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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.

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.

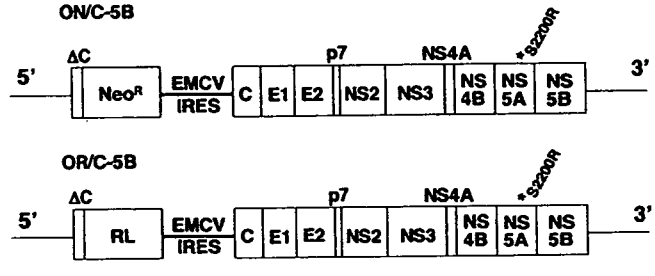

Fig. 1. Schematic gene organization of genome-length HCV RNAs used in this study. Open reading frames, untranslated regions, and EMCV IRES are depicted as open boxes, thin lines, and thick lines, respectively. ΔC indicates the 12N-terminal aa residues of the core as a part of IRES. ON/C-5B RNA and OR/C-5B 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).

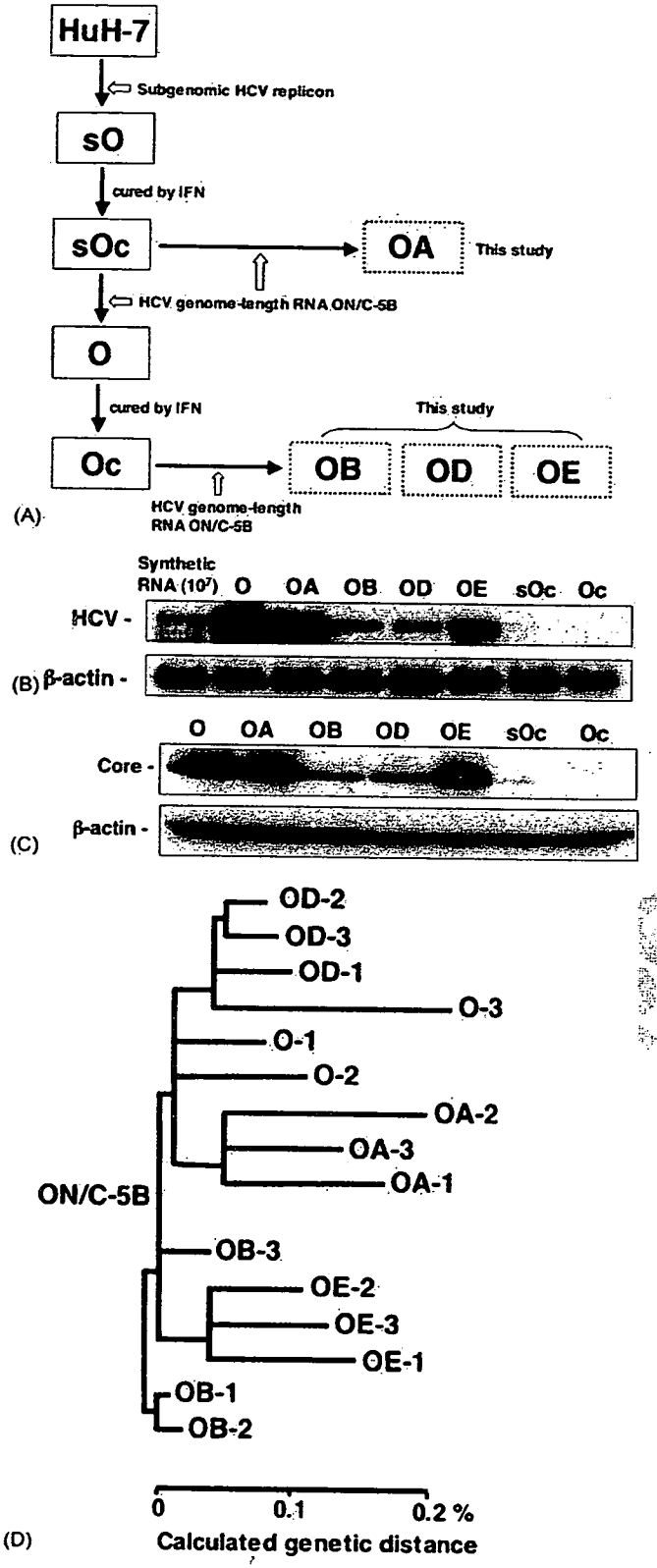

Fig. 2. Characterization of genome-length HCV RNA-replicating cell lines. (A) Lineage of genome-length HCV RNA-replicating cells. The sO and O cell lines were previously established (Ikeda et al., 2005; Kato and Sugiyama et al., 2003). (B) Northern blot analysis. Total RNAs from genome-length HCV RNA-replicating 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 *XbaI* 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.

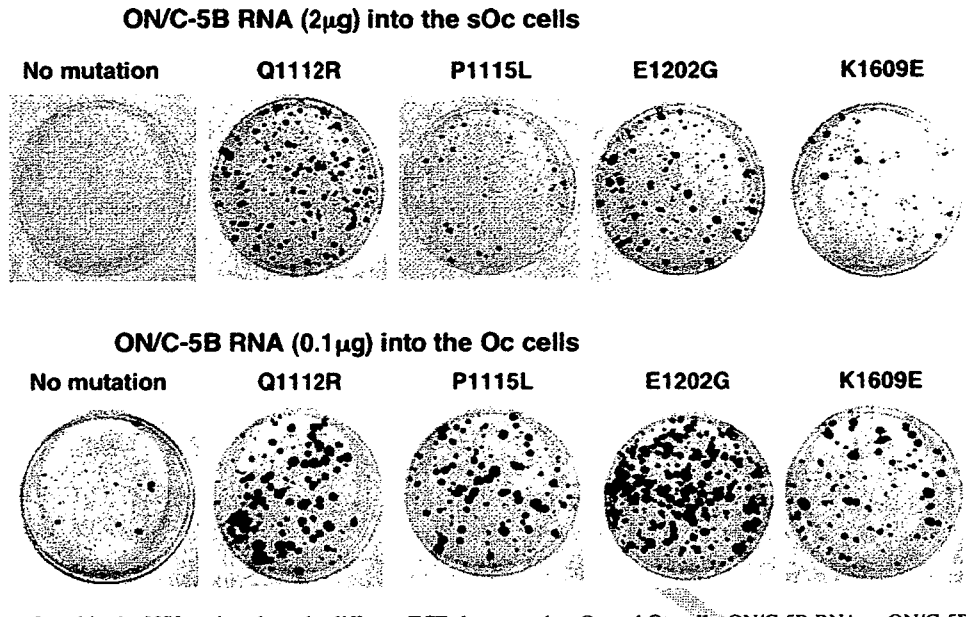

Fig. 3. Adaptive mutations found in the NS3 region show the different ECFs between the sOc and Oc cells. ON/C-5B RNA or ON/C-5B RNA with an additional NS3 mutation was transfected into the sOc cells (2 μg RNA per 10 cm dish; top panel) and the Oc cells (0.1 μg RNA per 10 cm dish; bottom panel). The panels 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

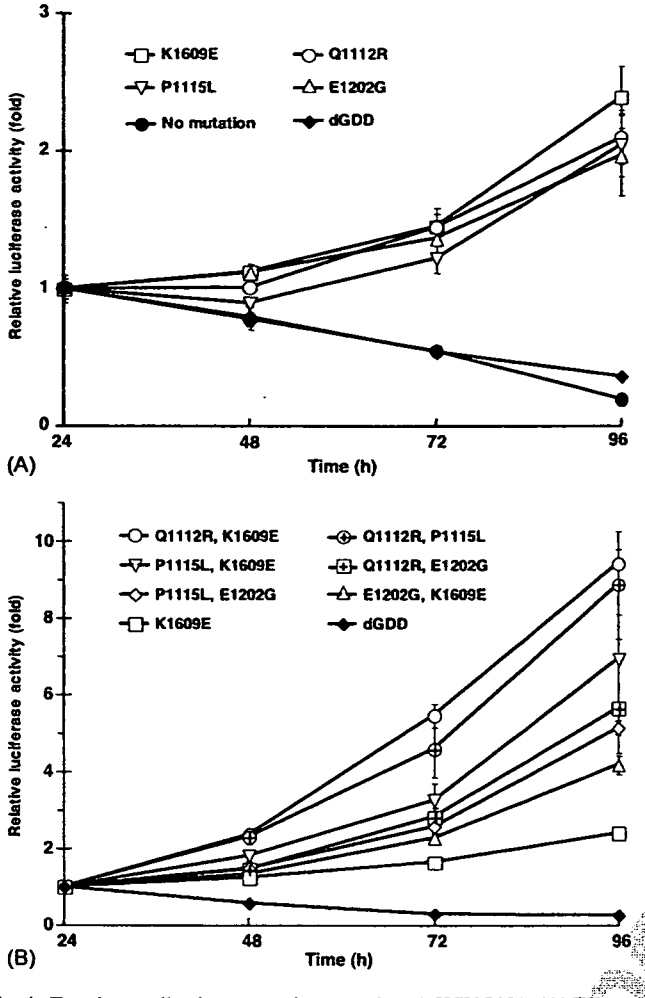

Fig. 4. Transient replication assay of genome-length HCV RNA. (A) Effects of adaptive NS3 mutations on transient replication of genome-length HCV RNA. OR/C-5B RNA or OR/C-5B RNA with an additional mutation was transfected into the Oc cells, and luciferase activity was determined in cell lysates that 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

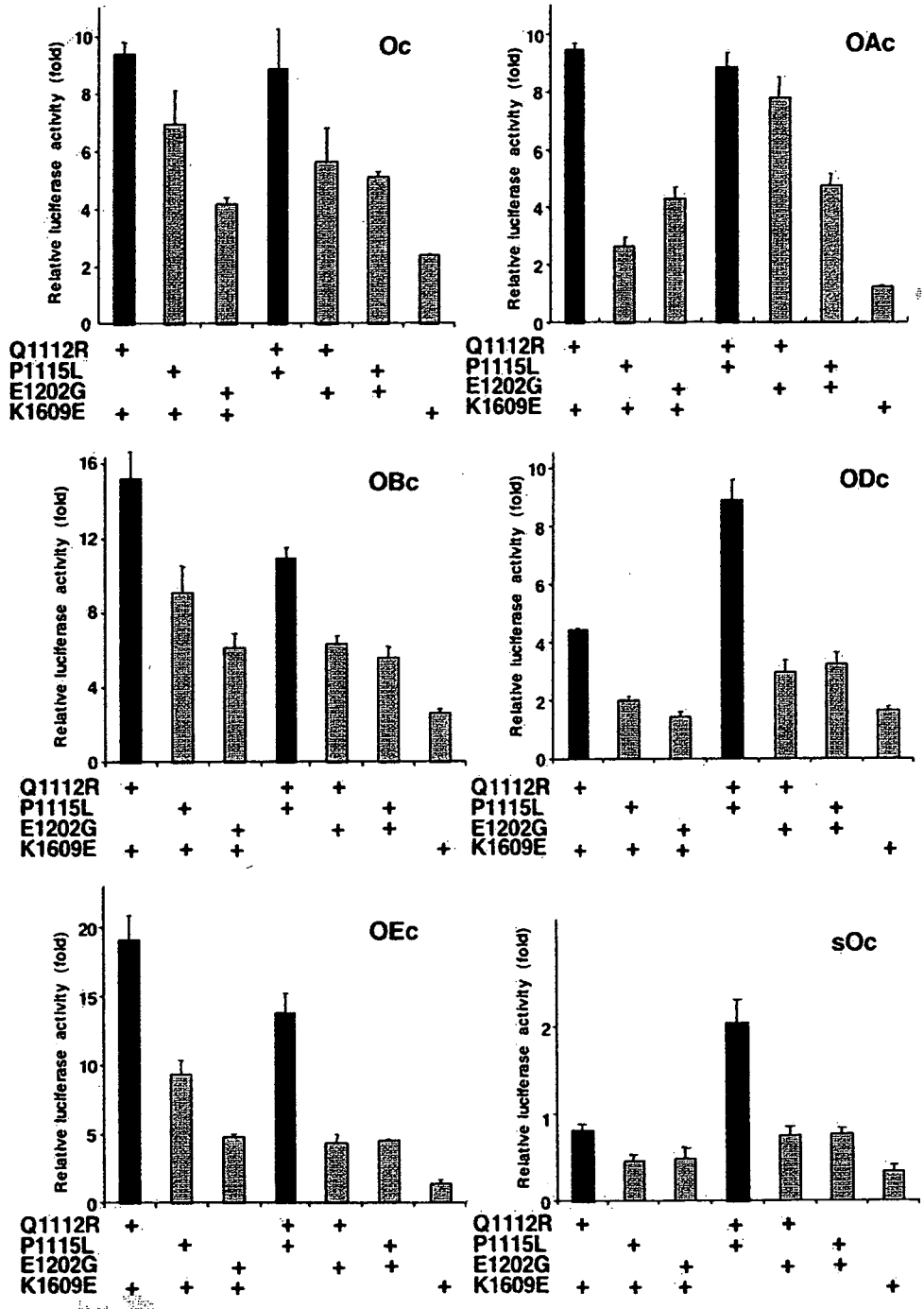

Fig. 5. Effects of combining adaptive NS3 mutations on transient genome-length HCV RNA replication in various cured cells. OR/C-5B RNAs with various combinations of adaptive NS3 mutations were transfected into various cured cells (Oc, OAc, OBc, ODc, OEc, and sOc cells), and luciferase activity was determined 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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JOURNAL OF VIROLOGY, Dec. 2007, p. 13922–13926 Vol. 81, No. 24
0022-538X/07/$08.00+0 doi:10.1128/JVI.01517-07

DDX3 DEAD-Box RNA Helicase Is Required for Hepatitis C Virus RNA Replication▽

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Received 11 July 2007/Accepted 5 September 2007

DDX3, a DEAD-box RNA helicase, binds to the hepatitis C virus (HCV) core protein. However, the role(s) of DDX3 in HCV replication is still not understood. Here we demonstrate that the accumulation of both genome-length HCV RNA (HCV-O, genotype 1b) and its replicon RNA were significantly suppressed in HuH-7-derived cells expressing short hairpin RNA targeted to DDX3 by lentivirus vector transduction. As well, RNA replication of JFH1 (genotype 2a) and release of the core into the culture supernatants were suppressed in DDX3 knockdown cells after inoculation of the cell culture-generated HCVcc. Thus, DDX3 is required for HCV RNA replication.

DEAD-box RNA helicases are involved in various RNA metabolic processes, including transcription, translation, RNA splicing, RNA transport, and RNA degradation (9, 20). DDX1 and DDX3, DEAD-box RNA helicases, have been implicated in the replication of human immunodeficiency virus type 1 (HIV-1). Both DDX1 and DDX3 interact with HIV-1 Rev and enhance Rev-dependent HIV-1 RNA nuclear export (10, 24).

On the other hand, DDX3 binds to the hepatitis C virus (HCV) core protein (17, 19, 25), and DDX3 expression is deregulated in HCV-associated hepatocellular carcinoma (HCC) (7, 8). However, the biological function of DDX3 in HCV replication is still not understood. To address this issue, we first used lentivirus vector-mediated RNA interference to stably knock down DDX3 in three HuH-7-derived cell lines: O cells, harboring a replicative genome-length HCV RNA (HCV-O, genotype 1b) (13); sO cells, harboring its subgenomic replicon of HCV RNA (14); or RSc cured cells, which cell culture-generated HCV (HCVcc) (JFH1, genotype 2a) (23) could infect and effectively replicate in (M. Ikeda et al., unpublished data). Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences against DDX3 in the lentivirus vector: for DDX3i#3, 5′-GATCCCCGGAGGAAATTATAACTCCCTTCAAGAGAGGGAGTTATAATTTCCTCCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAGGAGGAAATTATAACTCCCTCTCTTGAAGGGAGTTATAATTTCCTCCGGG-3′ (antisense); for DDX3i#7, 5′-GATCCCCGGTCACCCTGCCAAACAAGTTCAAGAGACTTGTTTGGCAGGGTGACCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAGGTCACCCTGCCAAACAAGTCTCTTGAACTTGTTTGGCAGGGTGACCGGG-3′ (antisense). These oligonucleotides were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (6). To construct pLV-DDX3i#3 and pLV-DDX3i#7, the BamHI-SalI fragments of the corresponding pSUPER plasmids were subcloned into the BamHI-SalI site of pRDI292 (5), an HIV-1-derived self-inactivating lentivirus vector containing a puromycin resistance marker allowing for the selection of transduced cells. The vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector system has been described previously (18). We used the second-generation packaging construct pCMV-ΔR8.91 (26) and the VSV-G-envelope plasmid pMDG2. The lentivirus vector particles were produced by transient transfection of 293FT cells with FuGene 6 (Roche).

Western blot analysis of the lysates demonstrated the only trace of DDX3 protein in DDX3 knockdown O cells (DDX3i#3) (Fig. 1A). In this context, the HCV core expression level was significantly decreased in the DDX3 knockdown O cells (Fig. 1A). To further confirm this finding, we examined the level of HCV RNA in these cells. We found that accumulation of genome-length HCV-O RNA was notably suppressed in DDX3 knockdown O cells (Fig. 1B). Furthermore, the efficiency of colony formation in DDX3 knockdown Oc cells (created by eliminating genome-length HCV RNA from O cells by interferon treatment) transfected with the genome-length HCV-O RNA with an adapted mutation at amino acid (aa) position 1609 in the NS3 helicase region (K1609E) (13) was also notably reduced compared with that in control cells (Fig. 1C). In contrast, highly efficient knockdown of an unrelated host factor, poly(ADP-ribose) polymerase 1 (PARP-1) (4), had no observable effects on HCV RNA replication, the efficiency of colony formation, or the core expression level (data not shown), suggesting that our finding was not due to a nonspecific event. Interestingly, accumulation of the subgenomic replicon RNA (HCV-sO) was also suppressed in DDX3 knockdown sO cells (Fig. 1D). Moreover, we examined the potential role of DDX3 in an HCV infection and produc-

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▽ Published ahead of print on 12 September 2007.

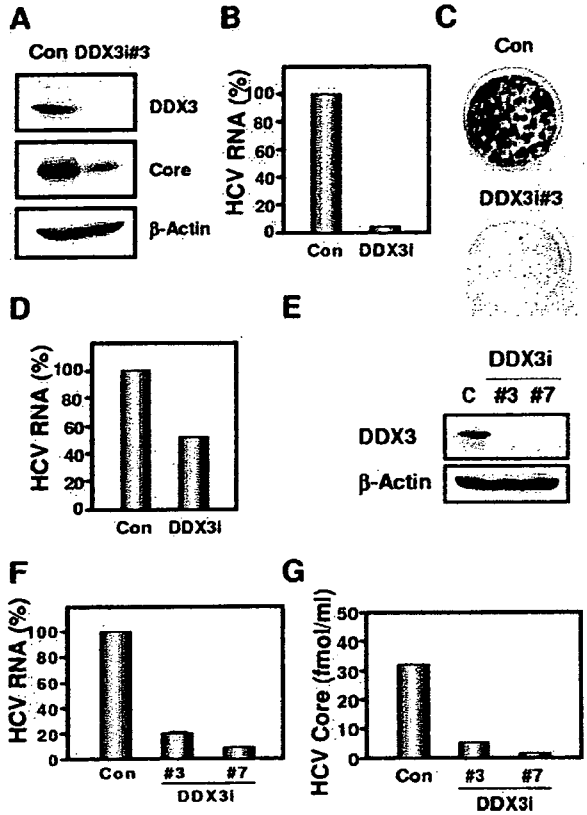

FIG. 1. Requirement of DDX3 for HCV replication. (A to D) Effect of DDX3 knockdown on HCV RNA replication. (A) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 (ProSci), anti-HCV core (CP-9; Institute of Immunology), or an anti-β-actin antibody (Sigma) in O cells expressing shRNA against DDX3 (DDX3i#3) as well as in O cells transduced with a control lentivirus vector (Con) are shown. (B) The level of genome-length HCV RNA was monitored by real-time LightCycler PCR (Roche). Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (C) Efficiency of colony formation in DDX3 knockdown cells. In vitro-transcribed ON/C-5B K1609E RNA (2 μg) was transfected into the DDX3 knockdown Oc cells (DDX3i#3) or the Oc cells transduced with a control lentivirus vector (Con). G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA. Experiments were done in duplicate, and representative results are shown. (D) The level of subgenomic replicon RNA was monitored by real-time LightCycler PCR. Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (E to G) Effect of DDX3 knockdown on HCV infection. (E) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 or an anti-β-actin antibody for RSc cells expressing the shRNA DDX3i#3 or DDX3i#7 and for RSc cells transduced with a control lentivirus vector (Con) are shown. (F) The level of genome-length HCV (JFH1) RNA was monitored by real-time LightCycler PCR after inoculation of the cell culture-generated HCVcc. Results from three independent experiments are shown. (G) The levels of the HCV core in the culture supernatants were determined by an enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories). Experiments were done in duplicate, and bars represent the mean HCV core protein levels.

tion system (23). We found 80 to 90% reductions in the accumulation of JFH1 RNA and 82 to 94% reductions in the release of the core into the culture supernatants in DDX3 knockdown HuH-7-derived RSc cured cells at 4 days after inoculation of HCVcc (Fig. 1E to G). Thus, DDX3 seems to be required for HCV RNA replication.

Previously, DDX3 was identified as an HCV core-interacting protein by yeast two-hybrid screening. This interaction required the N-terminal domain of the core (aa 1 to 59) and the C-terminal domain of DDX3 (aa 553 to 622) (17, 19, 25). To determine whether the core can interact with DDX3 regardless of the HCV genotype, we used the HCV-O core (genotype 1b) and the JFH1 core (genotype 2a) (Table 1). We first examined their subcellular localization by confocal laser scanning microscopy as previously described (3). Consistent with previous reports (17, 19, 25), both the HCV-O core and JFH1 core mostly colocalized with DDX3 in the perinuclear region (Fig. 2A). Then we immunoprecipitated lysates from 293FT cells in which hemagglutinin (HA)-tagged DDX3 and HCV-O core, JFH1 core, or their 40-aa N-truncated mutants were overexpressed with an anti-HA antibody. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.5% NP-40, 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared with 30 μl of protein G-Sepharose (GE Healthcare Bio-Sciences). Precleared supernatants were incubated with 1 μg of anti-HA antibody (3F10; Roche) at 4°C for 1 h. Following absorption of the precipitates

TABLE 1. Primers used for construction of the HCV core-expressing plasmids[a]

Plasmid name	Direction	Primer sequence
pCXbsr/core(HCV-O)	Forward	5′-GGAATTCCCACCATGAG CACGAATCCTAAACCTC-3
	Reverse	5′-ATAAGAATGCGGCCGCC TATCAAGCGGAAGCTGG GATGGT-3′
pcDNA3/core(HCV-O)	Forward	5′-CGGGATCCAAGATGAGC ACGAATCCTAAACCTCAA AGA-3′
pcDNA3/FLAG-core(HCV-O)	Reverse	5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′
pcDNA3/Δcore(HCV-O)	Forward	5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′
pcDNA3/FLAG-Δcore(HCV-O)	Reverse	5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′
pcDNA3/core(JFH1)	Forward	5′-CGGGATCCAAGATGAGC ACAAATCCTAAACCTCAA AGA-3′
pcDNA3/FLAG-core(JFH1)	Reverse	5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′
pcDNA3/Δcore(JFH1)	Forward	5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′
pcDNA3/FLAG-Δcore(JFH1)	Reverse	5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′

[a] To construct pCXbsr/core(HCV-O), a DNA fragment encoding the core was amplified by PCR from pON/C-5B (13) with the indicated primers. The PCR product was digested with EcoRI-NotI and subcloned into the same site of pCX4bsr (1). To construct pcDNA3/core(HCV-O), pcDNA3/FLAG-core(HCV-O), pcDNA3/Δcore(HCV-O), and pcDNA3/FLAG-Δcore(HCV-O), DNA fragments encoding the core were amplified by PCR from pON/C-5B (13) with the indicated primer sets. To construct pcDNA3/core(JFH1), pcDNA3/FLAG-core(JFH1), pcDNA3/Δcore(JFH1), and pcDNA3/FLAG-Δcore(JFH1), DNA fragments encoding the core were amplified by PCR from pJFH1 (23) with the indicated primer sets. The PCR products were digested with BamHI and XhoI and then subcloned into the same site of pcDNA3 (Invitrogen) or pcDNA3-FLAG (2).

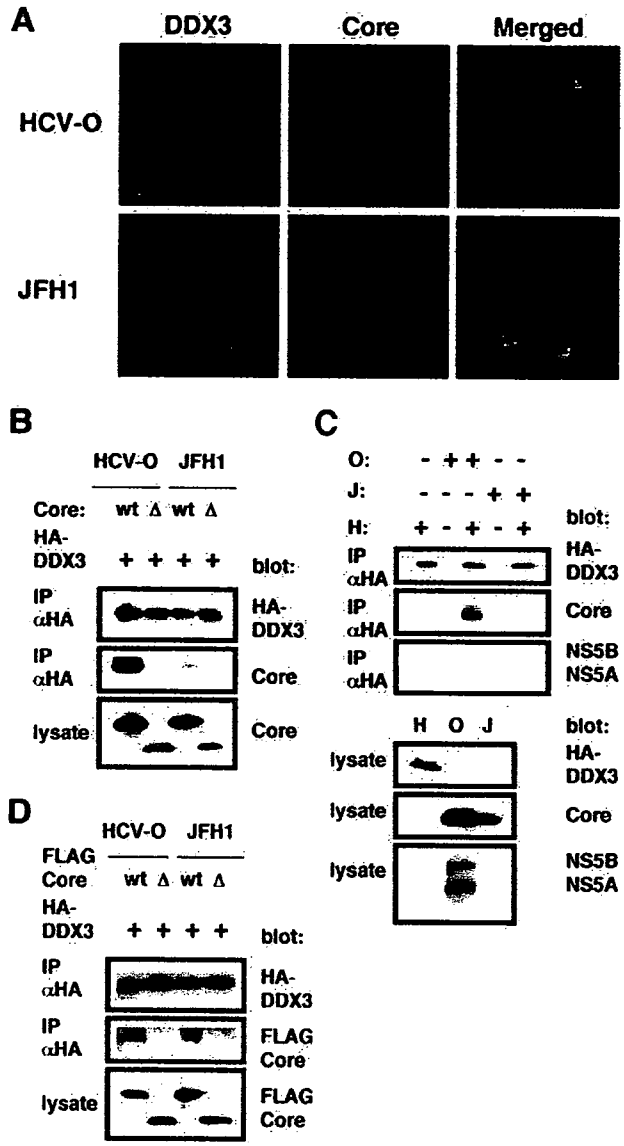

FIG. 2. Interaction of the HCV core with DDX3. (A) The HCV core colocalizes with DDX3. 293FT cells cotransfected with 100 ng of pCXbsr/core(HCV-O) or pcDNA3/core(JFH1) and 200 ng of pHA-DDX3 were examined by confocal laser scanning microscopy. Cells were stained with anti-HCV core (CP-9 and CP-11 mixture) and anti-DDX3 antibodies and were then visualized with fluorescein isothiocyanate (DDX3) or Cy3 (core). Images were visualized using confocal laser scanning microscopy (LSM510; Carl Zeiss). The right panels exhibit the two-color overlay images (Merged). Colocalization is shown in yellow. (B) The core binds to DDX3. 293FT cells were cotransfected with 4 μg of pHA-DDX3 and 4 μg of pCXbsr/core(HCV-O) (wt), pcDNA3/Δcore(HCV-O) (Δ), pcDNA3/core(JFH1) (wt), or pcDNA3/Δcore(JFH1) (Δ). The cell lysates were immunoprecipitated with an anti-HA antibody (3F10; Roche), followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). (C) 293FT cells transfected with 4 μg of pHA-DDX3 (H), O cells (O), or RSc cells 3 days after inoculation of HCVcc (JFH1) (J) were lysed and immunoprecipitated (IP) with 1 μg of anti-HA antibody (3F10), followed by immunoblotting with anti-HA (HA-7), anti-core (CP-9 and CP-11 mixture), or anti-HCV NS5A (no. 8926) and anti-HCV NS5B. (D) 293FT cells transfected with 4 μg of pHA-DDX3 and 4 μg of pcDNA3/FLAG-core(HCV-O) (wt), pcDNA3/FLAG-Δcore(HCV-O) (Δ), pcDNA3/FLAG-core(JFH1) (wt), or pcDNA3/FLAG-Δcore(JFH1) (Δ) were lysed and immunoprecipitated with 1 μg of an anti-HA antibody (3F10), followed by immunoblotting with an anti-HA (HA-7) or anti-core (CP-9 and CP-11 mixture) antibody.

on 30 μl of protein G-Sepharose resin for 1 h, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 1× Laemmli sample buffer. The proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). We observed that the HCV-O core but not its N-truncated mutant could strongly bind to HA-tagged DDX3 (Fig. 2B). In contrast, the binding activity of the JFH1 core to HA-tagged DDX3 seemed to be fairly weak (Fig. 2B). As well, immunoprecipitation of lysates of 293FT cells expressing HA-tagged DDX3, O cells, or JFH1-infected RSc cells, or mixtures of these lysates, with an anti-HA antibody revealed that HCV-O core but not JFH1 core could bind strongly to DDX3 (Fig. 2C). The anti-HCV core antibody we used could detect both HCV-O core and JFH1 core (Fig. 2), while both anti-HCV NS5A and anti-NS5B antibodies failed to detect JFH1 NS5A and NS5B (Fig. 2C). At least, we failed to detect an interaction between DDX3 and either HCV-O NS5A or NS5B under experimental conditions that permitted the core to interact with DDX3 by immunoprecipitation (Fig. 2C). In contrast, the FLAG-tagged JFH1 core could bind to HA-tagged DDX3 just as efficiently as the FLAG-tagged HCV-O core could (Fig. 2D). Thus, the binding affinity or stability of the complex formed between the JFH1 core and DDX3 might be weaker than that between the HCV-O core and DDX3.

Since DDX3 is required for HIV-1 and HCV replication, we hypothesized that the HCV core might affect the function of HIV-1 Rev when both proteins were coexpressed. To test this hypothesis, we used the Rev-dependent luciferase-based reporter plasmid pDM628, harboring a single intron that includes both the Rev-responsive element (RRE) and the luciferase coding sequences (Fig. 3A) (10). In this system, Rev binds to RRE on the unspliced reporter mRNA, allowing its export from the nucleus for luciferase reporter gene expression, while the intron containing the luciferase gene is excised during RNA splicing when cells are transiently transfected with pDM628 alone. As previously reported (10), the luciferase activity in 293FT cells transfected with this reporter plasmid was stimulated by Rev, which induced a fourfold increase in the reporter signal (Fig. 3B). Luciferase activity was increased eightfold by the combination of Rev and DDX3, whereas neither the HCV-O core nor the JFH1 core had any effect on this Rev function (Fig. 3B). Since the Rev-binding domain (the N-terminal domain) and the core-binding domain (the C-terminal domain) do not overlap in DDX3, the HCV core might not compete with HIV-1 Rev for binding with DDX3. However, the development of a novel DDX3 inhibitor might provide a powerful antiviral agent against both HIV-1 and HCV (15).

Taking these results together, this study has shown for the first time that DDX3 is required for HCV RNA replication.

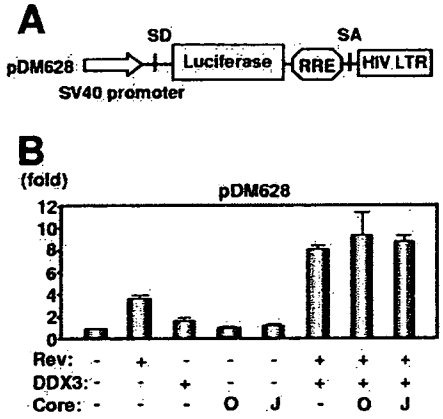

FIG. 3. HCV core does not affect the DDX3-mediated synergistic activation of Rev function. (A) Schematic representation of HIV-1 Rev-dependent luciferase-based reporter plasmid pDM628 harboring a splicing donor (SD), splicing acceptor (SA), and RRE. (B) 293FT cells were cotransfected with 100 ng of pDM628, 200 ng of pcRev, 200 ng of pHA-DDX3, and/or 100 ng of either pcDNA3/core(HCV-O) (O) or pcDNA3/core(JFH1) (J). A luciferase assay was performed 24 h later. All transfections utilized equal total amounts of plasmid DNA owing to the addition of the empty vector pcDNA3 to the transfection mixture. The relative stimulation of luciferase activity (*n*-fold) is shown. The results shown are means from three independent experiments.

Since helicases are motor enzymes that use energy derived from nucleoside triphosphate hydrolysis to unwind double-stranded nucleic acids, the DDX3–core complex might unwind the HCV double-stranded RNA and separate the RNA strands or might contribute to the function of HCV NS3 helicase. Since the replication of subgenomic replicon RNA was also partially suppressed in DDX3 knockdown cells (Fig. 1D), DDX3 might be associated with an HCV nonstructural protein(s) or HCV RNA itself. Indeed, Tingting et al. recently reported that DDX1 bound to both the HCV 3′ untranslated region (3′ UTR) and the HCV 5′ UTR and that short interfering RNA-mediated knockdown of DDX1 caused a marked reduction in the replication of subgenomic replicon RNA (22). Furthermore, Goh et al. demonstrated that DDX5/p68 associated with HCV NS5B and that depletion of endogenous DDX5 correlated with a reduction in the transcription of negative-strand HCV RNA (11). However, we failed to observe an interaction between DDX3 and NS5A or NS5B by immunoprecipitation under our experimental conditions in which the core could interact with DDX3 (Fig. 2C). Importantly, our DDX3 knockdown study demonstrated a more significant reduction in the accumulation of genome-length HCV RNA (95% reduction) than in the accumulation of subgenomic replicon RNA (52% reduction) (Fig. 1B and D). To date, it has been demonstrated that the 5′ UTR, the 3′ UTR, and the NS3-to-NS5B coding region are sufficient for HCV RNA replication (16); however, the core might be partly involved in the replication of genome-length HCV RNA. Importantly, DDX1 and DDX3 were specifically detected in the lipid droplets of core-expressing Hep39 cells by proteomic analysis (21), suggesting that DDX3 might be associated with HCV assembly or might incorporate into the HCV virion through interaction with the core to act as an RNA chaperone.

Recent studies have suggested a potential role of DDX3 and DDX5 in the pathogenesis of HCV-related liver diseases. DDX3 expression is deregulated in HCV-associated HCC (7, 8), and Huang et al. identified single-nucleotide polymorphisms in the DDX5 gene that were significantly associated with an increased risk of advanced fibrosis in patients with chronic hepatitis C (12). Interestingly, DDX3 might be a candidate tumor suppressor. DDX3 inhibits colony formation in various cell lines, including HuH-7, and up-regulates the $p21^{waf1/cip1}$ promoter (8). If DDX3 could in fact suppress tumor growth, then the core might overcome DDX3-mediated cell growth arrest and down-regulate $p21^{waf1/cip1}$ through interaction with DDX3, and it might also be involved in HCC development.

We thank D. Trono, K.-T. Jeang, V. Yedavalli, R. J. Pomerantz, J. Fang, R. Iggo, M. Hijikata, T. Akagi, and M. Kohara for pCMVΔR8.91, pMDG2, pHA-DDX3, pDM628, pcRev, pSUPER, pRDI292, 293FT cells, pCX4bsr, and the anti-NS5B antibody. We also thank A. Morishita and T. Nakamura for technical assistance.

This work was supported by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), by a Grant-in-Aid for Research on Hepatitis from the Ministry of Health, Labor, and Welfare of Japan, by the Naito Foundation, by the Ichiro Kanehara foundation, and by a research fellowship from the Japan Society for the Promotion of Science (JSPS).

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Modulation of host metabolism as a target of new antivirals ☆

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Received 28 March 2007; accepted 30 March 2007
Available online 11 August 2007

Abstract

The therapy for chronic hepatitis C (CH–C) started with interferon (IFN) monotherapy in the early 1990s and this therapy was considered effective in about 10% of cases. The present standard therapy of pegylated IFN with ribavirin achieves a sustained virologic response in about 50% of patients. However, about half of the CH–C patients are still at risk of fatal liver cirrhosis and hepatocellular carcinoma. The other significant event in hepatitis C virus (HCV) research has been the development of a cell culture system. The subgenomic replicon system enables robust HCV RNA replication in hepatoma cells. And recently, the complete life cycle of HCV has been achieved using a genotype 2a strain, JFH1. These hallmarks have provided much information about the mechanisms of HCV replication, including information on the host molecules required for the replication. Anti-HCV reagents targeting HCV proteins have been developed, and some of them are now in clinical trials. However, the RNA-dependent RNA polymerase frequently causes mutations in the HCV genome, which lead to the emergence of drug-resistant HCV mutants. Some of the cellular proteins essential for HCV RNA replication have already been discovered using the HCV cell culture system. These host molecules are also candidate targets for antivirals. Here, we describe the recent progress regarding the anti-HCV reagents targeting host metabolism.

Keywords: Hepatitis C virus; Replicon; Antiviral; Interferon; Host metabolism; Statin

Contents

Abbreviations: HCV, hepatitis C virus; CH, chronic hepatitis; HCC, hepatocellular carcinoma; IFN, interferon; SVR, sustained virological response; PEG-IFN, pegylated-IFN; GBV-B, GB virus B; uPA-SCID, urokinase plasminogen activator-severe combined immunodeficiency; NS, nonstructural; RdRp, RNA dependent RNA polymerase; CyPB, cyclophilin B; CsA, cyclosporine A; HSP90, heat shock protein 90; La, La auto antigen; PTB, polypyrimidine tract-binding protein; ALT, alanine aminotransferase; Neo, neomycin phosphotransferase; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; ORF, open reading frame; FKBP8, FK-506-binding protein 8; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; FTase, farnesyltransferase; GGTase-I, geranylgeranyltransferase type I; GGTI, GGTase-I inhibitor; HMG–CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LOV, lovastatin; ATV, atorvastatin; FLV, fluvastatin; PRV, pravastatin; SMV, simvastatin; EC_{50}, 50%; effective concentration to inhibit HCV RNA replication; PTV, pitavastatin; RSV, respiratory syncytial virus; CMV, cytomegarovirus; HIV, human immunodeficiency virus; ICAM-1, integrin intercellular adhesion molecule 1; LFA-1, lymphocyte function associated antigen-1; DRM, detergent resistant membrane; SPT, serine palmitoyltransferase; ER, endoplasmic reticulum; GSL, glycosphingolipid; SBD, sphingolipid-binding domain; IMPDH, inosine monophosphate dehydrogenase; XMP, xanthosine 5′ ; monophosphate; MPA, mycophenolic acid; RMP, ribavirin monophosphate; RDP, ribavirin diphosphate; RTP, ribavirin triphosphate; GTP, guanosine triphosphate; SARS, severe acute respiratory syndrome; HBV, hepatitis B virus; VLP, virus-like particle; PIAS1, protein inhibitor of activated STAT1; PRMT1, protein arginine methyltransferase 1; PP2Ac, catalytic subunit of protein phosphatase 2A; AdoMet, S-adenosyl-L-methionine; PUFAs, polyunsaturated fatty acids; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid.

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Toward Evidence Based Control of Hepatitis C Virus Infection".

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doi:10.1016/j.addr.2007.03.021

1. Introduction

Hepatitis C virus (HCV) was discovered in 1989 [1] as the causative agent of chronic hepatitis C (CH–C), liver cirrhosis and hepatocellular carcinoma (HCC) [2]. It is estimated that 170 million people worldwide are infected with HCV [3]. The ultimate goal of both clinical and basic HCV studies is the suppression of liver-related death caused by HCV infection. With respect to clinical studies, interferon (IFN) has played a major role in the treatment of patients with CH–C. IFN therapy started with IFN monotherapy in the early 1990s, and a sustained virologic response (SVR) was obtained in about 10% of patients [4]. IFN therapy was developed by the hepatologists, and the current therapy of pegylated IFN (PEG-IFN) with ribavirin has improved the SVR to about 50% [4]. Therefore, the next stage of the therapy for CH–C is to develop new anti-HCV reagents to improve the SVR.

During the development of IFN therapy, the most striking discovery in the basic research was the development of a cell culture system for robust HCV RNA replication. In 1999, Lohmann et al. [5] achieved subgenomic HCV RNA replication in a human hepatoma cell line, HuH-7. The advantages of this novel system (known as the replicon system) were that it provided not only a way to screen for anti-HCV reagents but also information about the mechanism of HCV RNA replication. This cell culture system has been further improved, and recently the complete life cycle of HCV was achieved using a genotype 2a HCV strain, JFH1 [6–8]. This newest system has extended the targets of the anti-HCV therapy to the virus infection and release.

The effects of anti-HCV reagents selected from the cell culture-based screening should be evaluated using an animal model system for HCV infection before they can be released to clinical trial. Chimpanzees were the only animal model in the early HCV studies [9]. However, the use of chimpanzees is limited for ethical and financial reasons. In addition to chimpanzees, a study using tree shrews (*Tupaia belangeri chinensis*) has been reported [10]. A different approach to the study of HCV using animal models was achieved using the related GB virus B (GBV-B). GBV-B belongs to the *Flaviviridae* family and can be transmitted to tamarins and marmosets [11,12]. These animal models may be valuable surrogate models for HCV study. Another approach was demonstrated in a study using urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice transplanted with human hepatocytes [13]. This chimeric mouse model can support chronic HCV viremia under the circumstance without immune system. Mass screening for anti-HCV reagents using cell culture systems will become a more powerful tool when combined with small animal model systems to evaluate the antiviral effects of selected reagents before clinical trial.

In considering a new strategy for CH–C to be used in place of or in combination with IFN, the main targets are HCV proteins and HCV RNA. With respect to the HCV proteins, two of these, nonstructural (NS) 3-4A and NS5B, have been well-characterized as protease and RNA-dependent RNA polymerase (RdRp), respectively [14,15]. Several reagents have been reported to be inhibitors of NS3-4A serine protease, including SCH6 [16,17], SCH503034 [18], VX-950 [19,20], and BILN-2061 [21]. Valopicitabine (NM283) was reported to inhibit NS5B RdRp [22]. HCV RNA itself is also a target of antivirals, and recent RNA interference technologies using siRNA or shRNA have targeted HCV RNA [23–25]. As RdRp lacks proofreading activity, the high mutation rate of RdRp allows the virus to escape from the reagents targeting HCV proteins and HCV RNA. These anti-HCV reagent-targeting viral proteins and genome will be reviewed in another section.

Other targets are the cellular proteins essential for HCV RNA replication and infection. The expression of HCV proteins is thought to affect the host cells' gene expression profiles and vise versa [26]. The interaction of the specific cellular proteins with HCV proteins is essential for HCV replication (Table 1). Cyclosporine A (CsA) is one of the best characterized inhibitors targeting the cellular proteins required for HCV replication [27–36]. The interaction of cyclophilin B (CyPB) with NS5B is required for HCV RNA replication [28]. CsA inhibits HCV RNA replication by interrupting the interaction between NS5B and CyPB. Heat shock protein 90 (HSP90) has also been reported to be an essential cellular protein for HCV RNA replication [37–39]. Knockdown or inhibition of HSP90 has been shown to result in the anti-HCV activity in cell culture and in uPA-SCID mouse systems [37].

Table 1
Host molecules as targets of anti-HCV

Target molecules	Reagents	References
HMG–CoA reductase	Statin	[68–71]
Serine palmitoyltransferase	NA255	[81]
	Myriocin	[82]
IMP dehydrogenase	VX-497	[98]
	Ribavirin	[74,86]
	Mizoribin	[74]
	MPA	[97]
Protein arginin methyltransferase	AdoMet, Betaine	[102]
α-Glucosidase	Deoxynojirimycin	[101]
Cyclophilins	CsA	[27–36]
	NIM811	[27,33]
	DEBIO-025	[34]
HSP90	Geldanamycin, Radicicol	[37–39]
FKBP8		[38]
Unknown	PUFAs	[70,103,108]

FKBP8, a member of the FK506-binding protein family, specifically interacts with NS5A and forms a complex with HSP90 [38]. The La autoantigen (La) and polypyrimidine tract-binding protein (PTB) are also candidate cellular proteins for the inhibition of HCV RNA replication [40], although no inhibitors for these proteins have been reported to date. Thus, inhibition of the metabolism has recently been reported as a target of the new antivirals. Here, we survey the recent progress on enzyme inhibitors of the cholesterol, sphingolipid, and guanosine triphosphate (GTP) synthesis pathways, as well as other metabolic pathways.

2. Current standard therapy for chronic hepatitis C

HCV was discovered to be the causative agent of non-A, non-B hepatitis by the Chiron Corporation in 1989 [1]. However, a treatment for patients with non-A, non-B hepatitis was established before the discovery of HCV. In 1986, Hoofnagle et al. reported that IFN-α treatment normalized the serum alanine aminotransferase (ALT) levels in patients with non-A, non-B hepatitis [41]. Since the initial discovery of its anti-HCV activity, IFN-α has become the major reagent for CH–C treatment [4]. The replication of HCV RNA itself seems to stimulate IFN production signaling, and our recent results have suggested that core and/or NS5B induce IFN-stimulated genes [42–44]. However, viral NS3-4A protease inhibits the IFN production, although it does not completely shut it off. Therefore, exogenous IFN administration is needed for patients with CH–C. The SVR is affected by multiple factors, such as genotype, viral load and duration of therapy. IFN-α monotherapy was begun in the early 1990s, but an SVR was achieved in only about 10% of patients. In the early 2000s, IFN-α and ribavirin combination therapy was developed and the SVR was improved to about 30-40%. Furthermore, IFN itself has been modified by the attachment of PEG, thereby enhancing its stability in the blood. The SVR of the current standard therapy by PEG-IFN and ribavirin is as high as 50% [4]. In the current PEG-IFN and ribavirin combination therapy, the genotype of HCV is one of the major determinants of the SVR. HCV genotypes are classified into 6 groups, and genotype 1 is currently considered a problem due to its IFN resistance [45]. For example, in genotype 1 HCV, 12 months of treatment resulted in an SVR in 50% of patients, while in genotype 2, 6 months of treatment achieved an SVR of 80-90% [46]. The precise mechanisms of the IFN resistance remain unclear. However, the recently developed IFN-resistant HCV replicon-harboring cells will be useful for studies examining ways to improve the SVR [47–49]. Therefore, the focus in the treatment of patients with CH–C has shifted to increasing the SVR in genotype 1 HCV.

3. Cell culture-based HCV RNA-replication system

Before the development of an HCV replicon system, screening of anti-HCV reagents was rather difficult. The HCV replicon system developed by Lohmann et al. [5] was the first milestone in HCV study using a cell culture system. The replicon system has provided a wealth of information concerning the replication machinery of HCV. We can make strategies for the Achilles' heel of HCV based on the information regarding HCV RNA replication. The HCV replicon has been improved to be a suitable system for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [50]. However, this system does not contain a structural region. Therefore, selectable genome-length HCV RNA-replicating cell culture systems have been developed [51–54]. The second milestone was the infectious virus production system established by the three groups using a genotype 2a HCV strain, JFH1 [6–8]. This system has extended the range of the HCV study to the viral entry and release. Therefore, the life cycle of HCV in the cells has been reconstructed *in vitro*. Since the development of the HCV replicon and infectious HCV production systems, many cellular proteins have been identified as essential host molecules for HCV RNA replication.

3.1. From HCV replicon to infectious HCV production

The HCV replicon reported by Lohmann et al. contained neomycin phosphotransferase (Neo) and encephalomyocarditis virus (EMCV) internal ribosome entry sites (IRES) instead of the HCV structural regions (Fig. 1) [5]. This HCV replicon consists of 2 cistrons. In the first cistron, Neo is translated by HCV-IRES and in the second cistron NS3-NS5B is translated by EMCV-IRES introduced in the region upstream of the NS region (Fig. 1). After the development of the HCV replicon system [52,53,55–58], genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups [51–54]. In these genome-length HCV RNA replication systems, a complete open reading frame (ORF) of HCV was introduced into the second cistron instead of the NS region (Fig. 1).

For the mass screening for anti-HCV reagents, evaluation of the levels of HCV RNA or HCV proteins requires time and complicated procedures. To facilitate the monitoring of the replication level of HCV RNA, the reporter gene (Renilla luciferase) was fused to the Neo gene. In this system, anti-HCV activity was evaluated by the value of the reporter instead of the

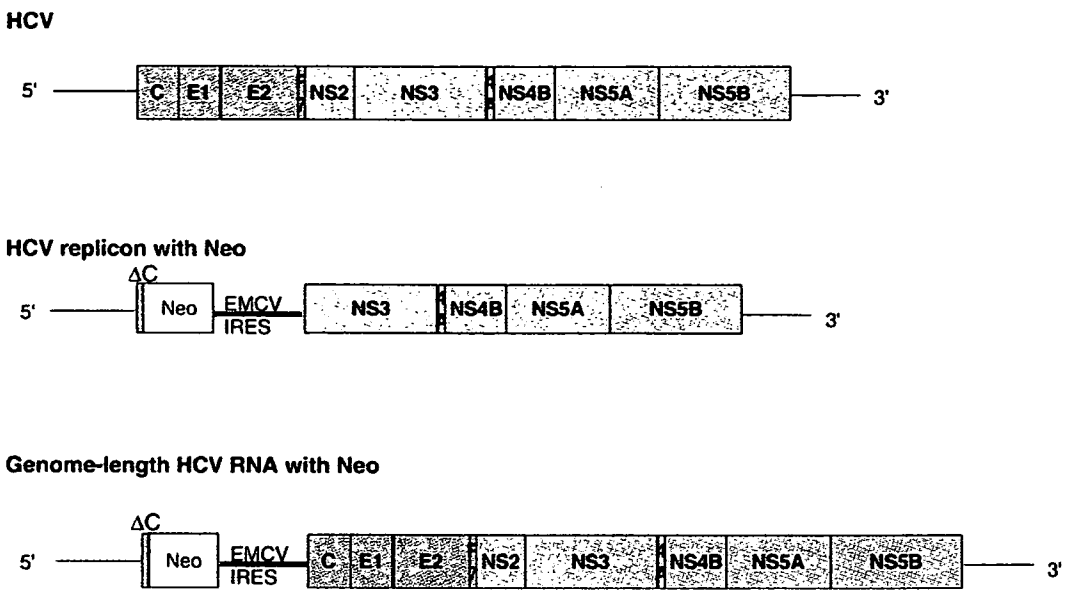

Fig. 1. Gene organizations of HCV and selectable HCVs. HCV ORF, untranslated regions, EMCV IRES, and Neo are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES.

quantification of HCV RNA or HCV proteins. As shown in Fig. 2A, ORN/C-5B/KE contains the fused Renilla luciferase and Neo genes in the first cistron [51]. One of the cloned cell lines, OR6, was established by the G418 selection after introduction of ORN/C-5B/KE RNA into HuH-7 cells. HCV RNA and HCV proteins were stably expressed in the OR6 cells, and the Renilla luciferase activity was correlated well with the level of HCV RNA [51]. Therefore, the antiviral effect of the reagents on HCV RNA replication could be monitored by the activity of Renilla luciferase. The OR6 assay system

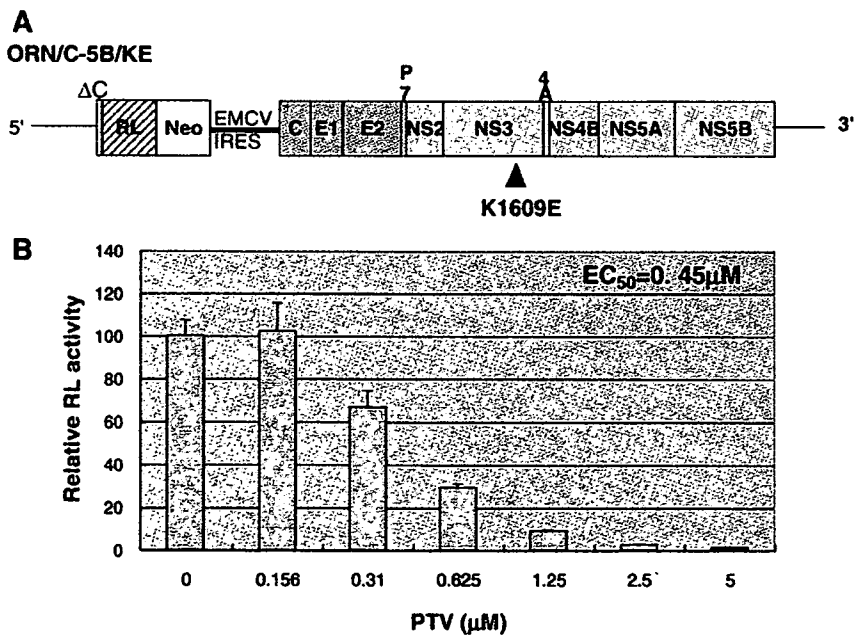

Fig. 2. Inhibitory effect of statin on HCV RNA replication in OR6 cells. (A) Schematic gene organization of genome-length HCV RNA (ORN/C-5B/KE) derived from genotype 1b, strain O. The Renilla luciferase gene, which is symbolized as RL, is depicted as a striped box and is expressed as a fusion protein with Neo. The adaptive mutation from lysine (K) to glutamine (E) at amino acids position 1609 was previously reported [51] and introduced into the genome-length HCV RNA. (B) Inhibition of HCV RNA by PTV. OR6 cells were cloned cell line selected by G418 [51]. OR6 cells were treated with PTV at a concentration of 0, 0.156, 0.31, 0.625, 1.25, 2.5, or 5 μM. After 72 hours of treatment the RL activities were determined. Shown here is the relative RL activity (%) calculated when the RL activity of untreated cells was assigned as 100%. The data indicate the means±standard deviation from three independent experiments. The EC_{50} of PTV was determined as 0.45 μM.