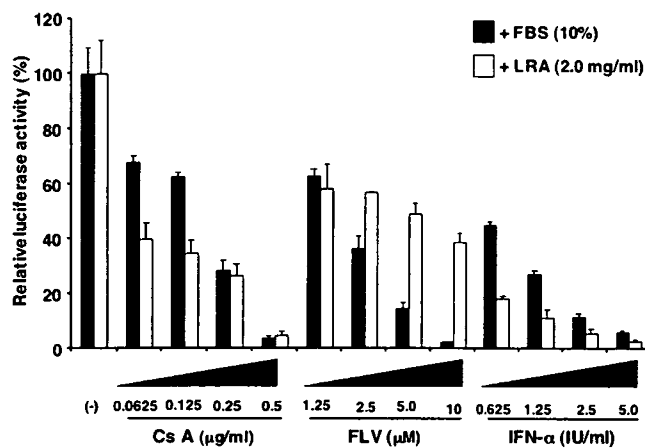

Fig. 5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells in serum-free medium supplemented with LRA or in 10% FBS medium. The OR6 cells were cultured in triplicate in 10% FBS medium with selenium (100 nM) or in serum-free medium with LRA (2 mg per ml) and selenium (100 nM). Under these cell culture conditions, the cells were treated with CsA (0.0625, 0.125, 0.25, and 0.5 μg per ml), FLV (1.25, 2.5, 5.0, and 10 μM), or IFN-α (0.625, 1.25, 2.5, and 5 IU per ml) for 72 h. Then, the cells were harvested and subjected to luciferase assay as described in Section 2. The luciferase activity of OR6 cells cultured under both medium conditions in the absence of anti-HCV compounds was assigned as 100% and is indicated by (−).

novel serum-free cell culture system supplemented with LRA that was able to support HCV RNA replication for more than 9 months. Although this cell culture system still contained animal proteins, the quantity of unknown cellular factors contained in the FBS was to a great extent reduced. The development of such a long-term cell culture is noteworthy, as it could be used for the stable mass-production of an HCV vaccine.

In a related previous study, it was reported that insulin, linoleic acid, and oleic acid enhance the growth of HuH-7 cells in serum-free cell culture (Nakabayashi et al., 1984). Therefore, we tested these supplements in HCV RNA replication experiments. Insulin was found to slightly enhance HCV RNA replication, but linoleic acid and oleic acid inhibited HCV RNA replication. In another recent study, Kapadia and co-workers reported that oleic acid enhanced HCV RNA replication in a serum-containing cell culture (Kapadia and Chisari, 2005). This discrepancy may have been due to the presence or absence of serum; moreover, some of the serum proteins may function in concert with oleic acid to support HCV RNA replication.

LDL is an exogenous source of cholesterol, and it was found to support HCV RNA replication in serum-free cell culture. When the cholesterol demand is satisfied, intrinsic mevalonate, which is a precursor of both cholesterol and non-sterol isoprenoid, is directed to non-sterol isoprenoid. Non-sterol isoprenoid is essential for the prenylation of cellular proteins that support HCV RNA replication (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). For this reason, LDL may support HCV RNA replication, even in serum-free cell culture. LRA was initially developed to reduce or replace the requirement of serum supplementation by chromatographic separation from bovine plasma (Invitrogen). Here, we found that LRA supported HCV RNA replication as well as cell growth in serum-free cell culture. LRA contains free fatty acids and cholesterol associated with albumin. Therefore, cholesterol may, at least to some extent, play a role in HCV RNA replication by the mechanism described above. However, care should be taken before coming to a conclusion regarding the effects of free fatty acids on HCV RNA replication, because LRA contains a mixture of fatty acids in different states of saturation. Recent reports have demonstrated that saturated fatty acids enhance HCV RNA replication, but polyunsaturated fatty acids inhibit HCV RNA replication (Kapadia and Chisari, 2005). In addition, these diverse effects of fatty acids on HCV RNA replication in serum-containing medium cannot be simply applied to serum-free culture systems, because oleic acid has been shown to exert different effects on HCV RNA replication under serum-containing and serum-free culture conditions. To clarify the roles played by fatty acids in this context, further studies will be needed.

Here, HCV RNA replication depended on the growth of HuH-7 cells, and it has previously been shown that expression levels of HCV proteins and RNA are low in confluent cells (Guo et al., 2001; Pietschmann et al., 2001). Therefore, we examined the time course of cell growth and found that cell growth in serum-free medium with LRA was slower than that in 10% FBS medium, although the replication levels of HCV RNA were similar under both culture conditions studied. As regards HCV RNA replication and cell growth, Windisch et al. reported that HCV RNA replication in HuH-6 cells was not dependent on cell growth (Windisch et al., 2005). They demonstrated that the expression of HCV proteins was not reduced, even when the HuH-6 cells became confluent. In serum-free culture supplemented with LRA, HCV RNA replication in HuH-7 cells proceeds in a manner independent of cell growth, as was previously observed in the case of replication in HuH-6 cells.

One disadvantage associated with the use of FBS-containing cultures in virology studies is the influence exerted by unknown serum proteins, because FBS is derived from the serum pool of a bovine population. To prevent discrepancies between experiments due to differences between FBS lots, it is desirable to include only the most simple components as possible in the culture media. To this end, serum-free cultures are preferable in terms of reproducibility. Along these lines, it is expected that the use of our serum-free culture system with LRA may lead to improvements in experimental conditions for experiments in cell biology, as our culture medium contained only very simple supplements: fatty acids, cholesterol, albumin, and selenium. We tested the anti-HCV reagents CsA, FLV, and IFN-α in our serum-free culture supplemented with LRA. CsA and IFN-α were found to inhibit HCV RNA replication more efficiently in serum-free medium with LRA than in 10% FBS medium. Surprisingly, FLV inhibited HCV RNA replication less effectively in serum-free medium supplemented with LRA than it did in 10% FBS medium. One explanation for these differences may be that only FLV is a lipid metabolism-related reagent, and therefore the anti-HCV effect appeared to be antagonized by LRA. To clarify this issue, further study will be needed.

The goal of a serum-free cell culture is to develop a cell culture system containing only compounds that are of non-animal origin. Recently, a serum-free cell culture for canine

pathogenic virus production was reported using Madin Darby canine kidney cells lacking animal protein (Mochizuki, 2006). In this system, soybean peptone was used for the serum-free culture without animal protein. Canine viruses were able to grow almost as efficiently in this serum-free medium as in serum-containing medium. This plant protein-containing culture system is of the second-highest quality in terms of controlling for animal-derived pathogens in vaccine development experiments. Assessments of this animal protein-free cell culture system in terms of its usefulness for HCV RNA replication are therefore warranted.

We found HuH-7 cells supported HCV RNA replication for more than 9 months in serum-free medium supplemented with LRA at 1.0 mg per ml and selenium at 100 nM. This is the first report to describe HCV RNA replication in a long-term, serum-free culture. Recently, an infectious virus-producing cell culture system was reported using genotype 2a strain JFH1 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). We are currently examining infectious virus production in a serum-free cell culture using the JFH1 virus. Our serum-free cell culture system may provide the useful information to the vaccine development.

In conclusion, we have established a serum-free cell culture system supplemented with LRA for the purpose of achieving HCV RNA replication. HCV proteins were detected during this series of experiments for more than 9 months. The present system has enabled an ongoing study of the production of an infectious HCV virion. Our serum-free cell culture system will yield relevant information for vaccine development, sustains only a relatively low risk of pathogenic contamination as compared to that of previous systems, and is expected to improve the reproducibility of similar experiments in the future.

Acknowledgements

The authors would like to thank Atsumi Morishita and Takashi Nakamura for their technical assistance. Kazuhito Naka (Kanazawa University) is to be thanked for valuable discussions of this study. This work was supported by a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control and by a Grant-in-Aid for research on hepatitis, both from the Ministry of Health, Labor, and Welfare of Japan. KA was supported by a Research Fellowship from the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

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Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA

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Received 11 September 2006; received in revised form 12 December 2006; accepted 14 December 2006
Available online 18 January 2007

Abstract

We recently established a genome-length HCV RNA-replicating cell line (O strain of genotype 1b; here called O cells) using cured cells derived from sO cells, in which HCV subgenomic replicon RNA with an adaptive NS5A mutation (S2200R) is replicated. Characterization of the O cells revealed a second adaptive NS3 mutation (K1609E) required for genome-length HCV RNA replication. To clarify the role of adaptive mutation in genome-length HCV RNA replication, we newly established one and three kinds of genome-length HCV RNA-replicating cell lines possessing the cell background of sO and O cells, respectively, and found additional adaptive NS3 mutations (Q1112R, P1115L, and E1202G) required for the robust replication of genome-length HCV RNA. We further found that specific combinations of adaptive NS3 mutations drastically enhanced HCV RNA replication, regardless of the cell lines examined. These findings suggest that specific viral factors may affect the replication level of genome-length HCV RNA.
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Keywords: Hepatitis C virus; Adaptive mutation; Genome-length HCV RNA replication; HCV RNA-replicating cell line

1. Introduction

Infection with the hepatitis C virus (HCV), of the family *Flaviviridae*, frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. Since more than 170 million individuals are estimated to be infected with HCV worldwide, this disease is a global health problem (Thomas, 2000). HCV is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1995). This polyprotein is processed by a combination of the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993). These HCV proteins function not only in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (Bartenschlager and Lohmann, 2000; Kato, 2001).

Although studies on the mechanism of HCV replication were for many years difficult due to the lack of efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000), such studies proliferated after the development of subgenomic HCV replicon (Con-1 of genotype 1b) that was capable of replication in human hepatoma (HuH-7) cells (Lohmann et al., 1999). The subgenomic replicon RNA is composed of the HCV 5′-untranslated region (UTR) fused to the first 12 aa of the core coding region, the neomycin phosphotransferase (Neo^R) gene as a selectable marker, and the HCV NS3-NS5B regions under the control of an encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES), followed by 3′-UTR. After the first replicon, several additional replicons derived from H77 (1a), N (1b), 1B-1 (1b), O (1b), and JFH-1 (2a) strains were developed, and tissue, genotype, and host ranges were also expanded (Ali et al., 2004; Blight et al., 2000; Date et al., 2004; Ikeda et al., 2002; Kato and Sugiyama et al., 2003; Kato and Date et al., 2003; Kishine et al., 2002; Zhu et al., 2003). Since intracellular replicon RNAs were easily detected by Northern blot analysis and the HCV proteins produced were detected by

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doi:10.1016/j.virusres.2006.12.011

Western blot analysis, these cell culture replication systems became valuable tools for basic studies of HCV, such as studies for viral replication and drug development (Bartenschlager, 2002, 2005; Lindenbach and Rice, 2005). However, in attempts to examine what happens in HCV-infected hepatocytes, subgenomic HCV replicons were insufficient because they lacked the effects of HCV structural proteins. For this reason, five kinds of genome-length HCV RNA-replicating cell lines, derived from H77 (1a), N (1b), Con-1 (1b), O (1b), and JFH-1 (2a) strains, have been established to date (Ikeda et al., 2002, 2005; Blight et al., 2002; Lindenbach et al., 2005; Pietschmann et al., 2002; Wakita et al., 2005; Zhong et al., 2005). Regarding the JFH-1 strain, the infectious virus was efficiently produced in cell culture (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

Studies in the past few years using subgenomic HCV replicon systems have revealed that most replicons possess cell culture-adaptive mutations, which enhance the efficiency of RNA replication and arise during G418 selection. Although these mutations have been found in most NS regions, they cluster in three distinct areas: the N-terminus of the NS3 helicase, two distinct positions of NS4B, and the center of NS5A (Appel et al., 2005; Blight et al., 2000, 2002, 2003; Grobler et al., 2003; Ikeda et al., 2002, 2005; Kato and Sugiyama et al., 2003; Kishine et al., 2002; Krieger et al., 2001; Lohmann et al., 2001, 2003; Yi and Lemon, 2004). To date, however, little information is available on the adaptive mutations obtained from a genome-length HCV RNA replication system. On the other hand, highly permissive cells (cured cells) for efficient RNA replication were also obtained by the elimination of replicons from the G418-selected cells by interferon (IFN) treatment (Blight et al., 2002; Kato and Sugiyama et al., 2003; Lohmann et al., 2003). These reports suggest that both viral and cellular factors determine the efficiency of RNA replication.

The sO replicon (O strain) that we developed also possesses a unique adaptive mutation (S2200R) in the center of NS5A (Kato and Sugiyama et al., 2003), and we recently established a genome-length HCV RNA-replicating cell line (O cells) by the transfection of genome-length HCV RNA with S2200R mutation into sOc cured cells, which were created by eliminating sO replicon from sO cells by IFN treatment (Ikeda et al., 2005). Sequence and functional analyses of HCV RNAs obtained from the O cells found a second adaptive mutation (K1609E) in the C-terminus of the NS3 helicase. We further found that the Oc cells, which were created by eliminating HCV RNA from O cells by IFN treatment, possessed more overwhelming advantages than the sOc cells in the replication of genome-length HCV RNA, even though the O cells were derived from sO cells (Ikeda et al., 2005). These results suggest that a second adaptive mutation, such as K1609E, is required for the robust replication of genome-length HCV RNA, and that the cell backgrounds regarding the potentials of genome-length HCV RNA replication differ greatly between Oc and sOc cells.

To evaluate these ideas, we newly established four kinds of genome-length HCV RNA-replicating cells possessing the cell background of sO or O cells, and then we characterized the genetic mutations detected in the replicating HCV RNAs. Here, we report the findings of adaptive NS3 mutations required for the robust replication of genome-length HCV RNA, and the drastic enhancement of HCV RNA replication by the specific combination of these adaptive NS3 mutations.

2. Materials and methods

2.1. Cell culture

sOc and Oc cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum as described previously (Ikeda et al., 2005). Cells supporting genome-length HCV RNAs were cultured in the presence of G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA) and passaged twice a week at a 5:1 split ratio. HCV RNA-replicating cells possess the G418-resistant phenotype because *Neo*^{R} was produced by the efficient replication of HCV RNA. Therefore, when HCV RNA is excluded from the cells or when its level is decreased, the cells are killed in the presence of G418.

2.2. Plasmid constructions

To introduce the mutations into the plasmid pON/C-5B (GenBank accession no. AB191333; Fig. 1; Ikeda et al., 2005), a PCR-based site-directed mutagenesis method was used. The *Spe*I–*Not*I fragment (corresponding to positions 3474-6159 of the HCV genome) and the *Not*I–*Kpn*I fragment (corresponding to positions 6159–9077 of the HCV genome) of pHCV-O (Ikeda et al., 2005) were subcloned into pBluescript II (Stratagene, La Jolla, CA), resulting in pBlue/34AB and pBlue/5AB, respectively. pBlue/34AB and pBlue/5AB were used as the templates for PCR-based site-directed mutagenesis. The introduced mutations were confirmed by the sequencing of the obtained plasmids. The *Spe*I–*Not*I and *Not*I–*Kpn*I fragments possessing the mutation(s) were each replaced with the corresponding region of pHCV-O. The pON/C-5B-possessing mutation or mutations were generated by replacing the *Eco*RI–*Spe*I fragment of the pHCV-O, into which one or more mutations were introduced.

To construct pOR/C-5B, the *Neo*^{R} gene was replaced with the *Renillia luciferase* (RL) gene at *Asc*I and *Pme*I sites in pON/C-5B.

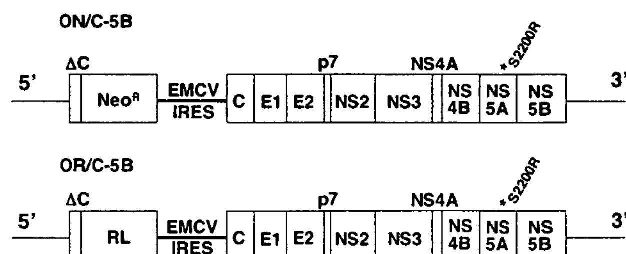

Fig. 1. Schematic gene organization of genome-length HCV RNAs used in this study. Open reading frames, untranslated regions, and EMCV IRES are depicted as open boxes, thin lines, and thick lines, respectively. ΔC indicates the 12N-terminal aa residues of the core as a part of IRES. ON/C-5B RNA and OR/C-5B RNA possess the *Neo*^{R} and RL genes, respectively. The asterisk indicates an adaptive mutation (S2200R) found in the sO subgenomic replicon (Kato and Sugiyama et al., 2003).

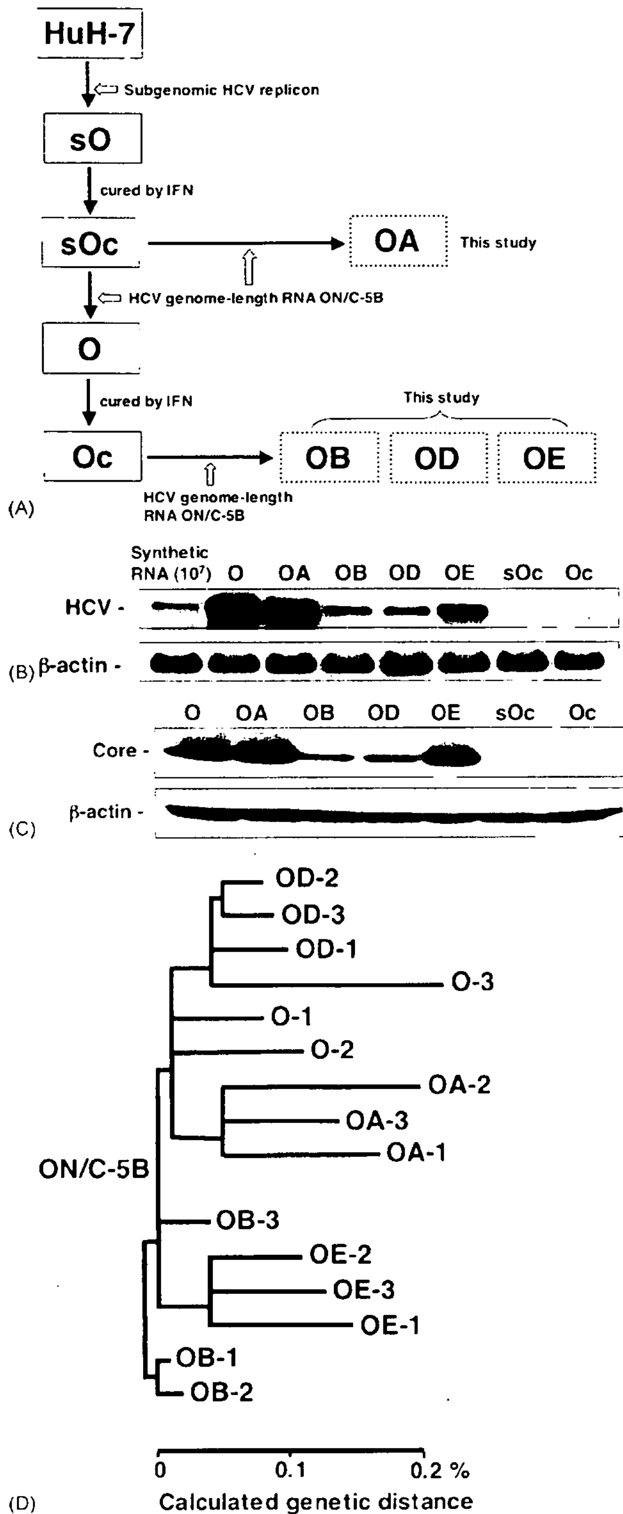

Fig. 2. Characterization of genome-length HCV RNA-replicating cell lines. (A) Lineage of genome-length HCV RNA-replicating cells. The sO and O cell lines were previously established (Ikeda et al., 2005; Kato and Sugiyama et al., 2003). (B) Northern blot analysis. Total RNAs from genome-length HCV RNA-replicating cells (O, OA, OB, OD, and OE cells), as well as total RNAs from the sOc and the Oc cells, were analyzed by Northern blot analysis using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific probe (lower panel). In vitro-synthesized ON/C-5B RNA (10^7 genome equivalents spiked into normal cellular RNA) was used for the comparison of the expression level. (C) Western blot analysis. The orders of specimens were the same as in (B). Production of the core in these cells was analyzed by immunoblotting using anti-core antibody. β-actin was used as a control for the amount of protein loaded per lane. (D) Phylogenetic tree of HCV-O clone populations obtained from genome-length HCV RNA-replicating cells. The phylogenetic tree is depicted on the basis of nucleotide sequences of all clones obtained from the O, OA, OB, OD, and OE cells.

2.3. RNA synthesis

Plasmid DNAs were linearized with *Xba*I and used for RNA synthesis with the T7 MEGAscript kit (Ambion, Austin, TX). Synthesized RNA was purified by lithium chloride precipitation and dissolved in nuclease-free water.

2.4. RNA transfection and selection of G418-resistant cells

RNA transfection and selection of G418-resistant cells were carried out as described previously (Ikeda et al., 2005). Briefly, for electroporation, sOc or Oc cells were suspended at 10^7 cells/ml in phosphate-buffered saline (PBS), and then RNA was mixed with 500 μl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad Laboratories, Hercules, CA). The mixture was immediately subjected to two electric pulses of 1.2 kV, 25 μF, and maximum resistance. The cells were then seeded into a 10 cm diameter dish. After 24 h, the cells were selected in complete DMEM with 300 μg/ml G418 for 3 weeks.

2.5. Northern blot analysis

Total RNA from the cultured cells was prepared using an RNeasy extraction kit (Qiagen, Hilden, Germany). Three micrograms of RNA was used to detect the HCV RNA and β-actin mRNA. Northern blotting and hybridization were carried out as described previously (Ikeda et al., 2002; Kato and Sugiyama et al., 2003). A digoxigenin-labeled, negative-sense RNA probe complementary to the NS5B region (positions 8935–9374 of the HCV genome) and β-actin-specific antisense RNA probe were used to detect the HCV RNA and β-actin mRNA, respectively (Kato and Sugiyama et al., 2003; Kato et al., 2005).

2.6. Western blot analysis

The preparation of cell lysates, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting analysis with a PVDV membrane were performed as described previously (Hijikata et al., 1993; Naganuma et al., 2000). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan) and β-actin (AC-15; Sigma–Aldrich, St. Louis, MO). Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin–Elmer Life Sciences, Wellesley, MA).

2.7. *Preparation of cured cells*

To prepare cured cells, HCV RNA-replicating cells were treated with IFN-α or IFN-γ as described previously (Abe et al., 2005; Ikeda et al., 2005; Naka et al., 2005). Briefly, the cells (each 1×10^6) were treated with IFN-α or IFN-γ (each 500 IU/ml) in the absence of G418. The treatment was continued for 3 weeks with the addition of IFN at 4-day intervals. The cured cells obtained from sO, O, OA, OB, OD, and OE cells (HCV RNA-replicating cells obtained in this study, see Fig. 2A) were named sOc, Oc, OAc, OBc, ODc, and OEc, respectively, and were cultured in DMEM supplemented with 10% fetal bovine serum in the absence of G418. RT-PCR confirmed the absence of HCV RNA in these cured cells.

2.8. *Reverse transcription-polymerase chain reaction (RT-PCR)*

To amplify HCV RNA, RT-PCR was carried out separately in two parts as described previously (Ikeda et al., 2005). Briefly, one part covered from HCV 5′-UTR to NS3, with a final product of approximately 5.1 kb, and the other part covered from NS2 to most of HCV 3′-UTR, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis for HCV open reading frame (ORF) after cloning into pBR322MC (Kishine et al., 2002). SuperScript II (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

2.9. *cDNA cloning and sequencing*

Two PCR products (5.1 and 6.1 kb) were digested with *Xba*I and then cloned into the *Xba*I site of pBR322MC, as described previously (Kato et al., 2005). Plasmid insertions were sequenced in both the sense and antisense directions using the Big Dye Terminator Cycle Sequencing kit (Perkin–Elmer Life Sciences) on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

2.10. *Transient replication assays*

The cells were transfected with 20 μg RNA by electroporation as described above. After electroporation, the cells were plated onto 24-well plates at 3×10^4 cells per well. The cells were harvested with Renilla lysis reagent (Promega, Madison, WI) at 24, 48, 72, and 96 h after the electroporation, and were subjected to luciferase assay according to the manufacturer's protocol.

2.11. *Molecular evolutionary analysis*

Nucleotide sequences (10,972 nucleotides of HCV 5′-UTR to NS5B) of clones obtained by RT-PCRs from O, OA, OB, OD, and OE cells were analyzed by neighbor-joining analysis using the program GENETYX-MAC (Software Development, Tokyo, Japan).

3. Results

3.1. *Establishment of genome-length HCV RNA-replicating cell lines*

We previously established a cloned sO cell line possessing a subgenomic HCV replicon (O strain) and found an adaptive mutation (S2200R) in NS5A (Kato and Sugiyama et al., 2003). More recently we further established a cloned O cell line replicating genome-length HCV RNA by the selection with G418 treatment following the electroporation of genome-length HCV RNA with the S2200R mutation into sOc cured cells (Ikeda et al., 2005). In that study, we found a second adaptive mutation (K1609E) in the NS3 helicase region of HCV RNA-replicating in the O cells, and we also observed that the Oc cured cells showed more overwhelming advantages than the sOc cells in the replication of genome-length HCV RNA (Ikeda et al., 2005). These results suggested that the K1609E mutation was required for the robust replication of genome-length HCV RNA, and that cell backgrounds regarding the potentials of RNA replication differ greatly between sOc and Oc cells. However, we could not clarify whether or not K1609E is the unique or best adaptive mutation for RNA replication. Furthermore, we obtained no information indicating whether viral or host factors are the main contributors to the robust RNA replication.

To obtain such information, we attempted to establish additional genome-length HCV RNA-replicating cell lines by the electroporation of ON/C-5B RNA, which possesses the S2200R mutation (Fig. 1), into sOc or Oc cells (Fig. 2A). After 3 weeks of G418 selection, we obtained one G418-resistant colony derived from sOc cells and three G418-resistant colonies derived from Oc cells. These G418-resistant colonies proliferated successfully; the sOc-derived colony was named OA cell line and Oc-derived three colonies were called OB, OD, and OE cell lines (Fig. 2A), and then these cell lines were used for further analysis. To examine the replication level of genome-length HCV RNA in these cell lines, total RNAs and proteins extracted from OA, OB, OD, and OE cells were subjected to Northern and Western blot analyses, respectively. Total RNAs and proteins extracted from the O, sOc, and Oc cells were also used for the comparison. Genome-length HCV RNAs approximately 11 kb long were detected in all specimens except those from the sOc and Oc cells (Fig. 2B). The number of copies of HCV RNAs in total RNA (each 3 μg) was estimated to be more than 10^7 by comparing these HCV RNAs with HCV RNA synthesized in vitro, although the levels in OB and OD cells were somewhat lower than those in O, OA, and OE cells. The core was also detected in all specimens except those from the sOc and Oc cells (Fig. 2C). The levels of the core in OB and OD cells were also somewhat lower than those in O, OA, and OE cells. These results suggest that the replication levels of HCV RNA in OA cells are equivalent to that in O cells and are higher than those in OB, OD, and OE cells. Although the expression of HCV RNAs and HCV proteins differed somewhat among these cell lines, these lines, including that of O cells, were maintained for at least several months in the presence of G418 (data not shown), suggesting the stable robust HCV RNA replication. The OA, OB, OD, and OE cells

were highly sensitive to IFN-α towards HCV RNA replication (data not shown), as were the O cells (Ikeda et al., 2005; Naka et al., 2005).

3.2. Genetic analysis of HCV RNAs replicating in the OA, OB, OD, and OE cells and comparison with that in the O cells

To learn whether or not HCV RNAs replicating in the OA, OB, OD, and OE cells possess the K1609E mutation, we performed sequence analysis of HCV RNAs replicating in these cell lines. Total RNAs extracted from these cells were subjected to RT-PCR, and then two fragments (5.1 and 6.1 kb) amplified by RT-PCR for ORF were subcloned into plasmid for sequence analysis, as described previously (Ikeda et al., 2005). The sequences of three independent clones were determined and compared with each other to avoid PCR error and to find conserved mutations. Based on the nucleotide sequence data of all clones sequenced in this study and the data obtained from the O cells (Ikeda et al., 2005), we constructed a phylogenetic tree for the HCV RNAs sequenced (10,972 nucleotides of HCV 5′-UTR to NS5B). The result (Fig. 2D) revealed that the three clones derived from each cell line were mostly clustered and located at similar genetic distances from the origin (ON/C-5B), although O-3 and OB-3 were not clustered completely in the expected positions, suggesting that O ∼ OE are independent cell lines. In our sequence analysis, the K1609E mutation found in the O cells was not detected in the OA, OB, OD, and OE cells. However, instead of the K1609E mutation, each cell line possessed a cell line-specific conserved mutation in the NS3 protease region (Table 1). The E1202G, P1115L, Q1112R, and P1115L mutations in the NS3 protease region were detected in the OA, OB, OD, and OE cells, respectively. These results indicated that K1609E was not a representative mutation in genome-length HCV RNA-replicating in the cells. Although Q1112R, P1115L, and E1202G mutations have been detected in other subgenomic HCV replicons (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2003), E1202G mutation seems to have little impact on adaptive mutations (Lohmann et al., 2003) and there is no information on whether or not Q1112R and P1115L are adaptive mutations. Since these mutations were detected as cell line-specific conserved NS3 mutations, we estimated that these NS3 mutations are required for genome-length HCV RNA replication. However, we estimated that the D2415G mutation (the carboxyl region of NS5A) detected in the OA cells is not an adaptive mutation, because this is a naturally observed aa substitution (Murphy et al., 2002; Tanaka et al., 1992). In addition, none of the conserved mutations in the upstream of the NS3 region were detected in the OA, OB, and OD cells or in the O cells, although three conserved mutations (I258K and Y361H in E1 and M939V in NS2) were detected in the OE cells (Table 1). Therefore, we focused on the NS3 mutations found in this analysis for further analyses described below.

3.3. Adaptive mutations found in the NS3 region are required for the robust replication of genome-length HCV RNA

To clarify whether or not conserved NS3 mutations (Q1112R, P1115L, E1202G) are adaptive mutations as are the K1609E mutation, we first carried out qualitative analysis regarding the effects of these mutations in ON/C-5B RNA on the efficiency of colony formation (ECF). The effect of the K1609E mutation derived from O cells was also examined for the comparison. We introduced ON/C-5B RNA with a single NS3 mutation into sOc and Oc cells. Since our previous study (Ikeda et al., 2005) indicated that Oc cells possessed overwhelming advantages in the replication of genome-length HCV RNA, one-twentieth of the ON/C-5B RNA used for sOc cells was used for Oc cells. The results revealed that the NS3 mutated RNA-introduced sOc and Oc cells produced a number of G418-resistant colonies, as did those in K1609E mutation-introduced cells, although no and a few G418-resistant colonies were obtained in the original ON/C-5B RNA-introduced sOc and Oc cells, respectively (Fig. 3). Although the K1609E or E1202G mutation was found in the sOc-derived O or OA cells, the effect of the K1609E or E1202G mutation was not different from that of the Q1112R or P1115L mutation found in the Oc-derived OB, OD, or OE cells. This result indicated that the effects of these mutations are not dependent on their cell origins, and that the Q1112R, P1115L, and E1202G mutations also worked as cell culture-adaptive mutations, as did the K1609E mutation in both sOc and Oc cells. Furthermore, these results indicated again that Oc cells are superior to sOc cells regarding the intracellular replication of genome-length HCV RNA, supporting the previous suggestion (Ikeda et al., 2005) that the cell backgrounds for the potentials of the RNA replication are rather different between Oc and sOc cells.

We next performed quantitative analysis whether or not the effects of these adaptive mutations on ECF are correlated with the effects in early events after RNA transfection into the cells, because of the possibility that additional adaptive mutations

Table 1
Summary of genetic analysis of HCV RNAs derived from O, OA, OB, OD, and OE cells

Cell	Cell background	Size (Nts)	Clone number sequenced	Conserved mutations
O	sOc	10972	3	K1609E (NS3)
OA	sOc	10972	3	E1202G (NS3) D2415G (NS 5A)
OB	Oc	10972	3	P1115L (NS3)
OD	Oc	10972	3	Q1112R (NS3)
OE	Oc	10972	3	I258K (E1) Y361H (E1) M939V (NS2) P1115L (NS3)

Only amino acid positions substituted in all three clones are indicated as the conserved mutations.

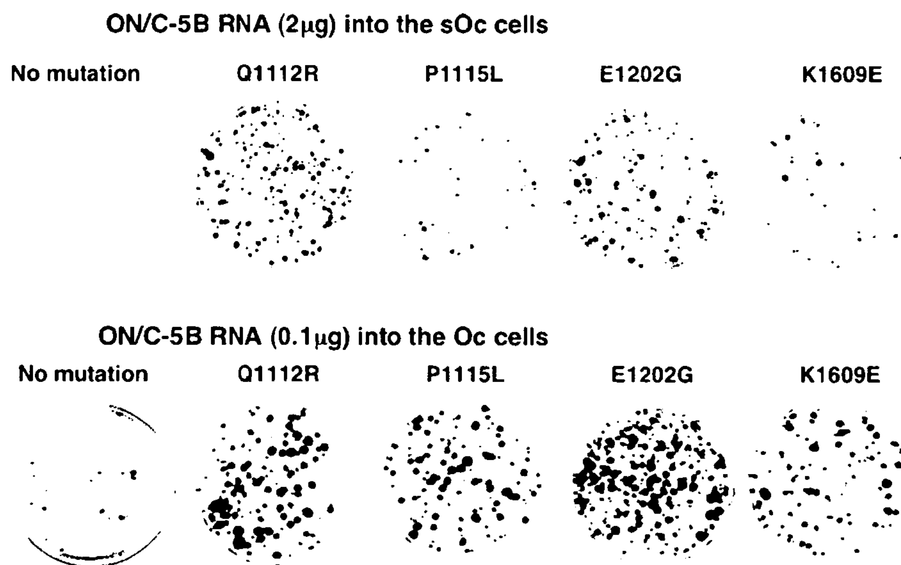

Fig. 3. Adaptive mutations found in the NS3 region show the different ECFs between the sOc and Oc cells. ON/C-5B RNA or ON/C-5B RNA with an additional NS3 mutation was transfected into the sOc cells (2 μg RNA per 10 cm dish; top panel) and the Oc cells (0.1 μg RNA per 10 cm dish; bottom panel). The panels show G418-resistant colonies that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection (Naganuma et al., 2004).

occur during G418 selection for 3 weeks. For the development of a transient replication assay using the RL reporter gene, we first constructed pOR/C-5B from pON/C-5B by replacing the RL and *Neo*^{R} genes (Fig. 1). Next, we made OR/C-5B RNA with the Q1112R, P1115L, E1202G, or K1609E mutation, and then introduced these RNAs into the Oc cells by electroporation. OR/C-5B RNA with dGDD was used as a negative control. The results revealed that the luciferase activity increased in a time-dependent manner when OR/C-5B RNA with an adaptive NS3 mutation was transfected, whereas the activity decreased with time when OR/C-5B RNA without an adaptive NS3 mutation or with dGDD was transfected (Fig. 4A). These results suggest that genome-length HCV RNA with an adaptive NS3 mutation is efficiently able to replicate immediately after transfection. At 96 h, the luciferase activities in the cases of the adaptive NS3 mutation were approximately twice those at 24 h after RNA transfection, and no significant differences in replication efficiency among the adaptive NS3 mutations were observed (Fig. 4A). In summary, we demonstrated a good correlation between the ECF assay (Fig. 3) and luciferase reporter assay for transient replication (Fig. 4A). Therefore, we conclude that Q1112R, P1115L, E1202G, and K1609E function as cell culture-adaptive mutations, and that at least one of them is required for efficient replication of genome-length HCV RNA.

3.4. Every combination of adaptive NS3 mutations in the Oc cells caused more effective genome-length HCV RNA replication than any single adaptive NS3 mutation

According to previous reports using subgenomic HCV replicons, some combinations of adaptive mutations drastically enhance ECF (Krieger et al., 2001; Lohmann et al., 2001, 2003). However, some combinations of adaptive mutations reduced ECF drastically (Lohmann et al., 2001, 2003), suggesting that some adaptive mutations are not compatible. To examine whether or not such conflicting effects of adaptive mutations are observed in genome-length HCV RNA replication, we tested the effects of combining the adaptive NS3 mutations identified in this study, using the luciferase reporter assay for transient replication. We prepared six kinds of OR/C-5B RNA with double adaptive NS3 mutations (i.e., Q1112R and P1115L), and then introduced these RNAs into the Oc cells by electroporation. OR/C-5B RNA with the K1609E mutation was used as a representative of a single adaptive NS3 mutation, and OR/C-5B RNA with dGDD was used as a negative control. The results revealed that the luciferase activities in every combination of adaptive NS3 mutations were remarkably increased, to approximately four- to nine-fold at 96 h, in comparison with the activities at 24 h after RNA transfection, although the enhancement of the luciferase activity by the K1609E mutation was only approximately two-fold (Fig. 4B). The combination of Q1112R and K1609E mutations was the most effective in the Oc cells, followed by that of Q1112R and P1115L mutations. These results suggest that all adaptive NS3 mutations identified in this study are compatible for genome-length HCV RNA replication. It is noteworthy that Q1112R and P1115L mutations are compatible, regardless of very near localization in the NS3 protease.

3.5. Specific combination of adaptive NS3 mutations drastically enhanced genome-length HCV RNA replication, regardless of cell lines

Although we found that every combination of adaptive NS3 mutations caused more effective genome-length HCV RNA replication than any single adaptive NS3 mutation and that some combinations of the mutations drastically enhanced RNA replication (Fig. 4B), the possibility remains that these findings are due to cell clonality, because a cloned Oc cell line was used

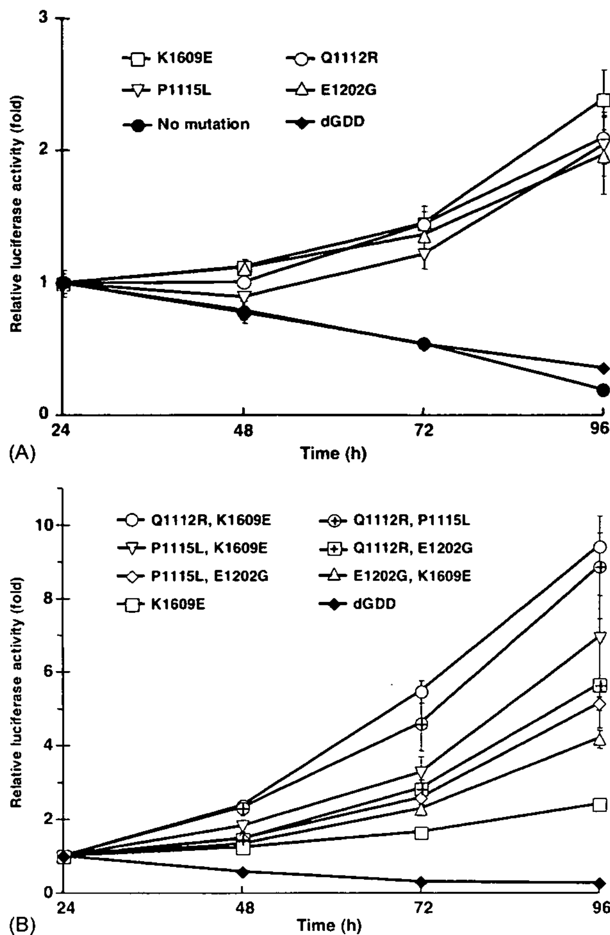

Fig. 4. Transient replication assay of genome-length HCV RNA. (A) Effects of adaptive NS3 mutations on transient replication of genome-length HCV RNA. OR/C-5B RNA or OR/C-5B RNA with an additional mutation was transfected into the Oc cells, and luciferase activity was determined in cell lysates that were prepared at given time points post-transfection. Values for each time point correspond to the mean and the error range of quadruplicate results. Note that, owing to the slightness of the variations, error bars are in some cases not viable in the graph. Values are corrected for transfection efficiency as determined by measuring the luciferase activity 24 h after transfection. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the genome-length HCV RNA with dGDD was used as a negative control. (B) Combination effects of adaptive NS3 mutations on transient replication of genome-length HCV RNA. OR/C-5B RNAs with various combinations of adaptive NS3 mutations were transfected into the Oc cells, and luciferase activity was determined in cell lysates that were prepared at given time points post-transfection, as shown in (A). For a comparison of the results, OR/C-5B RNA with K1609E was used as a representative of a single adaptive mutation.

for the analysis. To check this possibility, we tested the effects of combining adaptive NS3 mutations in the cured OAc, OBc, ODc, and OEc cells, using the transient replication assay as described above. sOc cells, which are rather inferior to the Oc cells in RNA replication (Ikeda et al., 2005; Fig. 3), were also used for the analysis. Interestingly, in the OAc, OBc, ODc, and OEc cells also, the results were similar to those obtained in the Oc cells. The combination of Q1112R and K1609E mutations or Q1112R and P1115L mutations was the most effective on the genome-length HCV RNA replication, although the combination of P1115L and K1609E mutations or E1202G and K1609E mutations was not effective in ODc cells (Fig. 5). These results suggest that the NS3 with specific adaptive mutations is the primary determinant of the replication level of genome-length HCV RNA, regardless of cell lines. In addition, we found that OEc cells possessed the best environment for RNA replication among examined cell lines, by demonstrating that the luciferase activity in the combination of Q1112R and K1609E mutations was approximately 20-fold higher at 96 h than that at 24 h after RNA transfection (Fig. 5). On the other hand, we observed that most combinations of adaptive mutations in the sOc cells did not enhance RNA replication, although luciferase activity was enhanced approximately two-fold in the combination of Q1112R and P1115L only (Fig. 5). These results suggest that the cellular environment is also involved in the efficient replication of genome-length HCV RNA.

4. Discussion

In this study, we established four kinds of genome-length HCV RNA (O strain of genotype 1b) replicating cell lines (OA, OB, OD, and OE), which were independent from the O cell line established previously (Ikeda et al., 2005). We also found several cell culture-adaptive NS3 mutations required for the replication of genome-length HCV RNA. We further found that specific combinations of these adaptive mutations remarkably enhanced the efficiency of the RNA replication, regardless of the cell lines obtained.

To establish genome-length HCV RNA-replicating cell lines, we introduced ON/C-5B RNA with the S2200R mutation (NS5A), which was identified as the adaptive mutation for the sO replicon, into two types (sOc and Oc) of cured cells. Since the ECF of the Oc cells was higher than that of the parental sOc cells (Ikeda et al., 2005), the Oc cells were also used to facilitate the establishment of genome-length HCV RNA-replicating cell lines. We initially estimated that adaptive mutations other than K1609E (NS3 helicase region) found in the O cells would be obtained from the sOc-derived OA cell line, and that the K1609E mutation would be obtained mainly from the Oc-derived OB, OD, and OE cell lines. Although a new E1202G adaptive mutation was obtained from the sOc-derived OA cells, the K1609E mutation was not obtained from the Oc-derived OB, OD, and OE cells. Instead of the K1609E mutation, the P1115L adaptive mutation (NS3 protease region) was obtained from the OB and OE cells, and the Q1112R adaptive mutation (NS3 protease region) was obtained from the OD cells. The ECF assay and transient replication assay showed that these adaptive mutations possessed similar potentials of genome-length HCV RNA replication in the Oc cells. These results suggest that the K1609E mutation is only one of the adaptive mutations that function in the Oc cells, and suggest that the combination of an NS3 mutation (Q1112R, P1115L, E1202G, or K1609E) with the NS5A mutation (S2200R) is required for efficient replication of genome-length HCV RNA, although only the S2200R mutation is enough to efficiently replicate the subgenomic sO replicon (Kato and Sugiyama et al., 2003). Therefore, our findings suggest that viral factors, which are not required for the robust

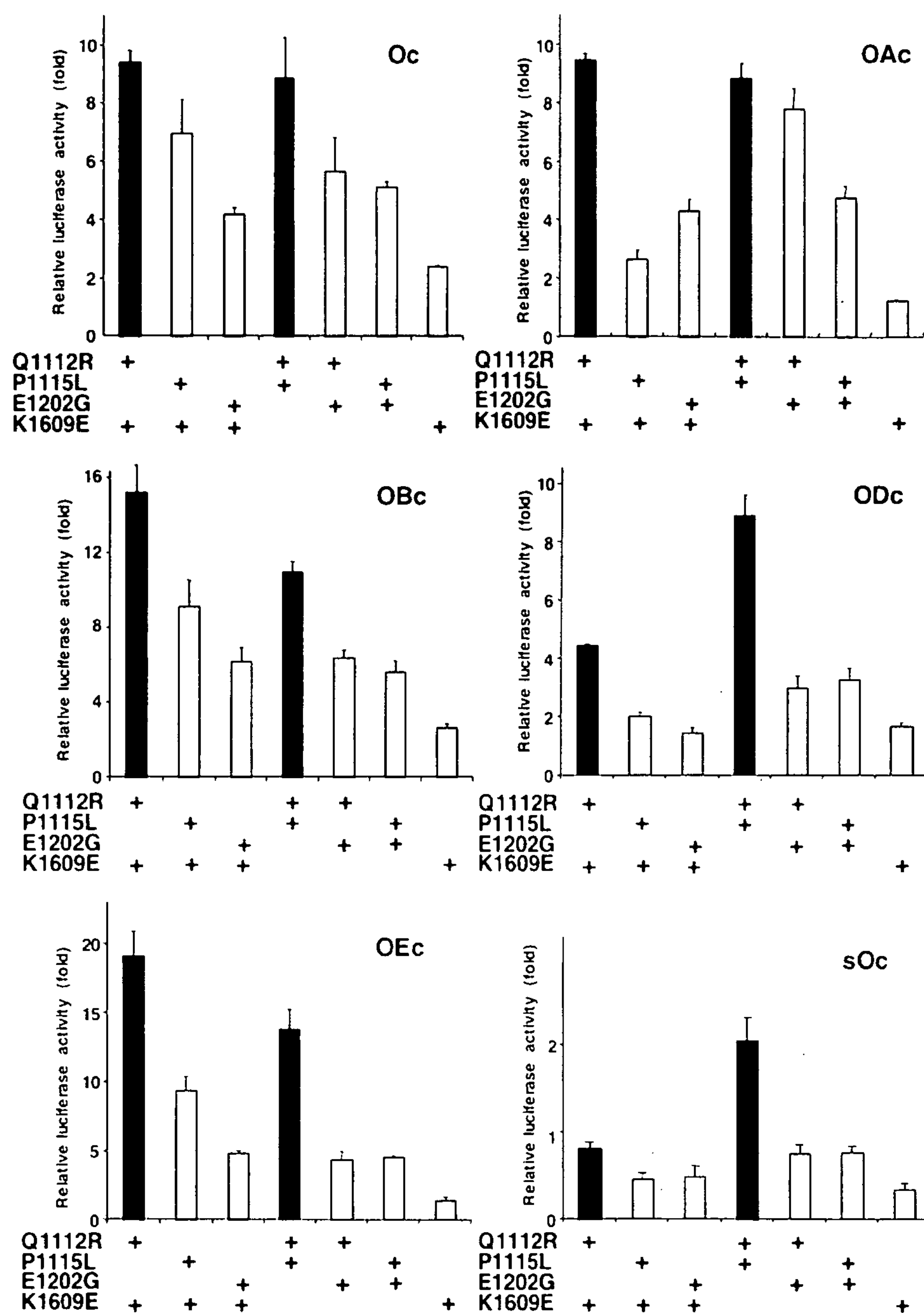

Fig. 5. Effects of combining adaptive NS3 mutations on transient genome-length HCV RNA replication in various cured cells. OR/C-5B RNAs with various combinations of adaptive NS3 mutations were transfected into various cured cells (Oc, OAc, OBc, ODc, OEc, and sOc cells), and luciferase activity was determined in cell lysates that were prepared at 96 h post-transfection, as shown in Fig. 4. Values are corrected for transfection efficiency as determined by measuring the luciferase activity 24 h after transfection.

replication of a subgenomic HCV replicon, are required for the robust replications of genome-length HCV RNA.

To date, it has been believed that cell culture-adaptive mutations that enhance HCV RNA replication do not exist in HCV-infected patients (Bartenschlager, 2005). However, Sarrazin et al. (2005) recently reported the existence of adaptive NS3 mutations in 5 of 26 HCV-infected patients. In that study, it is noteworthy that mutations (P1112R and P1115G) in positions 1112 and 1115 have been found, although Q1112R and P1115L have not been detected, and that specific adaptive mutations have been associated with a slower initial decrease in HCV RNA concentrations during IFN-α-based antiviral therapy (Sarrazin et al., 2005). Furthermore, a search of the Hepatitis Virus Database (Nagoya City University, Japan) found Q1112R, P1115L, and E1202G in the HCV sequences (accession nos. AY460204, D84262, and AF011751, respectively) derived from HCV-infected patients. Therefore, some of the cell culture-adaptive mutations may not be artificial mutations

but may reflect some phenomena that HCV-infected patients undergo.

Although adaptive NS3 mutations found in this study were the same as those detected in subgenomic HCV replicons derived from different HCV strains (Blight et al., 2000; Kishine et al., 2002; Krieger et al., 2001; Lohmann et al., 2001, 2003), the impacts of these mutations in subgenomic HCV replicons seem to be small. The impacts of E1202G and K1609E on ECF assay and transient replication assay, respectively, were only about four- and two-fold that of the wild-type subgenomic HCV replicon (Lohmann et al., 2001, 2003), and information regarding Q1112R and P1115L mutations has yet to be reported. However, we observed that Q1112R, P1115L, E1202G, and K1609E mutations remarkably enhanced the efficiency of genome-length HCV RNA replication in both ECF and transient replication assay (Figs. 3 and 4). The discrepancy in the results might be due to the differences in subgenomic and genomic lengths of HCV RNA, in HCV strains, or in host cell lines used. Recently, we showed time-dependent genetic mutations of subgenomic HCV replicons and time-dependent expansions of their genetic diversities in long-term culture (at least 1 year) of two cell lines harboring subgenomic HCV replicons (1B-1 and O strains) (Kato et al., 2005). In that study, we observed that the expansion of the replicons' genetic diversity was associated with the enhancement of RNA replication. It is noteworthy that the P1115L mutation has been detected as a conserved mutation after 6 months in cell culture of the 1B-1-derived replicon, although this mutation's contribution to the replication is not clear (Kato et al., 2005). Genetic analysis of HCV RNAs in long-term cultures of O ~ OE cells will provide useful information regarding the genetic advantages of adaptive mutations found in the NS3 region. For such analysis, long-term culture (at least 1 year) of O ~ OE cells is in progress.

From the analyses using subgenomic HCV replicons to date, the center of NS5A has been thought to represent a hot spot for cell culture-adaptive mutations, because most mutations are found in this region. Interestingly, adaptive mutations often affect serine residues involved in hyperphosphorylation of NS5A. Although the HCV-O-derived sO replicon also possesses a unique S2200R adaptive mutation in the center of NS5A, the serine residue at position 2200 is not thought to be involved in the hyperphosphorylation of NS5A (Tanji et al., 1995). To examine whether or not S2200R mutation is required for efficient replication of genome-length HCV RNA, we tested the effect of the S2200R mutation on RNA replication by the introduction of OR/C-5B RNA with Q1112R and K1609E, in which the arginine residue in position 2200 was restored to serine residue, into Oc cells. The results revealed that the replication of genome-length HCV RNA was abolished with the restoration only in position 2200 (data not shown), suggesting that the S2200R mutation plays an important key role in HCV RNA replication in HuH-7-derived cells. However, the mechanism underlying the mutations found in NS3 causes the replication of genome-length HCV RNA is unknown, as is the mechanism underlying the great enhancement of replication by the combination of NS3 mutations. One possibility is that the NS3 mutations found in this study are able to drastically enhance the protease or helicase activity of NS3. To evaluate this possibility, further experiments using the quantitative system that measures the NS3 protease or helicase activity will be needed.

The relation between the combination of adaptive NS3 mutations and the cloned cell lines is interesting. From information obtained in previous studies (Lohmann et al., 2003; Ikeda et al., 2005) and the present study, it clearly appeared that both viral and cellular factors contributed to HCV RNA replication in cell culture. For the replication of genome-length HCV RNA, we showed that adaptive NS3 mutations were viral factors and that the differences between sOc and Oc cells were cellular factors (Fig. 3). However, our results revealed that specific combinations of adaptive NS3 mutations (Q1112R and K1609E, or Q1112R and P1115L) were superior to the other combinations in all Oc, OAc, OBc, ODc, and OEc cell lines examined. In even sOc cells, the combination of Q1112R and P1115L, but not the combination of Q1112R and K1609E, was superior to the other combinations. These findings suggest that the effect of NS3 possessing a specific combination of mutations is superior to that of the host cell clonality. Recently, Yi et al. (2006) reported the production of infectious genotype 1a HCV in the cells transfected with genome-length HCV RNA (H77-S) possessing five adaptive mutations (two in NS3, one in NS4A, and two in NS5A), suggesting that robust replication of HCV RNA is also necessary for the production of infectious viruses (Yi et al., 2006). Therefore, identification of the best combination of adaptive mutations for efficient RNA replication may be useful in the development of a system to produce infectious genotype 1b HCV, and for understanding the HCV replication mechanism.

Acknowledgments

We thank T. Nakamura and A. Morishita for their technical assistance. This work was supported by a grant-in-aid for the third-term comprehensive 10-year strategy for cancer control and by a grant-in-aid for research on hepatitis, both from the Ministry of Health, Labor, and Welfare of Japan. K.A. was supported by a Research Fellowship from the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2007, p. 3756–3759 Vol. 51, No. 10
0066-4804/07/$08.00+0 doi:10.1128/AAC.00233-07
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Identification of Novel Epoxide Inhibitors of Hepatitis C Virus Replication Using a High-Throughput Screen▽†§

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Received 15 February 2007/Returned for modification 7 May 2007/Accepted 28 July 2007

Using our high-throughput hepatitis C replicon assay to screen a library of over 8,000 novel diversity-oriented synthesis (DOS) compounds, we identified several novel compounds that regulate hepatitis C virus (HCV) replication, including two libraries of epoxides that inhibit HCV replication (best 50% effective concentration, < 0.5 μM). We then synthesized an analog of these compounds with optimized activity.

Hepatitis C virus (HCV) infects over 170 million people worldwide and frequently leads to cirrhosis, liver failure, and hepatocellular carcinoma (1). Currently, the best therapy for the treatment of chronic hepatitis C is a combination of pegylated interferon and ribavirin, which has suboptimal efficacy and has an unfavorable side effect profile (14). The identification of more-effective and better-tolerated agents is therefore a high priority.

We have recently reported the successful adaptation of the Huh7/Rep-Feo replicon cell line (18) to a high-throughput screening assay system (8). Using this system, we previously screened a library of 2,568 well-known compounds whose biological activity is fully characterized (8). In order to discover novel regulators of HCV replication, we then screened a library of 8,064 diversity-oriented synthesis (DOS) compounds (15, 16). This library, known as the DOS set, is a

TABLE 1. Hits by library from the primary high-throughput screening with the DOS set[a]

Library	Increased luciferase signal hit libraries			Antiviral hit libraries		
	Hits	Members	Reference(s)	Hits	Members	Reference(s) or sources
FPA	11	319	5			
BUCMLD	4	880	10, 17	4	880	10, 17, Fig. 1, Table 2
JMM	4	544	13			
UGISS	1	319	2			
BUCMLD epoxyquinol				12	34	10, 17, Fig. 1 and 2, Table 2
SM				9	27	Fig. 1 and 2, Table 2
SpOx				6	612	6, 12
BEA				3	238	3
ICCB6				3	352	4
YKK				2	281	9
RTE				2	159	19

[a] The total number of compounds which comprise each library is listed in the "Members" columns.

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† This publication is dedicated to Yoshito Kishi on the occasion of his 70th birthday.

§ Supplemental material for this article may be found at http://aac.asm.org/.

‡ L.F.P. and S.S.K. contributed equally to this project.

▽ Published ahead of print on 6 August 2007.

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TABLE 2. Results of secondary screening with antiviral hit compounds from the SM and BUCMLD libraries[a]

Compound name	EC_{50}	CC_{50}
BUCMLD-B10A11	<0.5 (<0.5–0.5)	19.5 (19.4–22.4)
BUCMLD-B10A3	0.7 (<0.5–5.2)	9.0 (7.1–10.0)
BUCMLD-XL-184	1.4 (0.8–3.9)	>50
BUCMLD-B10A5	1.5 (<0.5–5.4)	39.3 (28.6–>50)
BUCMLD-XL-190	2.5 (<0.5–10)	>50
BUCMLD-B10A1	2.6 (1.0–5.0)	18.0 (15.9–19.5)
SM_A14B5	3.5 (2.7–4.4)	27.1 (18.0–44.6)
BUCMLD-XL-189	3.8 (2.2–7.0)	>50
SM_A6B5_2P100	6.6 (4.0–15.3)	>50
BUCMLD-B10A8	7.0 (0.9–30.0)	>50
BUCMLD-B10A10	7.0 (5.4–30.0)	>50
BUCMLD-B10A14	7.6 (1.0–23.0)	>50
BUCMLD-B10A7	7.75 (1.0–30.0)	35.3 (33.6–36.7)
SM_A4B6_2P123	8.0 (6.3–10.0)	>50
SM_A5B5_2P118	9.1 (2.5–16.7)	>50
SM_A7C2_2P155	12.7 (7.0–24.0)	>50
BUCMLD-B13A2	14.2 (6.4–45.0)	>50
SM_A1B2_1P32	19.6 (11.7–28.2)	>50
SM_A1B5_2P24	19.7 (6.25–50.0)	>50
BUCMLD-B13A1	21.1 (7.5–36.7)	>50
SM_A5B3_2P141	25.7 (18.3–39.8)	>50
SM_A5B2_2P142	26.7 (9.1–50.0)	>50
BUCMLD-NTM-EN2-67A	30.0 (0.7–46.1)	>50
SM_A12B3	>30	>50
BUCMLD-B10A13	42.9 (25.2–59.4)	>50
BUCMLD-XL-130	>100	>50

[a] Note that structure-activity relationship SM library compounds are also included. The EC_{50} and 50% cytotoxic concentration (CC_{50}) are reported in μM with 95% confidence intervals in parentheses. A value of <0.5 indicates a concentration of less than 0.5 μM; >30 indicates a concentration of greater than 30 μM; >50 indicates a concentration of greater than 50 μM; and >100 indicates a concentration of greater than 100 μM.

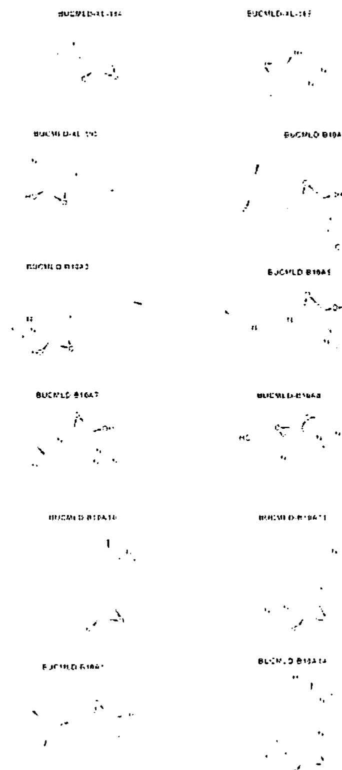
meta-library comprised of DOS libraries from chemists throughout the United States and Canada. Information about the DOS set is available at http://www.broad.harvard.edu/chembio/platform/screening/compound_libraries/index.htm.

The high-throughput primary screen and the secondary validation assays were performed as described in our previous publication (8).

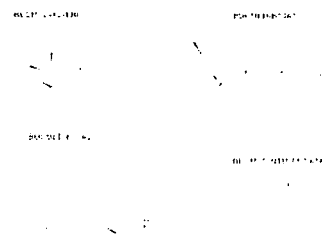
Computational data analysis of the primary screening results was performed as previously described (8) except for the hit criteria. As the characteristics of this data set are different from those generated by our previous screen (8), different threshold values were chosen to assure optimal hit selection. Compounds were considered hits for inhibiting replication if they had a composite Z score of <−2.57 in the reporter gene screen, a reproducibility of >0.9 or <−0.9 in that screen, and a composite Z score of >−2.00 in the cell viability screen. Compounds were considered hits for stimulating luciferase production if they had a composite Z score of >2.50 in the reporter gene screen, a reproducibility of >0.9 or <−0.9 in that screen, and a composite Z score of <1.00 in the cell viability screen.

Full synthetic experimental procedures and spectroscopic data for the SM library compounds discussed in this publication are provided in the supplemental material. The synthesis of the full SM library, including compounds not discussed here, will be the subject of an upcoming report.

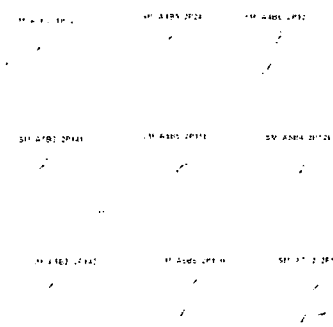
BUCMLD Epoxyquinol Hit Compounds



BUCMLD Non-Epoxyquinol Hit Compounds



SM Library Hit Compounds



SM Library SAR Compounds

FIG. 1. Structures of antiviral hit compounds from the BUCMLD and SM libraries. SAR, structure-activity relationship.

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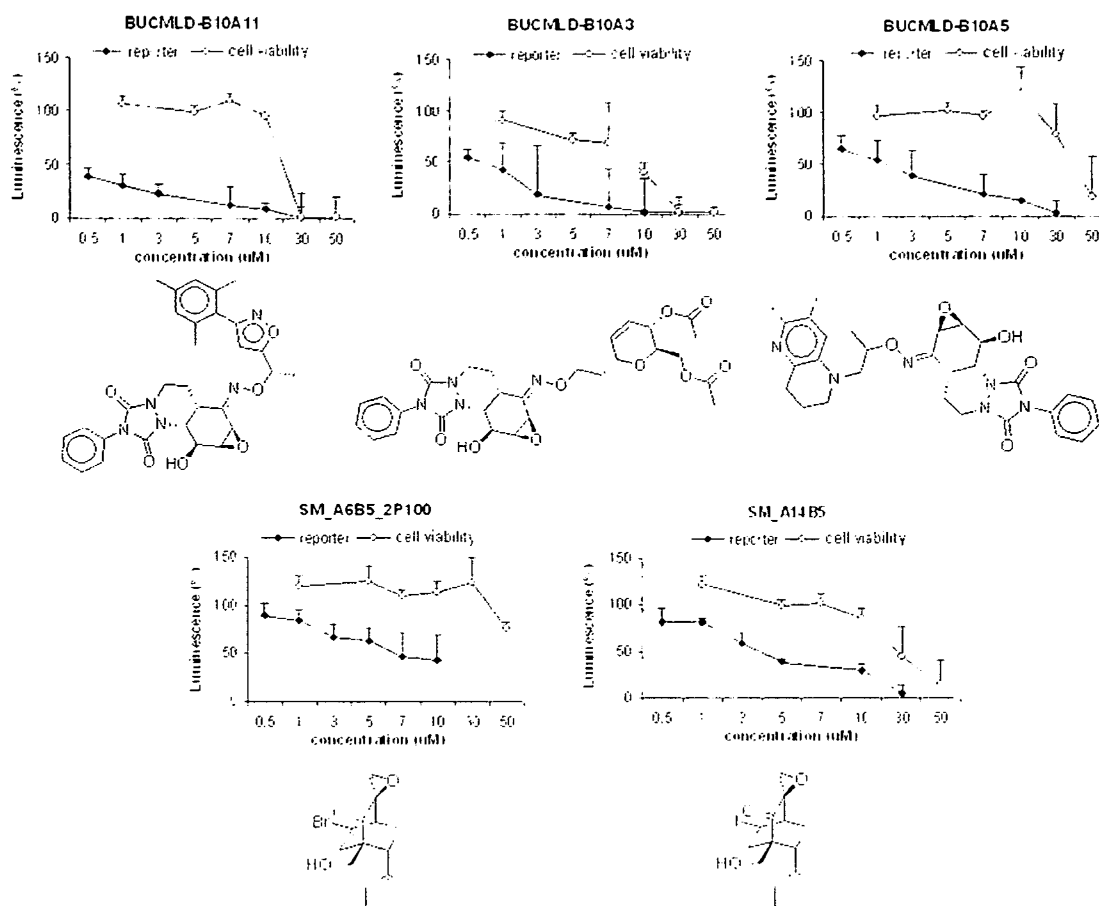

FIG. 2. Selected graphical results of secondary screening with antiviral hit compounds from the SM and BUCMLD epoxyquinol libraries. Luciferase activity for HCV RNA replication levels is shown as a percentage of control. Cell viability is also shown as a percentage of control. Each point represents the average of triplicate data points with standard deviation represented as the error bar.

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The synthesis of the BUCMLD epoxyquinol library has been previously described (10, 17).

Full experimental details regarding the JFH1 HCVcc system (11) are provided in the supplemental material. We identified 41 antiviral compounds that inhibited HCV replication and 20 proviral compounds that increased luciferase production (Table 1). In our analysis of the antiviral hit compounds from the DOS set, a striking finding was that 21 of the 41 compounds contained an epoxide moiety. Moreover, the most potent of these compounds were epoxides. Further analysis revealed that these epoxides came from only two DOS libraries, SM and BUCMLD epoxyquinol (10, 17), with very high sublibrary hit rates of 35% and 33%, respectively (Table 1). Of note, the non-hit members of these two libraries did exhibit antiviral activity but failed to meet the formal hit criteria.

As we were especially intrigued by these epoxide-bearing compounds, we restricted our hit validation to these compounds (Table 2 and Fig. 1). SM_A6B5_2P100 was the most active member of the SM library, while BUCMLD-B10A11 was the most potent member of the BUCMLD epoxyquinol library (Table 2 and Fig. 2).

Structure-activity relationship analysis of the SM library reveals the structural elements most important for antiviral activity (Table 2 and Fig. 1). Comparing SM_A5B5_2P118 to SM_A1B5_2P24, iodinated compounds are more active than brominated ones. Comparing SM_A5B5_2P118 to SM_A5B3_2P141 and SM_A5B2_2P142, compounds with a phenyl substituent are more active than those with aliphatic chains. Finally, the most active compounds, SM_A4B6_2P123 and SM_A6B5_2P100, have a bridgehead substituent. Thus, we hypothesized that the most active compound should bear an iodine, a phenyl substituent, and a bridgehead substituent.

SM_A14B5, which incorporates all of these elements, was therefore synthesized, as it was reasoned to be the most active SM library compound. Indeed, SM_A14B5 had a 50% effective concentration (EC_{50}) of approximately 3.5 μM, which is about half that of SM_A6B5_2P100 (Table 2 and Fig. 2).

The most potent compounds from each library, SM_A14B5 and BUCMLD-B10A11, underwent further validation in the infectious JFH1 HCVcc system (11). They were tested at concentrations of 5 μM and 1 μM, respectively, and inhibited HCV replication 48.4% ± 5.9% and 45.1% ± 5.2%, respectively, relative to the level of inhibition achieved by interferon at a concentration of 1 ng/ml. These data roughly approximate the EC_{50} validation data derived from the OR6 system (7) in

which inhibition was also measured relative to that of interferon at a concentration of 1 ng/ml.

Our observations suggest that the epoxide moiety is essential for potent antiviral activity. Analyzing the BUCMLD compounds, those compounds that bear an epoxide moiety are, in general, more-potent antivirals than those that do not (Table 2 and Fig. 1). Furthermore, all of the compounds from the SM library bear epoxides. SM_A12B3, an analog of SM_A5B3_2P141, which bears a tetrahydrofuran moiety in place of an epoxide, was therefore synthesized to further test this hypothesis. SM_A12B3 had negligible antiviral activity (Table 2), while SM_A5B3_2P141 displayed modest antiviral activity. Other analogs of SM compounds bearing tetrahydrofuran rings in place of epoxides showed similar attenuation of antiviral activity relative to their parent compounds. Unfortunately, attempts to synthesize the tetrahydrofuran analog of the most potent SM compound, SM_A14B5, have so far been unsuccessful.

It is interesting to note that it is the urazole-containing epoxyquinol constituents of the BUCMLD epoxyquinol library, rather than the maleimide-derived ones, that demonstrated anti-HCV activity in the primary screen. It is therefore likely that the combination of a urazole with the epoxide is necessary for the activity of the BUCMLD epoxyquinol compounds.

Although none of our most potent antiviral DOS compounds showed significant cytotoxicity at their EC_{50}s, all of them ultimately proved to be cytotoxic at higher concentrations (Table 2 and Fig. 2). Therefore, future modifications should not only aim to improve anti-HCV activity but should also attempt to decrease cytotoxicity, in order to widen the therapeutic window.

It is tempting to hypothesize that these epoxides exert their antiviral effects through a common pathway. Presumably, they act as electrophiles, with the nucleophilic target making a covalent bond by attacking and opening the epoxides. Studies to elucidate their mechanism of action are under way.

We thank the National Cancer Institute and the Initiative for Chemical Genetics, who provided support for this publication, and the Chemical Biology Platform of the Broad Institute of Harvard and MIT for their assistance in this work.

The project has been funded in whole or in part with federal funds from the National Cancer Institute's Initiative for Chemical Genetics, National Institutes of Health, under contract no. N01-CO-12400.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

Financial support was provided by the following: The American Gastroenterological Association FDHN/TAP Pharmaceuticals FFT Award (L.F.P.), The GlaxoSmithKline Research Fund of the Korean Association for The Study of The Liver (S.S.K.), NIH 5T32DK07191-31 (L.F.P.), NIH NS050854-01 (R.T.C.), and the NIGMS CMLD Initiative P50 GM067041 (J.A.P.).

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Microbiol. Immunol., 51(1), 117–125, 2007

Editor-Communicated Paper

Tandem Repeats of Lactoferrin-Derived Anti-Hepatitis C Virus Peptide Enhance Antiviral Activity in Cultured Human Hepatocytes

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Communicated by Dr. Masanobu Ohuchi: Received November 8, 2006. Accepted November 13, 2006

Abstract*: Previously, we found that bovine and human lactoferrin (LF) specifically inhibited hepatitis C virus (HCV) infection in cultured non-neoplastic human hepatocyte-derived PH5CH8 cells, and we identified 33 amino acid residues (termed C-s3-33; amino acid 600–632) from human LF that were primarily responsible for the binding activity to the HCV E2 envelope protein and for the inhibiting activity against HCV infection. Since the anti-HCV activity of C-s3-33 was weaker than that of human LF, we speculated that an increase of E2 protein-binding activity might contribute to the enhancement of anti-HCV activity. To test this possibility, we made two repeats [$(\text{C-s3-33})_2$] and three repeats [$(\text{C-s3-33})_3$] of C-s3-33 and characterized them. Far-Western blot analysis revealed that the E2 protein-binding activities of $(\text{C-s3-33})_2$ and $(\text{C-s3-33})_3$ became stronger than that of the C-s3-33, and that the binding activity of $(\text{C-s3-33})_3$ was stronger than that of $(\text{C-s3-33})_2$. Using an HCV infection system in PH5CH8 cells, we demonstrated that the anti-HCV activities of $(\text{C-s3-33})_2$ and $(\text{C-s3-33})_3$ became stronger than that of the C-s3-33. Furthermore, using a recently developed infection system with a VSV pseudotype harboring the green fluorescent protein gene and the native E1 and E2 genes, we demonstrated that the antiviral activities of $(\text{C-s3-33})_2$ and $(\text{C-s3-33})_3$ were stronger than that of C-s3-33. These results suggest that tandem repeats of LF-derived anti-HCV peptide are useful as anti-HCV reagents.

Key words: **Hepatitis C virus, Lactoferrin, Anti-HCV peptide, E2 protein-binding activity**

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (28). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae*. The HCV genome encodes a large polyprotein precursor of about 3,000 amino acids (aa), which is cleaved by the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, and non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (7, 8, 18). These HCV proteins function not only in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (1, 17).

Approximately 170 million people worldwide are infected with HCV (32). The combination of a pegylated interferon with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (6). This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma.

Although the entry mechanism of HCV remains unclear, to date, several candidates for HCV receptors

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Abbreviations: aa, amino acids; DMEM, Dulbecco's modified Eagle's medium; E2, envelope 2; GFP, green fluorescent protein; HCV, hepatitis C virus; LF, lactoferrin; MBP, maltose-binding protein; NS2, non-structural 2; TF, transferrin; VSV, vesicular stomatitis virus.

have been reported: CD81, the scavenger receptor class B type I, the mannose-binding lectins DC-SIGN and L-SIGN, low-density lipoprotein receptors, etc. (4). Most of them have been identified as interacting materials with a soluble and truncated form of the HCV E2 protein, because of the lack of efficient HCV proliferation in cell cultures, although several culture systems using PCR for detection of HCV infection have been reported (20). However, a major advance in investigating HCV entry has been achieved by the development of pseudotype viruses bearing HCV E1 and E2 proteins assembled onto retrovirus particles (2, 9) or vesicular stomatitis virus (VSV) particles (3, 23, 30). Extensive characterization of the pseudotype viruses has shown that these mimic the early steps of the HCV life cycle. This system has allowed the study of the role of candidate receptors in the early steps of HCV infection (4).

We previously found that bovine and human lactoferrins (LFs) specifically prevented HCV infection in cultured human non-neoplastic hepatocyte PH5CH8 cells using the PCR method for detection of HCV infection (10, 12). Regarding these findings, some clinical studies have demonstrated that monotherapy with bovine LF improves the serum HCV RNA and/or alanine aminotransferase levels in patients with chronic hepatitis C (15, 16, 27, 31).

LF is an 80-kDa mammalian iron-binding glycoprotein and consists of two homologous globular lobes (an N-lobe and a C-lobe), each with a single iron (Fe^{3+}) binding site. It is structurally related to the plasma iron-transport protein transferrin (TF). LF's biological roles include activities in the host defense mechanism as well as in iron metabolism (21, 22). Unlike TF, LF is a primary defense protein against microbial infection. LF possesses strong bacteriostatic and bactericidal activities against pathogenic bacteria, as well as inhibitory activity against pathogenic viruses (5, 21, 22, 33).

LF's preventive mechanism against HCV infection has been thought to be the direct interaction between LF and HCV; indeed, by Far-Western blot analysis using thioredoxin-fused LF fragments expressed in *Escherichia coli* (*E. coli*) and the soluble E2 protein expressed in Chinese hamster ovary cells, we demonstrated that the 93 carboxyl aa of LF (human, bovine, and horse), termed C-s3, specifically bound to the E2 protein (25). On the other hand, Yi et al. (34) independently reported that the E1 and E2 proteins could bind to human and bovine LFs, although the binding region of LF was not identified. Furthermore, we identified the 33 aa of human LF (termed C-s3-33; aa 600–632), which was primarily responsible for the E2 protein-binding activity, and demonstrated that maltose-binding protein (MBP)-fused C-s3-33 prevented HCV infection in PH5CH8 hepatocyte cells (25). However, the E2 protein-binding activity and the anti-HCV activity of C-s3-33 were obviously weaker than those of human LF. Therefore, we presumed that the increase of the E2 protein-binding activity would lead to the enhancement of anti-HCV activity.

To evaluate this idea, we made tandem repeats of C-s3-33, and compared their E2 protein-binding activities and anti-HCV activities with those of the C-s3-33. Here, we report our findings that the anti-HCV activity of the tandem repeats were stronger than that of the monomer when accompanied by the enhancement of the E2 protein-binding activity, by analyses using not only the HCV infection system but also the infection system of a VSV pseudotype bearing the native forms of HCV E1 and E2 proteins.

Materials and Methods

Cell cultures. Simian virus 40 large T antigen-immortalized non-neoplastic human PH5CH8 hepatocytes were maintained as described previously (11, 24). Human hepatoblastoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Construction of expression plasmids for E. coli. The pMAL-c2X (hLF600–632) (25) expression plasmid for the MBP-fused C-s3-33 LF fragment, was used as a template for the PCR using a primer set of hLFB6 5'-TGATAGGATCCGTGGTGTCTCGGATGATAAGG-3' containing the *Bam*HI recognition site (underlined) (25) and 632R6A 5'-ATCCATCCGAGACACCA-CAAACTTGTCCGGGCAGTCAGATCC-3' containing an extra 18 nts (underlined) encoding the amino-terminal 6 aa of the C-s3-33 LF fragment. After PCR (20 cycles) using KOD-plus DNA polymerase (Toyobo, Osaka, Japan), the amplified PCR product was used as a template for a second PCR using the primer set of hLFB6 and 632R 5'-TAATAAAGCTTT-TAAAACTTGTCCGGGCAGTCAGATCC-3' containing the *Hin*dIII recognition site (underlined) (25). After PCR (35 cycles) using KOD-plus DNA polymerase, amplified PCR products (approximately 200 bp for the two-repeat form and approximately 300 bp for the three-repeat form) were subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and were used as expression plasmids for the production of the MBP-fused (C-s3-33)$_2$ and (C-s3-33)$_3$.

To prepare an expression plasmid for the production of the MBP-fused C-s3-33-relevant fragment (aa 587–619) of human TF, pCXbsr/huTF (29) encoding full-length human TF was used as a template for the PCR using a primer set of hTF587F 5'-TGATAG-

GATCCGTGGTCACACGG-3' containing the *Bam*HI recognition site (underlined) and hTF619R 5'-TAATAAAGCTTTTAAAAGTTGCCCG-3' containing the *Hin*dIII recognition site (underlined). After PCR (35 cycles) using KOD-plus DNA polymerase, the amplified PCR product was subcloned into the *Bam*HI and *Hin*dIII sites of pMAL-c2X, and was used as the expression plasmid.

Expression and purification of the MBP-fused protein. Expression and purification of the MBP-fused LF fragment [C-s3-33, $(C\text{-}s3\text{-}33)_2$, or $(C\text{-}s3\text{-}33)_3$] or the MBP-fused C-s3-33-relevant fragment of human TF were carried out as described previously (25). Briefly, the expression plasmid for MBP-fused protein was transformed into the *E. coli* strain JM109. The transformants were cultured at 37 C for several hours, and the harvested cells were sonicated. After removal of insoluble cellular debris by centrifugation, the supernatant obtained as the soluble fraction was applied onto an amylose resin affinity column (New England Biolabs) to obtain the MBP-fused protein. The purity of the obtained MBP-fused protein was evaluated to be more than 95% by electrophoresis on 10% SDS-PAGE gels. The concentration of the purified MBP-fused protein was determined by using Coomassie protein assay reagent (Pierce). The MBP2 (43 kDa) produced from the pMAL-c2X with a stop codon inserted into the *Xmn*I site was used as a control protein.

Far-Western blot analysis. Far-Western blot analysis was carried out as described previously (25). Briefly, 0.5 μg of human LF, MBP2, and MBP-fused LF fragments were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with N-buffer (25), a binding reaction was carried out using the secreted form of the E2 protein (E2-681) consisting of aa 384–681 expressed in Chinese hamster ovary cells as a probe (14), and then rat monoclonal antibody, MO-12 (13), against E2 protein was used for the detection of E2 protein-bound MBP-fused LF fragments.

Assay for anti-HCV activity of MBP-fused protein. An assay for anti-HCV activity of the MBP-fused LF fragment was carried out by the method described previously (25). Briefly, 2 μl (2×10^4 HCV) of the HCV-positive serum HCV-O (previously described as 1B-2 (19)) (genotype 1b) and the MBP-fused LF fragment (final concentration, 0.5, 1.0, and 2.0 mg/ml) were pre-incubated for 60 min at 4 C and then inoculated onto to the PH5CH8 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed three times with PBS and further cultured for 1 day at 32 C. Cellular RNA (0.5 μg) prepared by ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan) was used for the quantitative analysis of HCV RNA using LightCycler PCR as described previously (26). As the positive and negative controls for anti-HCV activity, human LF and MBP2, respectively, were used.

Assay for anti-VSV pseudotype activity of MBP-fused protein. For this assay, the VSV pseudotype VSVΔG*(HCV), bearing the native forms of HCV E1 and E2 proteins from the O strain (19), was used. VSVΔG*(HCV) was prepared by introducing the native form of E1 and E2 proteins into recombinant VSV, VSVΔG*, which harbors the green fluorescent protein (GFP) gene instead of the VSV G envelope protein gene (30). An assay for the anti-VSV pseudotype activity of the MBP-fused LF fragment was carried out by a method described previously (30). Briefly, VSVΔG*(HCV) (Approximately 100 IU/assay) was pre-incubated with the MBP-fused LF fragment (final concentration, 0.1–1.0 mg/ml) at 37 C for 60 min and inoculated onto PH5CH8 or HepG2 cells (1.5×10^4 cells were cultured for 2 days before viral inoculation on a 96-well plate). After incubation of the cells for 90 min at 37 C, the cells were washed with DMEM three times and incubated with fresh culture medium. VSVΔG*G was used as a control in this assay. After 24 hr of incubation, each infectious titer was determined by counting the number of GFP-expressing cells under a fluorescence microscope. As the positive and negative controls for the assay, human LF and MBP2 were used, respectively. Human TF and an MBP-fused C-s3-33-relevant fragment of human TF were also used for the assay.

Results

Two and Three Repeats of the Human LF Fragment (C-s3-33) Strengthened the E2 Protein-Binding Activity

Previously we found that bovine and human LFs prevented HCV infection in PH5CH8 cells via direct interaction between LF and HCV (10, 12), and we further identified 33 aa residues (C-s3-33; aa 600–632 of human LF) as an essential and minimum domain possessing binding activity for the HCV E2 protein (secreted form consisting of aa 384–681) and inhibiting activity against HCV infection (25). This result suggested that the E2 protein-binding activity contributes to the anti-HCV activity. However, the E2 protein-binding activity of C-s3-33 was somewhat weaker than that of human LF (25), and the anti-HCV activity of C-s3-33 (IC_{50}=20 μM) in the infection system using PH5CH8 cells was also weaker than that of human LF (IC_{50}=5 μM) (25). To improve these points, we first tried to