidation could be blocked by lipid-soluble antioxidants such as vitamin E (23). Conversely, several antioxidants, such as vitamin C, vitamin E, and NAC, enhanced HCV replication in the present study (Fig. 6A and B) (65). Thus, we suggest that ATO inhibited HCV RNA replication by modulating the glutathione redox system and oxidative stress. In contrast to the above findings with HCV, NAC has been shown to suppress HIV-1 replication by preventing the activation of HIV-1 long terminal repeat transcription by NF-κB, suggesting a correlation between a decrease in glutathione levels and activation of HIV-1 replication (46, 53, 54). In this context, ATO has shown opposite effects on HIV-1 and HCV replication, stimulating the former and inhibiting the latter. Considering all of these results together, ATO can be regarded as a useful, novel anti-HCV reagent. In addition, the host redox system may be critical for HCV replication and may represent a pivotal target for the clinical treatment of patients with chronic hepatitis C.

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A new living cell-based assay system for monitoring genome-length hepatitis C virus RNA replication

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ABSTRACT

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

1. Introduction

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

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

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

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

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2. Materials and methods

2.1. Reagents

IFN-α, IFN-γ, and CsA were purchased from Sigma–Aldrich (St. Louis, MO). IFN-β was a gift from Toray Industries (Tokyo, Japan). Fluvastatin (FLV) was purchased from Calbiochem (San Diego, CA).

2.2. Cell culture

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

2.3. Construction of plasmids and RNA synthesis

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

2.4. RNA transfection and selection of G418-resistant cells

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

2.5. Visualization of the fluorescence by EGFP

The fluorescence of EGFP was directly visualized by a fluorescence microscope (Axiovert 25CFL; Carl Zeiss) or a confocal laser-scanning microscope (LSM510; Carl Zeiss). The cells were fixed with 4% paraformaldehyde and were photographed under a fluorescence microscope or a confocal laser-scanning microscope as described previously (Dansako et al., 2003).

2.6. Integration analysis

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

2.7. Northern blot analysis

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

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

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

2.9. Western blot analysis

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

2.10. Quantitative RT-PCR analysis

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

3. Results

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

We previously developed a dicistronic genome-length HCV RNA (O strain of genotype 1b) replication system that stably expresses *Renilla* luciferase as a reporter in order to monitor the level of HCV RNA replication (OR6 assay system) (Ikeda et al., 2005; Naka et al., 2005). To further facilitate mass screening of potential candidates for anti-HCV reagents, we attempted to develop a novel assay system for monitoring the level of HCV RNA replication without lysis of cells. For this purpose, we chose EGFP as a reporter, and we first tried to establish cloned cell lines that efficiently replicate genome-length HCV RNA encoding EGFP. All of the constructed plasmids (Fig. 1) were used as templates for RNA synthesis in vitro, and then the transcribed RNAs were transfected into OR6c cells by the electroporation method, as described in Section 2. After 3 weeks of G418 selection, we obtained several G418-resistant colonies from the OGN/C-5B/KE RNA, ON/GC-5B/KE RNA, ON/C-5B G2390/KE RNA, or OGN/GC-5B/KE RNA-introduced cells, and most of the G418-resistant colonies were successfully established as cell lines (Fig. 1). In contrast, no G418-resistant colonies were obtained from the OGN/C-5B G2390/KE RNA, ON/GC-5B G2390/KE RNA, or OGN/GC-5B G2390/KE RNA-introduced cells (Fig. 1).

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

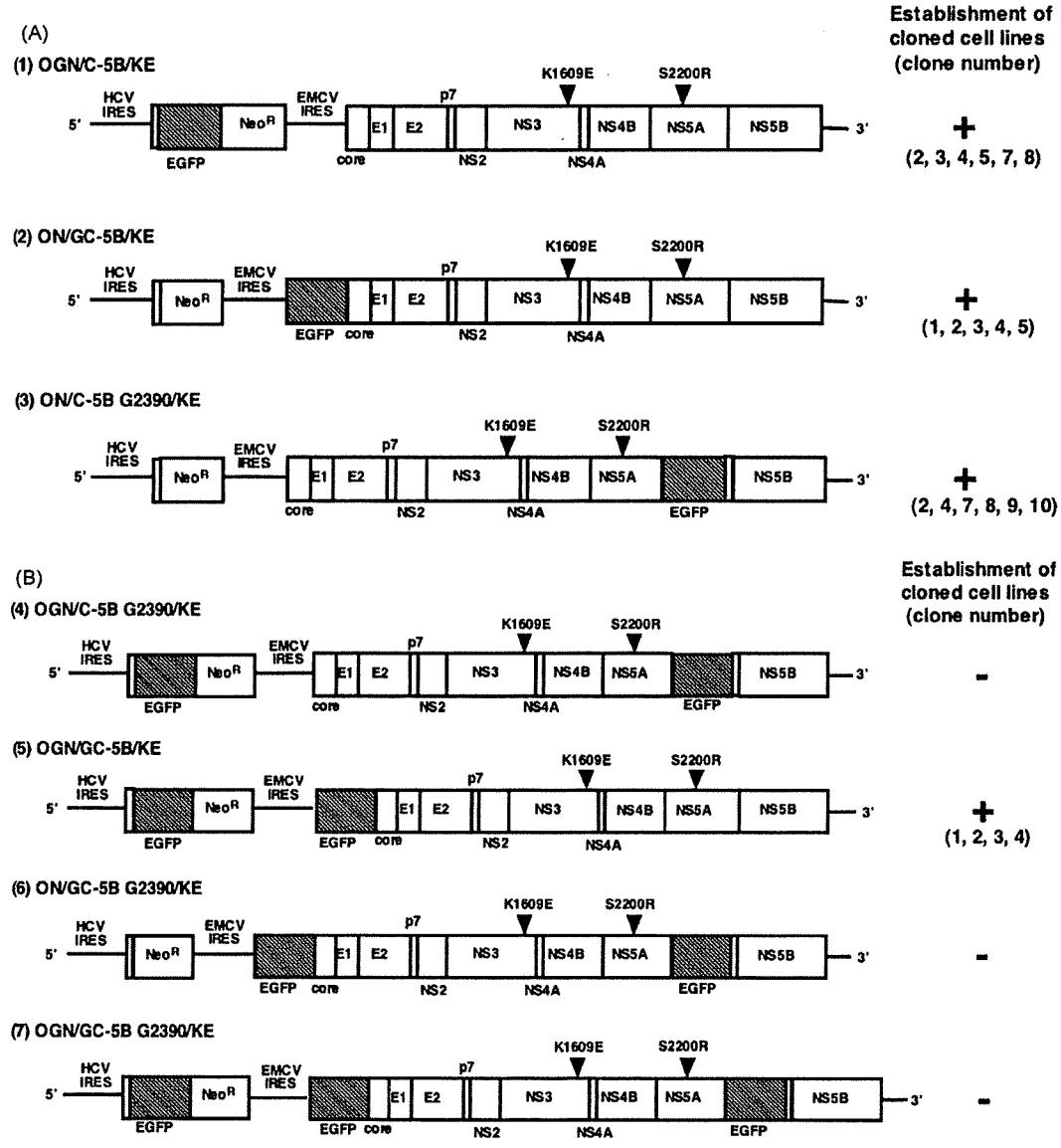

Fig. 1. Schematic presentation of various genome-length HCV RNAs (HCV-O strain) containing an EGFP-encoding sequence. (A) Genome-length HCV RNAs containing one copy of the EGFP-encoding sequence. The basic construct is described in our previous study (Ikeda et al., 2005). The EGFP-encoding region is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