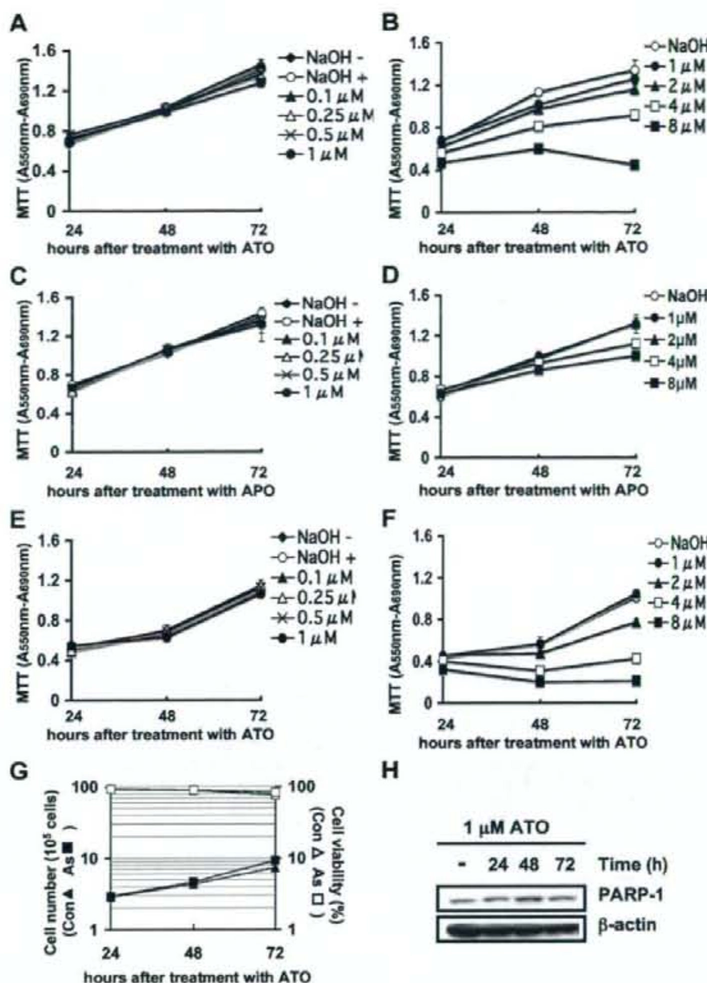

FIG. 3. Effect of ATO on cell growth and viability. (A and B) MTT assay of O cell lysates at the indicated times after treatment with ATO at various concentrations. NaOH (10 μM) was used as the solvent for ATO. The results shown are means from three independent experiments. Error bars indicate standard 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

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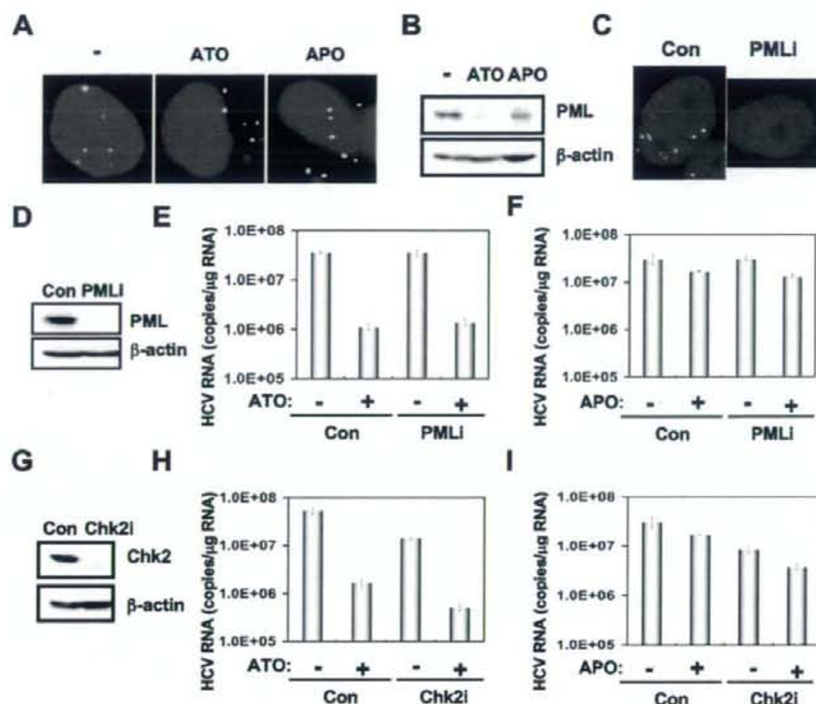

FIG. 4. PML and Chk2 are not required for the anti-HCV activity of ATO. (A) Subcellular localization of PML in O cells at 72 h after treatment with 10 μM NaOH (−), 1 μM ATO, or 1 μM APO. PML was detected by indirect immunofluorescence analysis with anti-PML antibody (PM001). DAPI staining of the nuclear 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.

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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

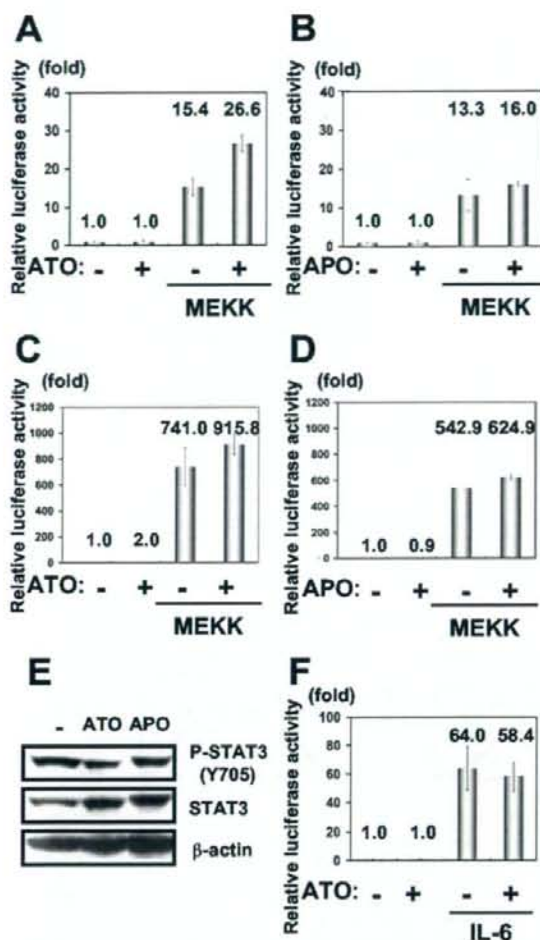

FIG. 5. Effect of ATO on the stress-signaling pathways. (A and B) Effect of ATO or APO on the NF-κB signaling pathway. O cells were transfected with 100 ng of reporter plasmid, pNF-κB-Luc, and/or 100 ng of pFC-MEKK (Stratagene, La Jolla, CA). Cells were treated with either 1 μM ATO (A) or 1 μM APO (B), 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, Ref1, 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-

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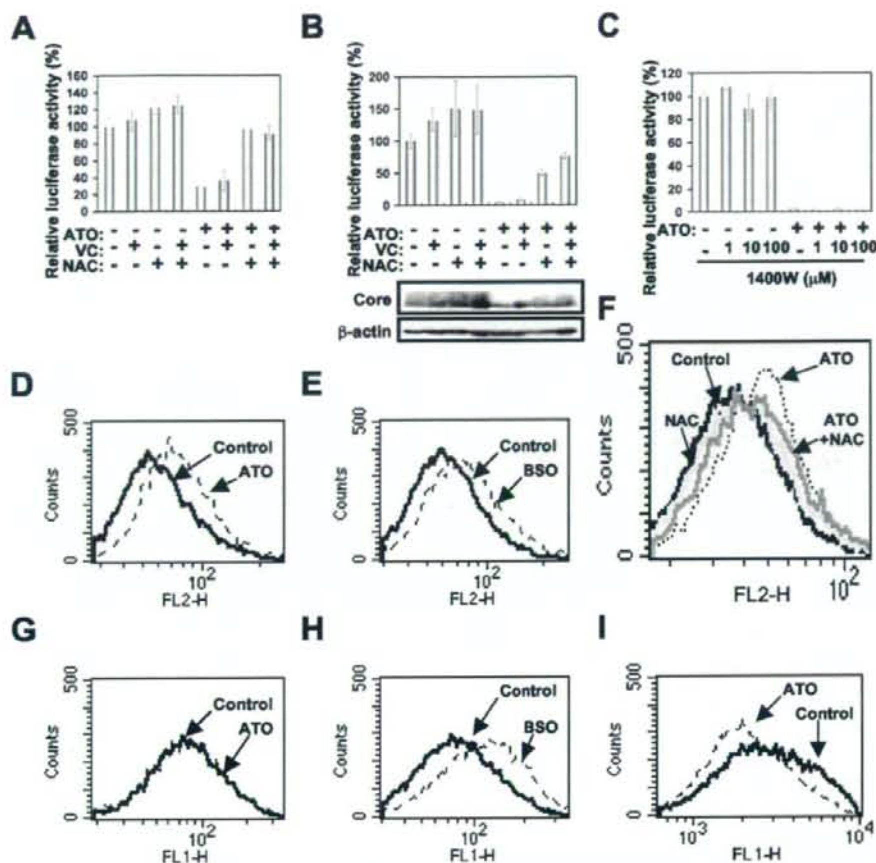

FIG. 6. The anti-HCV activity of ATO is associated with the glutathione redox system and oxidative stress. (A and B) The anti-HCV activity of ATO is eliminated by treatment with the antioxidant NAC. OR6 cells were treated with 1 μM ATO alone and in combination with 100 μM vitamin C (VC), with or without 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

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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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ORIGINAL ARTICLE

Genetic variability and diversity of intracellular genome-length hepatitis C virus RNA in long-term cell culture

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Abstract Hepatitis C virus (HCV) is known to circulate persistently in vivo as a complex population of different but closely related viral variants. To understand the quasispecies nature of HCV, we performed genetic analysis of intracellular HCV RNAs obtained in long-term cell culture of genome-length HCV-RNA-replicating cells. The results revealed that genetic mutations in HCV RNAs accumulated in a time-dependent manner, and that the mutation rates of HCV RNAs were $3.5–4.8 \times 10^{-3}$ base substitutions/site/year. The mutation rates of nonstructural regions that are essential for RNA replication were lower than those of structural regions. The genetic diversity of HCVs was also enlarged in a time-dependent manner. Furthermore, we found that the GC content of HCV RNA was increased in a time-dependent manner. These results suggest that an HCV-RNA-replicating cell culture system would be useful for analysis of the evolutionary dynamics and variations of HCV.

Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. Such persistent infection has now become a serious health problem, with more than 170 million people worldwide currently infected with HCV [23]. HCV is an enveloped, positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae*, and the HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues [9]. 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 protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [5, 6, 8].

The most characteristic feature of the HCV genome is its remarkable diversity and variation. To date, more than six genotypes and multiple subtypes, which show more than 20% difference at the nucleotide level compared with any of the other subtypes, have been identified worldwide [4, 19]. An approximately 5–8% difference at the nucleotide level is observed within a single genotype [8]. Furthermore, an approximately 1% difference at the nucleotide level is also observed among HCV genomes in an individual [20]. Regarding variations of the HCV genome, three reports using specimens from chimpanzees [16, 18] and a human patient [17] have estimated that the mutation rate of the HCV genome was $1.4–1.9 \times 10^{-3}$ base substitutions/site/year. Since the selective pressure of the immune system functions in vivo [10, 24], an experimental system of HCV replication is needed to define the actual mutation frequency of HCV RNA.

We considered that the cell-culture-based HCV replicon system developed in 1999 [15] would be useful as an experimental system for analysis of the genetic variations and diversity of HCV, since it has been shown that HCV subgenomic RNA (so-called replicon RNA) containing the NS3–NS5B regions could autonomously and efficiently replicate in a human hepatoma cell line, HuH-7, using this

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HCV replicon system [3]. The replicon RNA is a selectable, bicistronic HCV RNA with the first cistron, the neomycin phosphotransferase (Neo^R) gene, being translated under control of the HCV internal ribosome entry site (IRES) and the second cistron, the NS3–NS5B regions, being translated under control of the encephalomyocarditis virus (EMCV) IRES. Therefore, we previously performed genetic analyses of HCV variation and diversity using HCV replicon systems [11, 13] developed using two HCV strains, 1B-1 and HCV-O [12]. In that study, HCV-replicon-harboring cells were cultured for 18 months (1B-1 strain) or 12 months (HCV-O strain), and, using these cell cultured specimens, the mutation rates of both HCV replicons were estimated to be approximately 3.0×10^{-3} base substitutions/site/year. The genetic diversity of both replicons was also enlarged during long-term cell cultures [12]. However, it is unclear that the obtained results reflect the variations and diversity of the whole HCV genome, since the HCV replicon lacks the core–NS2 regions (half of the HCV genome). Furthermore, information regarding the genetic variation and diversity of the core–NS2 regions is needed in order to understand the dynamics of the whole HCV genome. To clarify this point, recently established genome-length HCV RNA (HCV-O strain)-replicating cell lines, HuH-7-derived O, OA, OB, OD, and OE [1, 7], were used for this study. There is no evidence that infectious HCV particles are released into the supernatants of genome-length HCV-RNA-replicating cells (O–OE). Since genome-length HCV-RNAs possessing cell-line-specific adaptive mutations that enhance the efficiency of RNA replication efficiently replicated in these five kinds of cells, we cultured these cells for 2 years and comprehensively analyzed the variations and diversity of the whole intracellular HCV genome. Here, we report the evolutionary HCV dynamics occurring in long-term replication of genome-length HCV RNAs.

Materials and methods

Cell cultures

The O, OA, OB, OD, and OE cells supporting genome-length HCV RNAs were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%) and G418 (0.3 mg/ml). These cells were passaged every 7 days for 2 years.

Northern blot analysis

Total RNAs from the cultured cells were prepared using an RNeasy extraction kit (Qiagen, Hilden, Germany). Total RNA (3 μg) was used to detect the genome-length HCV RNA and β-actin mRNA (for check the amount of RNA). Northern blotting and hybridization were performed using a positive-stranded HCV-genome-specific RNA probe (NS5B region) and a β-actin-specific probe, as described previously [12].

Quantification of HCV RNA

The reverse transcription (RT)-quantitative PCR (RT-qPCR) analysis for HCV RNA was performed using LightCycler PCR as described previously [8]. Experiments were done in triplicate.

Western blot analysis

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis with a PVDF membrane were performed as described previously [6]. The antibodies used to examine the expression levels of HCV proteins were those against core [CP9, CP11, and CP14 monoclonal antibodies (Institute of Immunology, Tokyo); a polyclonal antibody (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan)], E1 and NS5B (generous gifts from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). The epitopes of CP9, CP11, and CP14 were located within aa positions 39–74, 21–40, and 5–40 of the core protein, respectively. Anti-β-actin antibody (AC-15; Sigma, St. Louis, MO) was also used to detect β-actin as an internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences, Boston, MA).

RT-PCR and sequencing

To amplify genome-length HCV RNA, RT-PCR was performed separately in two fragments as described previously [7]. Briefly, one fragment covered from 5′-UTR to NS3, with a final product of approximately 5.1 kb, and the other fragment covered from NS2 to NS5B, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis of the HCV open reading frame (ORF) after cloning into pBR322MC [11]. SuperScript II (Invitrogen, Carlsbad, CA) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively. Plasmid inserts were sequenced in both the sense and antisense directions using Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences of each of the three independent clones obtained were determined.

Molecular evolutionary analysis

Nucleotide and deduced amino acid sequences of the clones obtained by RT-PCRs were analyzed by neighbor-joining analysis using the program GENETYX-MAC (Software Development, Tokyo, Japan).

Results

Efficient replication of genome-length HCV RNA is maintained in long-term cell culture

To prepare the specimens for the genetic analysis of HCV, genome-length HCV-RNA-replicating O, OA, OB, OD, and OE cells were cultured for 2 years. The cell-line-specific and conserved adaptive mutations, K1609E, E1202G, P1115L, Q1112R, and P1115L, in the NS3 region were detected in the O, OA, OB, OD, and OE cells, respectively, when these cell lines were established [1, 7]. Using the specimens obtained at 0, 1, and 2 years in culture of O, OA, OB, OD, and OE cells, the levels of genome-length HCV RNAs were examined by Northern blot analysis (Fig. 1a) and RT-qPCR analysis (Fig. 1b). As shown in Fig. 1a, genome-length HCV RNAs approximately 11 kb long were detected in all specimens except that from HuH-7 parental cells, although the strength of the detected bands was weak in some cases. However, RT-qPCR analysis revealed that at least approximately 2×10^7 copies/µg RNA were present in the cultured cells (Fig. 1b). The results of RT-qPCR were well correlated with those of Northern blot analysis. The levels of HCV proteins (core, E1, and NS5B) were also examined by Western blot analysis. The E1 and NS5B proteins were also detected in all specimens except that from HuH-7 cells, although the levels of E1 protein were rather different among the specimens (Fig. 1c). In contrast, core protein was not detected in OB1, OB2, and OE2 cells, when the mixture of three kinds of monoclonal antibodies (CP9, CP11, and CP14) was used for the analysis. Even when polyclonal anti-core antibody was used, core protein was still not detected in OB2 cells. In addition, the strength of bands detected in the Western blot analysis was decreased in a time-dependent manner. These results suggest that sequence variations within the epitopes of the anti-core or E1 antibody, but not the anti-NS5B antibody, have occurred during the long-term cell culture.

Genetic variations of genome-length HCV RNAs during long-term cell culture

The determined nucleotide sequences of genome-length HCV RNAs were compared with those of the original ON/C-5B RNA (Gene Bank accession no. AB191333) [7] used for the establishment of the O, OA, OB, OD, and OE cell lines. The results revealed that the numbers of base substitutions in genome-length HCV RNAs increased in a time-dependent manner (Fig. 2). These substitutions were considered to be mutations that occurred during the intracellular replication of genome-length HCV RNA. Based on the results after 2 years in culture, the apparent mutation rates of genome-length HCV RNAs in O, OA, OB, OD, and OE cells were calculated to be 3.5 ± 0.4, 4.5 ± 1.4, 4.8 ± 0.6, 4.3 ± 0.5, $4.2 \pm 0.4 \times 10^{-3}$ base substitutions/site/year, respectively. These values suggest that the genetic evolution of HCV in these different cell lines occurs at similar rates during long-term RNA replication. The deduced aa substitution rates in HCV ORFs among these cell lines are well correlated with the mutation rates of HCV RNAs (Fig. 2). We further examined whether or not the mutation rates are similar throughout the HCV genome. For this analysis, genome-length HCV RNA was divided into three parts: the 5′-terminus to the EMCV IRES region (1,938 nts), the core to the NS2 region (3,078 nts), and the NS3 to the NS5B region (5,955 nts). The results revealed that the mutation rates in the NS3–NS5B regions were lower than those of the other regions, although the 5′-terminus to the EMCV IRES region in the OA and OE cell lines showed mutation rates similar to that for the NS3–NS5B regions (Fig. 3). These results suggest that the NS3–NS5B regions, which are essential for RNA replication, are evolutionally limited. The conserved aa substitutions (mutated in all three clones sequenced) are summarized in Table 1 (core–p7 regions) and Table 2 (NS2–NS5B regions). Eight aa substitutions (K12N, Q1112R, P1115L, K1609E, A1738T, K2280E, D2292E, and D2415G) were commonly detected in at least two different cell lines. Approximately 57% of aa substitutions detected in this study were found in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp; Nagoya City University, Japan).

Classification of mutations occurring in genome-length HCV RNAs during the long-term cell culture

We examined the numbers of synonymous and non-synonymous mutations with transition or transversion in three divided regions (Neo^R, core–NS2, and NS3–NS5B regions). The results revealed that the frequencies of aa substitutions in the NS3–NS5B regions were lower than those in the core–NS2 regions, and that the rate of transition mutations in genome-length HCV RNA was greater than the rate of transversion mutations (Supplementary Table S1), as previously reported for the replicon system [12].

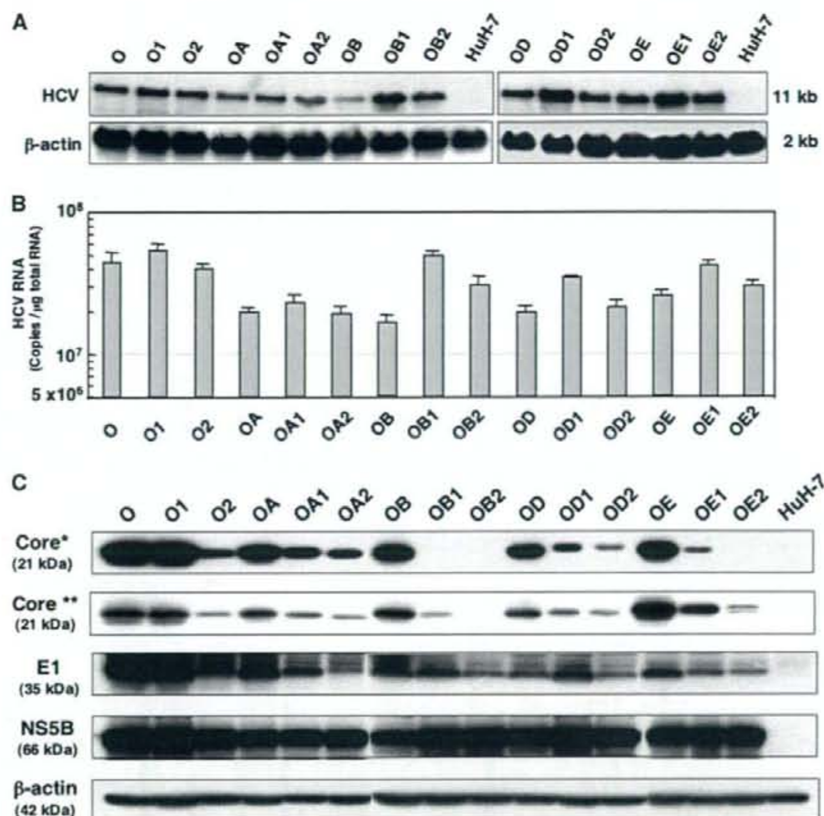

Fig. 1 Characterization of cells containing replicating genome-length HCV RNA in long-term cell culture. **a** Northern blot analysis. Total RNAs from O, OA, OB, OD, and OE cells after 1 year (O1, OA1, OB1, OD1, and OE1) and 2 years (O2, OA2, OB2, OD2, and OE2) in culture, as well as total RNAs from the parental O, OA, OB, OD, and OE cells were used for the analysis. HuH-7 cells were used as a negative control. In vitro-synthesized ON/C-5B RNA [1] was used as a size marker (11 kb). **b** Quantitative analysis of intracellular genome-length HCV RNA. The total RNAs from the cells used for Northern blot analysis were also used for comparison. The levels of intracellular genome-length HCV RNA were quantified by LightCycler PCR. **c** Western blot analysis. The cellular lysates from the cells used for Northern blot analysis were also used for comparison. Core, E1, and NS5B were detected by Western blot analysis. β-actin was used as a control for the amount of protein loaded per lane. A *single star* indicates that the mixture of three kinds (CP9, CP11, and CP14) of anti-core monoclonal antibodies was used for detection. A *double star* indicates that the anti-core polyclonal antibody was used for detection

Also regarding the mutation patterns, U → C and A → G mutations were the most and second-most frequent mutations, and these mutations were two to three times more frequent than C → U and G → A mutations (Supplementary Table S2) as previously reported in the replicon analysis [12]. The rarest mutation was C → G in 1- and 2-year cultures (Supplementary Table S2). As a result, we observed that the GC content of HCV RNA gradually increased in a time-dependent manner. The increase in GC content was observed in all genome-length HCV RNAs obtained from cultured cell lines (Fig. 4).

Genetic diversity of genome-length HCV RNA arising during long-term cell culture

Based on the sequence data of all clones obtained after 2-year culture, we examined the genetic diversities of genome-length HCV RNAs by the construction of phylogenetic trees. The results revealed that the genetic diversities of genome-length HCV RNAs were expanded at both the nucleotide and aa sequence levels, as previously reported in the replicon analysis [12], and that the three clones derived from each cell line were clustered and located at similar genetic distances from the origin

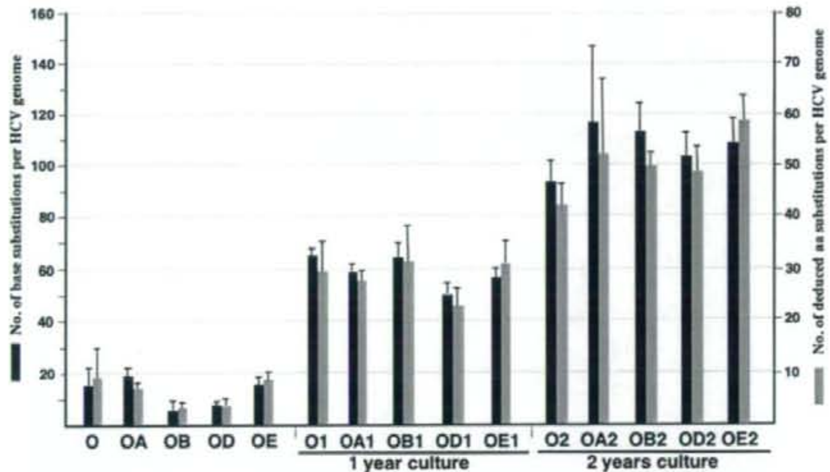

Fig. 2 Genetic variations occurring in long-term replication of genome-length HCV RNAs. The *left vertical line* indicates the mean numbers of base substitutions detected in three clones of genome-length HCV RNA, by comparison with the original sequences (ON/C-5B) [7]. The *right vertical line* indicates the mean numbers of aa substitutions deduced from each of three clones of genome-length HCV RNA, by comparison with the original aa sequences (ON/C-5B) [7]

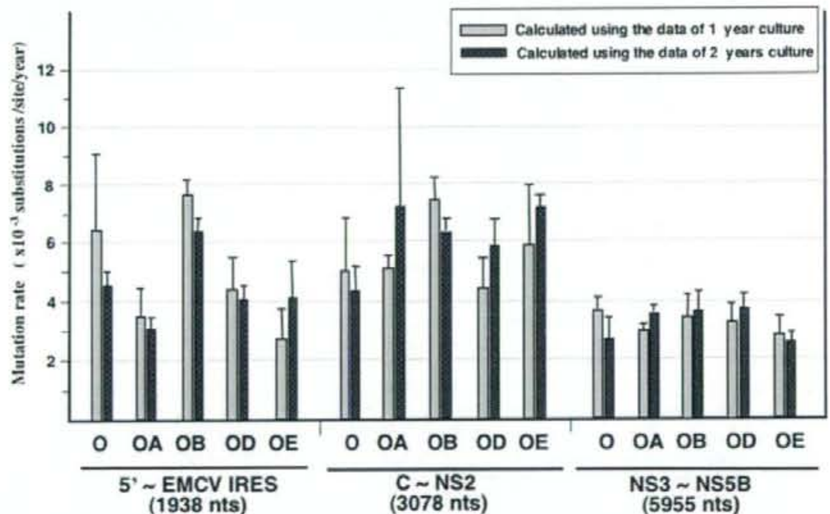

Fig. 3 Mutation rates of genome-length HCV RNAs in long-term cell culture. The mutation rates of three regions (5′–EMCV-IRES, Core–NS2, and NS3–NS5B) of genome-length HCV RNAs (O, OA, OB, OD, and OE) were calculated using the sequence data obtained from 1- or 2-year cell culture. The *vertical line* indicates the means of the mutation rates calculated using the nucleotide sequences of three clones of genome-length HCV RNAs, by comparison with the original sequences (ON/C-5B) [7]

(ON/C-5B) at both the nucleotide and aa sequence level (Supplementary Fig. S1). These results indicate that the quasispecies nature of genome-length HCV RNA has been steadily acquired over long-term intracellular RNA replication.

Discussion

In the present study, we analyzed the genetic evolution and dynamics of HCV in long-term culture of five kinds of genome-length HCV-RNA-replicating cells, and demonstrated that the genetic mutations of HCV accumulated in a time-dependent manner, and the genetic diversity of HCV also increased with time. These results will be useful for understanding the quasispecies nature of HCV in patients with chronic hepatitis C.

Previously, we reported that the genetic mutation rate of HCV replicons (subgenomic RNA) was approximately 3.0×10^{-3} base substitutions/site/year in both the 5′ terminus-EMCV IRES region and the NS3–NS5B regions [12]. The NS3–NS5B regions in this study showed mutation rates ($2.8–3.8 \times 10^{-3}$ base substitutions/site/year) similar to those of the replicons in the previous study; however, the mutation rates of the 5′ terminus to the EMCV IRES region in O and OB cells were over 6.0×10^{-3} base substitutions/site/year, suggesting that genetic mutations in this region occur independently

Table 1 Conserved aa substitutions occurring during long-term replication of genome-length HCV RNAs (I)

Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells
Core	S2G	OB2	Core	N163S	O2	E2	A457T[a]	OB1, OB2
	K6N	OB2		N163T	OE1, OE2		D463H[a]	OE, OE1, OE2
	K10R[a]	OB2		L169S	OD1, OD2		W469R	OE2
	K10E	OA2		F174S	O2		Y485H	OA1, OA2
	K12N[a]	OA1, OA2		F177S	OB1, OB2		Y485C	OD2
		OE2					G504S	OE2
	N16D	OE1, OE2	E1	N205T	O2		Y507H	OA2
	F24V	OB2		D218G[a]	OB2		L537P	OB2
	V34A	OD2		M219V[a]	OB1, OB2		M555V[a]	OB1
	R40G	O2		I220V[a]	OE1, OE2		T595A[a]	OA2
	L44M[a]	OE2		C226R[a]	OE2		K595A[a]	OA2
	T49A[a]	OE2		L242I[a]	OE2		K596N	OD1, OD2
	K67M[a]	O2		T257A[a]	OA1, OA2		L603S	OE2
	P71S[a]	O2		I258K	OE, OE1, OE2		C607S	OA1, OA2
	A75P	OE2		L264S[a]	O2		G649E	OE2
	A77T	OB2		C272R[a]	OE2		D658G[a]	OD1, OD2
	A77P	OD2		S273P[a]	O2		T670A[a]	OB2
	E89V[a]	O2		R296H[a]	OD1		I674T[a]	O2
	R101C	OE2		Q302R[a]	OA1, OA2		L689S	OA1, OA2
	T110M[a]	OE1, OE2		V313A[a]	OB2		N695S	OE2
	L119S	OB2		Y361C[a]	OA2		V713L	OA1, OA2
	K121R	OD1, OD2		Y361H	OE, OE1, OE2		Y718H	OB1, OB2
	I123T[a]	O2		V381D	OA2		Y718C	OD1
	F130L[a]	OA1, OA2					L722P	O2
	L133F	OB2	E2	G389R	OE2			
	L139P	OE1, OE2		F437S	OD1, OD2	p7	F771S	OE2
							L796P	O2

[a] aa Substitutions found in the Hepatitis Virus Databasse (http://s2as02.genes.nig.ac.jp; Nagoya City University, Japan)

among these cell lines. It was also noticed that the mutation rates ($4.3–7.4 \times 10^{-3}$ base substitutions/site/year) in the core–NS2 regions became higher than those in the NS3–NS5B regions due to frequent mutations in the core, E1, and E2 regions (Table 1). It was particularly difficult to detect the core protein by Western blot analysis due to the genetic changes within epitopes for anti-core antibodies (Fig. 1c). These results suggest that the structural region including the core, E1, and E2 regions is not required for persistent intracellular RNA replication, although approximately 42% of the aa substitutions detected in this study were observed in HCV-infected persons (Table 1). However, since we have recently found that DDX3 DEAD-box RNA helicase, which binds to the N-terminal domain (aa 1–59) of the core protein, is required for efficient replication of genome-length HCV RNA in O cells [2], none of the mutations detected in the core region should impair the interaction with DDX3. Furthermore, 6 and 9N-glycosylation sites in the E1 and E2 proteins, respectively, were completely conserved even after 2 years in culture, indicating that the E1 and E2 proteins may also affect the efficiency of RNA replication. Therefore, we speculate that the aa substitutions detected in the structural region do not reflect all of the random mutations occurring in long-term RNA replication. In contrast to the numerous aa substitutions in the structural region, the hypervariable region (HVR) 1 located in the N-terminal region of the E2 protein showed only one aa substitution (G389R in OE2 cells). This finding supports our previous proposition that an immunosurveillance system is involved in the genetic mutation in HVR1 [10]. In addition, no aa substitutions were detected in HVR2 (aa 474–480) of the E2 protein.

We showed that the mutation rates of HCV RNAs were $3.5–4.8 \times 10^{-3}$ base substitutions/site/year. However, our observed mutation rates of the HCV RNAs were 1.8–3.4 times higher than those previously obtained in chimpanzees [16, 18] and a human patient [17] with chronic hepatitis C. Since the selective pressures of the humoral immune responses [10] targeting the envelope proteins and cellular immune responses [24] targeting all HCV proteins

Table 2 Conserved aa substitutions occurring during long-term replication of genome-length HCV RNAs (II)

Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells	Region	aa Substitution	Observed cells
NS2	M814T[a]	OE1, OE2	NS4B	L1724I[a]	OB2	NS5A	D2377G[a]	OA2
	I885V[a]	O2		A1738T[a]	OA2		V2385H[a]	OD2
	F886L	OB2			OD1, OD2		S2387P	OD1, OD2
	E887G[a]	O2			OE2		L2391P	OA2
	T889A	OB2		I1797V[a]	OB2		W2405R[a]	OE2
	I891V[a]	O2		P1822S	OE2		E2414G[a]	OB2
	L902F[a]	OB2		V1880A	OA1, OA2		D2415G[a]	OA, OA1, OA2
	M939V[a]	OE, OE1, OE2						OD1, OD2
			NS5A	S1975T[a]	OE2		C2418R[a]	OA2
NS3	Q1112R[a, b]	O1, O2		H2218R[a]	OE2			
		OB2		H2219R[a]	OB2	NS5B	N2529S[a]	OB2
		OD, OD1, OD2		S2221F[a]	OA2		N2536S[a]	OB2
	P1115L[a, b]	OB, OB1, OB2		N2248D[a]	OB2		V2757A[a]	OB2
		OE, OE2		K2280E[a]	OB2		K2860R[a]	OB2
	N1148S[a]	OE2			OD1, OD2		R2963Q[a]	OB2
	E1202G[a, b]	OA, OA1, OA2		A2284T	OB2		W2990R	O2
	T1531A[a]	OA2		D2292E[a]	OB2		V3002A	O2
	D1581E[a]	OA2			OE2			
	K1609E[a, b]	O, O1, O2		V2340M[a]	OA2			
		OE2		S2342P[a]	OD2			
	I1612T[a]	OD1, OD2		G2371A[a]	OE2			
	I1641M[a]	OD1, OD2		G2376S[a]	OD1, OD2			

[a] aa Substitutions found in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp; Nagoya City University, Japan)

[b] Adaptive mutations detected in O, OA, OB, OD, and OE cells when these cell lines were established [1, 7]

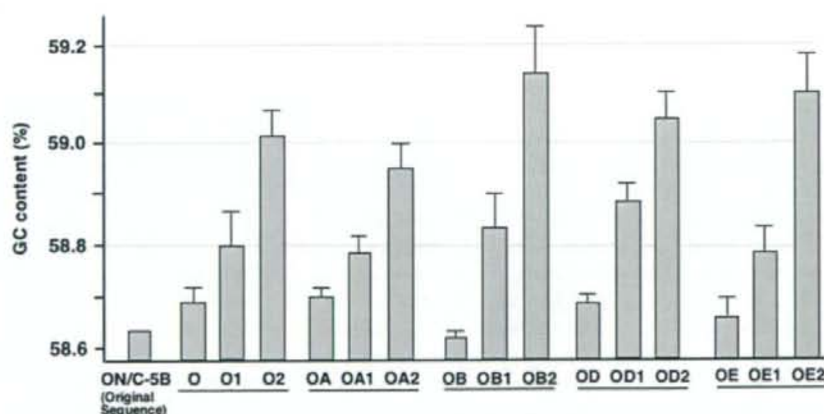

Fig. 4 Increased GC content of genome-length HCV RNAs in long-term cell culture. The GC content of genome-length HCV RNAs (O–O2, OA–OA2, OB–OB2, OD–OD2, and OE–OE2) was calculated. The values indicate the means of three clones of each genome-length HCV RNA

function in vivo, the mutation rates obtained in this study using the cell culture system without the immunological pressure would be reasonable values as a potential mutation rate of HCV in RNA replication.

It is noteworthy that none of the aa substitutions were detected in the N-terminal half (242 aa of aa 1,976–2,217) of the NS5A protein after 2 years in cell cultures. This finding suggests that this region is the most critical for maintenance of RNA replication. It is interesting that this region corresponds to domain I (aa 1,973–2,185), which has been shown to complex with a zinc ion [21] and exists as a dimer [22]. Since the mutation of four cysteine residues essential for binding to zinc ions results in the complete inhibition of RNA replication [21], the complete

conservation of domain I in this study suggests that the intact form of domain I is required for efficient RNA replication. Genetic analysis in further long-term cell cultures will specifically clarify the critical domains required for the maintenance of RNA replication.

The unexpected phenomenon in this study was the time-dependent increase of the GC content of the HCV genome. After 1 year in culture, the GC content increased 0.14% (mean of five cell culture lines), corresponding to 15 nts per HCV genome, and during the next 1 year in culture, the GC content increased an additional 0.24% (mean of five cell culture lines), corresponding to 26 nts per HCV genome. Consequently, approximately 40 nts per HCV genome changed to a G or C residue during the 2 years in culture. The HCV genome may gradually change to an energetically stable form during RNA replication. The other possibility is that the increase in GC content may be due to an increase in G- and C-ending codons, except AGG and TTG codons, for efficient expression in human cells (codon optimization) [14]. However, our study revealed that the increase of G- and C-ending codons other than codons AGG and TTG was only 16–18% of the increase of GC content observed during the 2-year cultures of O–OE cells. To understand the mechanism underlying the increase of the GC content of genome-length HCV RNA during long-term RNA replication, further long-term cell cultures will be needed.

This study demonstrated that a single HCV genome could exhibit a quasispecies nature after 2 years in cell culture with RNA replication. Such quasispecies populations of HCV obtained by long-term cell culture may be useful not only for further analysis of the genetic variations and diversity of HCV but also for analysis of the sensitivity of reagents such as interferon against HCV.

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The DNA Damage Sensors Ataxia-Telangiectasia Mutated Kinase and Checkpoint Kinase 2 Are Required for Hepatitis C Virus RNA Replication▿

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Cellular responses to DNA damage are crucial for maintaining genome integrity, virus infection, and preventing the development of cancer. Hepatitis C virus (HCV) infection and the expression of the HCV nonstructural protein NS3 and core protein have been proposed as factors involved in the induction of double-stranded DNA breaks and enhancement of the mutation frequency of cellular genes. Since DNA damage sensors, such as the ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), poly(ADP-ribose) polymerase 1 (PARP-1), and checkpoint kinase 2 (Chk2), play central roles in the response to genotoxic stress, we hypothesized that these sensors might affect HCV replication. To test this hypothesis, we examined the level of HCV RNA in HuH-7-derived cells stably expressing short hairpin RNA targeted to ATM, ATR, PARP-1, or Chk2. Consequently, we found that replication of both genome-length HCV RNA (HCV-O, genotype 1b) and the subgenomic replicon RNA were notably suppressed in ATM- or Chk2-knockdown cells. In addition, the RNA replication of HCV-JFH1 (genotype 2a) and the release of core protein into the culture supernatants were suppressed in these knockdown cells after inoculation of the cell culture-generated HCV. Consistent with these observations, ATM kinase inhibitor could suppress the HCV RNA replication. Furthermore, we observed that HCV NS3-NS4A interacted with ATM and that HCV NS5B interacted with both ATM and Chk2. Taken together, these results suggest that the ATM signaling pathway is critical for HCV RNA replication and may represent a novel target for the clinical treatment of patients with chronic hepatitis C.

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV infection has now become a serious health problem, with at least 170 million people currently infected worldwide (28). HCV is an enveloped virus with a positive single-stranded 9.6-kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (12, 13, 27).

Studies have shown that various viruses with distinct replication strategies—including the DNA viruses Epstein-Barr virus, herpes simplex virus 1, adenovirus, and simian virus 40 and the retrovirus human immunodeficiency virus type 1 (HIV-1)—can activate DNA damage response pathways and utilize these damage responses to facilitate their own viral reproduction and promote the survival of infected cells (2, 16, 17). In the case of HCV, it has been proposed that HCV infection causes double-stranded DNA (dsDNA) breaks and enhances the mutation frequency of cellular genes and that these effects are mediated by nitric oxide (18, 19). In addition, the HCV core, E1, and NS3 proteins have been suggested to be potent reactive oxygen species inducers, leading to DNA damage (19). Furthermore, we previously demonstrated that HCV NS5B-expressing PH5CH8 immortalized human hepatocyte cells were susceptible to DNA damage in the form of dsDNA breaks (23). Thus, HCV seems to be associated with the dsDNA damage response pathways.

Since the DNA damage sensors, such as ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), poly(ADP-ribose) polymerase 1 (PARP-1), and checkpoint kinase 2 (Chk2; a direct downstream target of ATM), play central roles in response to genotoxic stress (10), we hypothesized that these sensors might affect HCV replication.

To investigate the possible involvement of these cellular factors in HCV replication, we examined the level of HCV RNA in cells rendered defective for DNA damage sensors by RNA interference or by pharmacological inhibition.

MATERIALS AND METHODS

Cell culture. 293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). The HuH-7-derived O cells harboring a replicative genome-length HCV RNA and the HuH-7-derived sO cells harboring the subgenomic replicon RNA of HCV-O were cultured in DMEM with 10% FBS and G418 (300 μg/ml geneticin; Invitrogen) as described previously (11, 14). Oc and sOc cells, which were created by eliminating HCV RNA from O cells and sO cells by interferon (IFN) treatment (11, 14), respectively, were also cultured in DMEM with 10% FBS.

RNA interference. Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding se-

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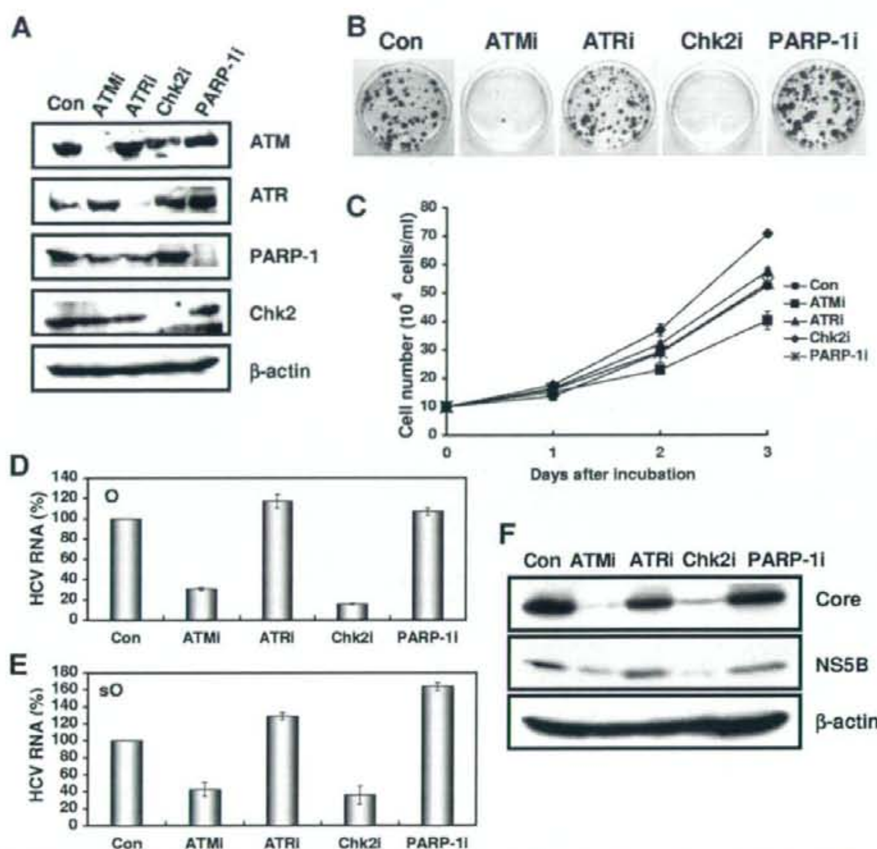

FIG. 1. The ATM signaling pathway is required for HCV RNA replication. (A) Inhibition of ATM, ATR, Chk2, or PARP-1 expression by shRNA-producing lentiviral vectors. The results of the Western blot analysis of cellular lysates with anti-ATM, anti-ATR, anti-Chk2, anti-PARP-1, or anti-β-actin antibody in Oc cells expressing shRNA targeted to ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in Oc cells transduced with a control lentiviral vector (Con) are shown. (B) ECF in ATM-, ATR-, Chk2-, or PARP-1-knockdown cells. In vitro transcribed ON/C-5B K1609E RNA (2 μg) was transfected into the ATM-, ATR-, Chk2, or PARP-1-knockdown Oc cells or the Oc cells transduced with a control lentiviral vector (Con). G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA. Experiments were done in duplicate, and a representative result is shown. (C) The cell growth curve of ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i)-knockdown Oc cells or the Oc cells transduced with a control lentiviral vector (Con). Results from three independent experiments are shown. (D) The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR (Roche). Experiments were done in triplicate, and columns represent the mean percentage of HCV RNA. (E) The level of subgenomic replicon (sO cells) RNA was monitored by real-time LightCycler PCR. Results from three independent experiments are shown as described in panel D. (F) The HCV core or NS5B protein expression level in ATM-, ATR-, Chk2-, or PARP-1-knockdown cells. The results of Western blot analysis of cellular lysates with anti-HCV core protein, anti-HCV NS5B, or anti-β-actin antibody in O cells expressing shRNA targeted to ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in O cells transduced with a control lentiviral vector (Con) are shown.

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quences targeted to Chk2 in lentiviral vector: 5'-GATCCCCGGGGGAGAGCTGTTTGACATTCAAGAGATGTCAAACAGCTCTCCCCCTTTTTGGAAA-3' (sense) and 5'-AGCTTTTCCAAAAAGGGGGAGAGCTGTTTGACATCTCTTGAATGTCAAACAGCTCTCCCCCGGG-3' (antisense). The oligonucleotides above were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (5), generating pSUPER-Chk2i. To construct pLV-Chk2i, the BamHI-SalI fragments of the pSUPER-Chk2i 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 (4). pLV-ATMi, pLV-ATRi, and pLV-PARP-1i were constructed as described previously (1).

Lentiviral vector production. The vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector system has been described previously (24). The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 (30) and the VSV-G envelope plasmid pMDG2 as well as the lentiviral vector into 293FT cells with FuGene6 (Roche Diagnostics, Mannheim, Germany).

Quantitative reverse transcription-PCR analysis. Quantitative reverse transcription-PCR analysis for HCV RNA was performed by real-time LightCycler PCR as described previously (11).

Western blot analysis. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting analysis using anti-ATM (2C1; GTX70103 [GeneTex, San Antonio, TX]), anti-ATR (GTX70133; GeneTex), anti-Chk2 (NT; ProSci, Poway, CA), anti-Chk2 (DCS-273; Medical and Biological Laboratories, Nagoya, Japan), anti-phospho-Chk2 (Thr68) (Cell Signaling,

Danvers, MA), anti-PARP-1 (C-2-10; Calbiochem, Merck Biosciences, Darmstadt, Germany), anti-hemagglutinin (HA) (HA-7; Sigma, St. Louis, MO), anti-core protein (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan), anti-NS3 and anti-NS5B (no. 14; a generous gift from M. Kohara, the Tokyo Metropolitan Institute of Medical Science, Japan), anti-NS5A (no. 8926; a generous gift from A. Takamizawa, The Research Foundation for Microbial Diseases of Osaka University, Japan), and anti-β-actin (Sigma) Antibodies.

Immunofluorescence and confocal microscopic analysis. Cells were fixed in 3.5% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in 0.1% NP-40 in PBS at room temperature. Cells were incubated with anti-ATM antibody (5C2; GTX70107 [GeneTex] or PM026 [MBL]), anti-HA antibody (3F10), anti-NS5B antibody and/or anti-NS3 antibody at a 1:300 dilution in PBS containing 3% bovine serum albumin at 37°C for 30 min. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) or anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch) at a 1:300 dilution in PBS containing bovine serum albumin at 37°C for 30 min. Following extensive washing in PBS, cells were mounted on slides using a mounting medium of 90% glycerin–10% PBS with 0.01% *p*-phenylenediamine added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (LSM510; Zeiss, Jena, Germany).

Immunoprecipitation. Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.5% NP-40, 10 mM NaF, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared with 30 μl of protein G-Sepharose (GE Healthcare Biosciences, Uppsala, Sweden). Precleared supernatants were incubated with 5 μg of anti-HA antibody (3F10; Roche), 10 μl of anti-NS5B antibody, 5 μg of anti-Chk2 antibody (DCS-273; MBL), 5 μg of anti-FLAG antibody (M2; Sigma), or 5 μg of anti-ATM antibody (2C1) (GTX70103; GeneTex) at 4°C for 1 h. Following absorption of the precipitates on 30 μl of protein G-Sepharose resin for 1 h, the resin was washed four times with 700 μl of lysis buffer. Proteins were eluted by boiling the resin for 5 min in 2× Laemmli sample buffer. The proteins were then subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting analysis using anti-ATM, anti-Chk2, anti-HCV core protein (CP-9 and CP-11 mixture), anti-NS5A, anti-NS5B, anti-HA (HA-7; Sigma), or anti-NS3 antibody.

RESULTS

ATM and Chk2 are required for HCV RNA replication. To determine the potential role of DNA damage sensors in HCV replication, we first used lentiviral vector-mediated RNA interference to stably knockdown ATM, ATR, PARP-1 (1), or Chk2 in the following human hepatoma HuH-7-derived cell lines: O cells harboring a replicative genome-length HCV RNA (HCV-O, genotype 1b) (11), Oc cells derived from O cells (created by eliminating genome-length HCV RNA from O cells by IFN treatment) (11), sO cells harboring the subgenomic replicon of HCV-O (14), or RSc cells that cell culture-generated HCV (HCVcc) (JFH1, genotype 2a) (29) could infect and effectively replicate (3). To express shRNAs targeted to ATM, ATR, PARP-1 (1), or Chk2, we used a VSV-G-pseudotyped HIV-1-based vector system (24). We used puromycin-resistant pooled cells 10 days after the lentiviral transduction in all experiments. Western blot analysis of the lysates demonstrated very effective knockdown of ATM, ATR, Chk2, and PARP-1 in Oc cells (Fig. 1A). The effective knockdown of ATM, ATR, Chk2, or PARP-1 in O cells or sO cells was also confirmed by Western blot analysis (data not shown). In this context, the efficiency of colony formation (ECF) in ATM- or Chk2-, but not ATR- or PARP-1-, knockdown Oc cells transfected with the genome-length HCV-O RNA with an adapted mutation at amino acid position 1609 in the NS3 helicase region (ON/C-5B K1609E RNA) (11) was notably reduced compared with the control cells (Fig. 1B) even though Chk2-knockdown cells had a slightly faster growth rate than the control cells (Fig. 1C), suggesting that both ATM and Chk2 are crucial for HCV RNA replication. To further confirm this

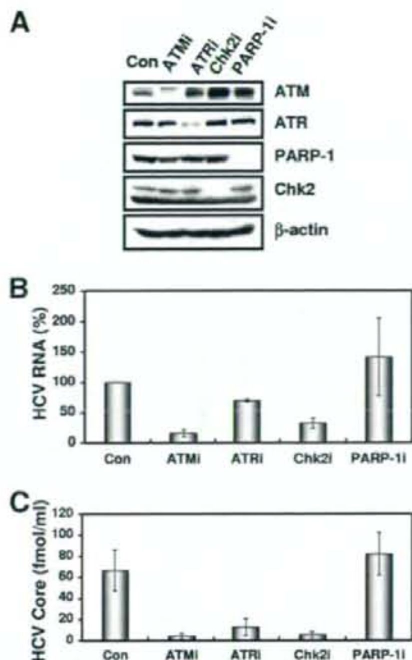

FIG. 2. ATM affects HCV infection. (A) Inhibition of ATM, ATR, Chk2, or PARP-1 expression by shRNA-producing lentiviral vectors. The results of Western blot analysis of cellular lysates with anti-ATM, anti-ATR, anti-PARP-1, anti-Chk2, or anti-β-actin antibody in RSc cured cells expressing shRNA targeted to ATM (ATMi), ATR (ATRi), Chk2 (Chk2i), or PARP-1 (PARP-1i) as well as in RSc cells transduced with a control lentiviral vector (Con) are shown. (B) The level of genome-length HCV (JFH1) RNA was monitored by real-time LightCycler PCR after inoculation of the HCVcc. Results from three independent experiments are shown as described in the legend of Fig. 1D. (C) The levels of the core protein in the culture supernatants were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories). Experiments were done in triplicate, and columns represent the mean core protein levels.

observation, we quantitatively examined the level of HCV RNA in the O cell- or sO cell-derived knockdown cells. Consequently, we found that replication of both genome-length HCV RNA (HCV-O) and its subgenomic replicon RNA (sO) were notably suppressed in ATM- or Chk2-knockdown cells but not in ATR- or PARP-1-knockdown cells (Fig. 1D and E). Consistent with this finding, the expression levels of core and NS5B proteins were also significantly decreased in the cell lysates of ATM- or Chk2-knockdown O cells (Fig. 1F). We next examined the replication level of HCV-JFH1 in ATM-, ATR-, Chk2-, or PARP-1-knockdown RSc cells (Fig. 2A). The results revealed that RNA replication of HCV-JFH1 and release of core protein into the culture supernatants were suppressed in only ATM- or Chk2-knockdown RSc cells after inoculation with HCVcc (Fig. 2B and C). Interestingly, the release of core protein into the culture supernatant was also significantly suppressed in ATR-knockdown RSc cells, while HCV RNA replication was slightly suppressed in these cells

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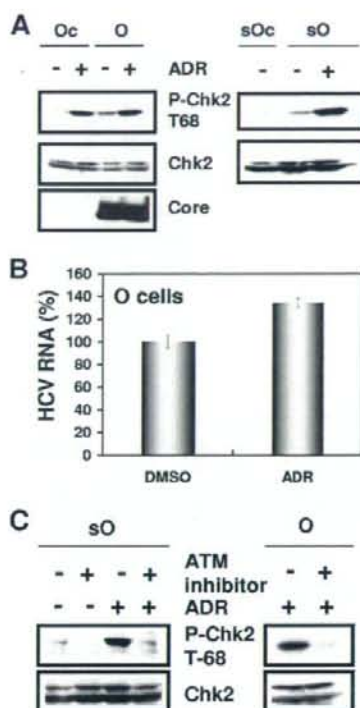

FIG. 3. ATM-dependent DNA damage response in HCV RNA-replicating cells. (A) Stimulation of Chk2 phosphorylation in the HCV RNA-replicating cells. The Oc, O, or sO cells were treated with 100 nM adriamycin (Sigma) for 2 h. The results of Western blot analysis of cellular lysates with anti-phospho-Chk2 (Thr68) (P-Chk2 T68), anti-Chk2, or anti-core protein antibody are shown. (B) Effect of adriamycin on HCV RNA replication. The O cells were treated with 100 nM adriamycin for 24 h. The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR. Results from three independent experiments are shown as described in the legend of Fig. 1D. DMSO, dimethyl sulfoxide. (C) Effect of ATM kinase inhibitor on Chk2 phosphorylation. The sO or O cells were pretreated with 10 μM ATM kinase inhibitor (KU-55933) (Calbiochem) for 2 h, followed by treatment with 100 nM adriamycin for 2 h. The results of Western blot analysis of cellular lysates with anti-phospho-Chk2 (Thr68) or anti-Chk2 antibody are shown.

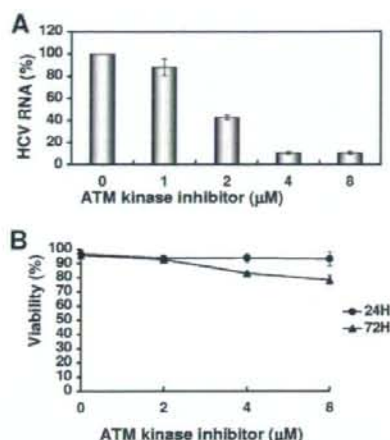

FIG. 4. Suppression of HCV RNA replication by ATM kinase inhibitor. (A) The level of genome-length HCV-O RNA was monitored by real-time LightCycler PCR after treatment with the indicated concentration of ATM kinase inhibitor for 72 h. Results from three independent experiments are shown as described in the legend of Fig. 1D. (B) Cell viabilities after treatment with the indicated concentration of ATM kinase inhibitor for 24 h or 72 h are shown.

(Fig. 2B and C), suggesting that ATR participates in the production of HCV virion.

In contrast, highly efficient knockdown of PARP-1 had no observable effects on the ECF (Fig. 1B), HCV RNA replication (Fig. 1D and E and 2B), or core protein expression in the cell lysate or in the supernatant (Fig. 1F and 2C), suggesting that our finding was not due to a nonspecific event. Thus, we have demonstrated for the first time that DNA damage sensors, ATM and Chk2, are required for HCV RNA replication.

ATM kinase activity in HCV RNA-replicating cells. Although it has been proposed that HCV causes dsDNA breaks (18, 19), little is known about whether HCV activates or inhibits the ATM-dependent damage response pathway. In this regard, it is worth noting that we observed weak but significant Chk2 phosphorylation at threonine 68, the specific marker for ATM activation (20, 21), in the HCV RNA-replicating cells (O and sO cells) but not in the HCV-negative Oc and sOc cells (created by eliminating replicon RNA from sO cells by IFN treatment) (Fig. 3A), suggesting that the persistent HCV RNA replication stimulated the ATM-dependent DNA damage response. Furthermore, a 2-h treatment with 100 nM adriamycin, a dsDNA break inducer, markedly induced Chk2 phosphorylation in Oc, O, and sO cells (Fig. 3A). Importantly, Chk2 phosphorylation was not inhibited even in the HCV RNA-replicating cells (O and sO cells) (Fig. 3A), suggesting that the persistent HCV RNA replication and the HCV proteins are not able to suppress the ATM-dependent DNA damage response. To examine whether such a DNA damage response activates HCV RNA replication, we quantified the level of HCV RNA in the O cells treated with 100 nM adriamycin for 24 h. The results show that HCV RNA replication was increased (approximately 1.3-fold) after treatment with adriamycin (Fig. 3B), suggesting that the DNA damage response activates HCV RNA replication.

Suppression of HCV RNA replication by a small-molecule inhibitor of the ATM kinase. We next examined the effect of a specific small-molecule inhibitor of the ATM kinase (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one [KU-55933]) (16) on HCV RNA replication. As expected, the ATM kinase inhibitor effectively inhibited Chk2 phosphorylation after adriamycin treatment in both sO and O cells (Fig. 3C). In this context, the ATM kinase inhibitor could efficiently suppress genome-length HCV RNA replication with an in vitro 50% effective concentration (EC_{50}) of approximately 2 μM at 72 h after treatment with adriamycin (Fig. 4A). Although this ATM kinase inhibitor did not affect cell viability at 24 h after the treatment, there was a slight decrease in the cell viability at 72 h after treatment (Fig. 4B). Thus, this or other ATM kinase inhibitors may be

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