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Original article

Efficient formation of vesicular stomatitis virus pseudotypes bearing the native forms of hepatitis C virus envelope proteins detected after sonication

Kazushi Tamura [a,b], Atsushi Oue [a], Atsushi Tanaka [a], Nobuaki Shimizu [a], Hitoshi Takagi [c], Nobuyuki Kato [d], Akihiro Morikawa [b], Hiroo Hoshino [a,*]

[a] *Department of Virology and Preventive Medicine, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*
[b] *Department of Pediatrics and Developmental Medicine, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*
[c] *Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*
[d] *Department of Molecular Biology, Institute of Cellular and Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan*

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Abstract

Hepatitis C virus (HCV) causes chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in addition to acute hepatitis. The HCV genome encodes two envelope glycoproteins, E1 and E2. To investigate the role of E1 and E2 in HCV infection, we used a recombinant vesicular stomatitis virus (VSV), VSVΔG*, harboring the green fluorescent protein gene instead of the VSV G envelope protein gene. It was complemented with the native form of E1 and E2, or E1 or E2 alone, to make HCV pseudotypes VSVΔG*(HCV), VSVΔG*(E1), and VSVΔG*(E2). Neither E1 nor E2 expression was detected on the cell surface, as reported. Unlike previous reports, infectious activities of VSVΔG*(HCV), VSVΔG*(E1) and VSVΔG*(E2) pseudotypes were detected under conditions where VSV was completely neutralized by anti-VSV. We could enhance the infectious titers 100-fold by sonication upon virus harvest. Bovine lactoferrin efficiently inhibited infection by VSVΔG*(HCV) as well as VSVΔG*(E2), as the interaction between E2 and lactoferrin has been thought to contribute to the inhibition of HCV infectivity. VSVΔG*(HCV) infected many adherent cell lines, including hepatic cell lines, but not most hematopoietic cell lines. Treatment of cells with trypsin, tunicamycin, or sulfated polysaccharides before infection reduced the infectivity of VSVΔG*(HCV) by about 90%, suggesting that a cell surface protein(s) with sugar chains plays an important role in HCV infection. The VSV pseudotypes developed here would be useful for analyzing the early stages of HCV infection.
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Keywords: HCV; Pseudotype; Envelope; Sonication; Glycosylation

1. Introduction

Hepatitis C virus (HCV) has been one of the major causative agents of posttransfusion and sporadic hepatitis [1]. At present, transfusion-associated hepatitis C has been virtually eliminated in developed countries, and risk factors that most are strongly correlated with HCV infection there are illegal drug use and high-risk sexual behavior. Current worldwide estimations suggest that more than 200 million people are infected with HCV [2]. The infection frequently develops into chronic hepatitis, which further leads to the development of liver cirrhosis and hepatocellular carcinoma [3,4]. The mechanisms involved in HCV infection and HCV-mediated disease progression are not well understood, and a therapy effective for most HCV-infected patients is not yet available.

HCV is an enveloped, positive-stranded RNA virus belonging to the Flaviviridae family [2]. The viral genome contains

* Corresponding author. Tel.: +81 27 220 8000; fax: +81 27 220 8006.
E-mail address: hoshino@med.gunma-u.ac.jp (H. Hoshino).

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a single open-reading frame of approximately 9.5 kb that codes for a large polyprotein precursor of 3000 amino acids (aa) [5,6] (Fig. 1a). Structural proteins are located in the N-terminal of the precursor polyprotein, which is to be cleaved by cellular signal peptidases. The core protein (C) is followed by two putative envelope proteins, E1 and E2. A small protein, p7, is produced by the cleavage of the E2 protein. Downstream of the structural proteins, non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are located [7,8]. The E1 and E2 envelope proteins form a non-covalently linked heterodimer, which probably represents the native pre-budding complex, in the endoplasmic reticulum (ER) [9].

The binding of the virus to the host cell surface receptor(s) is the first step in the infection process. As in most enveloped viruses, E1 and E2 are believed to be the major viral attachment proteins in HCV. There has been no clear evidence as to which protein, E1 or E2, defines the interaction with human cells, because of the lack of a suitable experimental system for HCV entry. It has been reported that a truncated, soluble form of E2 binds to human CD81 (hCD81) and human scavenger receptor class B type I (SR-IB), suggesting that

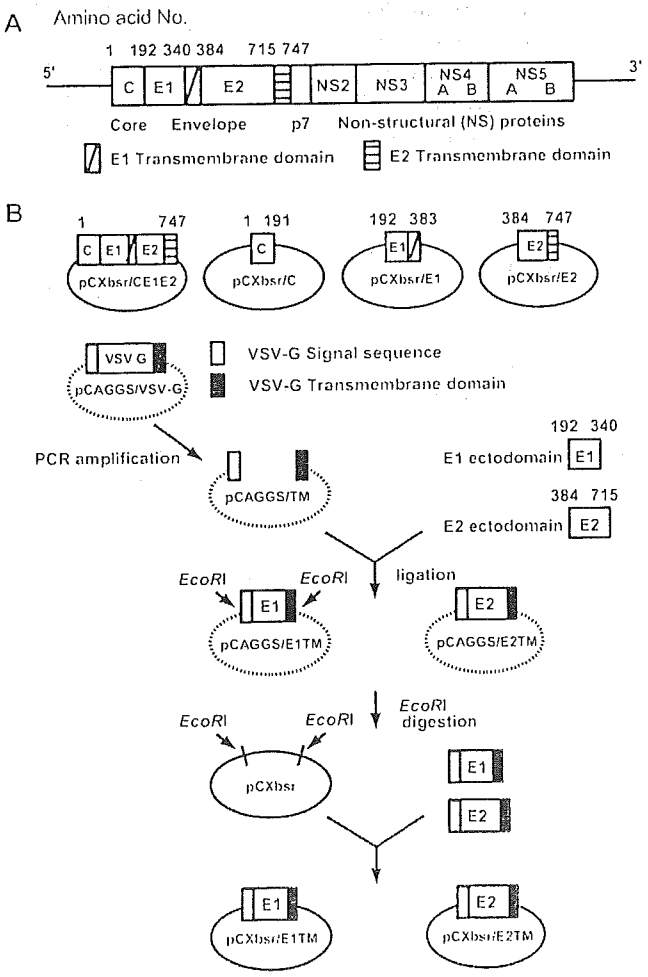

Fig. 1. Preparation of plasmids expressing HCV envelope proteins. (a) Structure of HCV genome. (b) Schematic representation of the plasmids and the chimeric gene constructs for the expression of HCV envelope glycoproteins. TM represents the signal sequence and the transmembrane domain of VSV G protein.

hCD81 or SR-IB is a candidate cellular receptor for HCV [10,11]. Furthermore, HCV particles have also been reported to utilize the LDL receptor for binding and their entry into the cells [12,13]. But it is still unknown whether they serve as functional receptors, since the expression of neither hCD81 nor the LDL receptor is restricted in hepatocytes, and hCD81 transgenic mice are resistant to HCV infection [14]. There may also be a functional difference between the native form of HCV envelope protein and the soluble form of E2. The main obstacle to clarifying these points is the lack of suitable tools with which to evaluate the attachment to and entry into the target cells quantitatively. Recently, to analyze virus entry mediated by HCV envelopes, systems for the production of vesicular stomatitis virus (VSV) pseudotypes bearing modified HCV envelope proteins have been reported [15–17]. To express the HCV envelope proteins on the cell surface to incorporate them into VSV virions, chimeric HCV E1 and E2 proteins containing the transmembrane domain and cytoplasmic tail of VSV G glycoprotein (VSV G) were generated. Otherwise, E1 or E2 is not expressed on the cell surface. There is the possibility that these pseudotypes may show a different infectivity from the viruses bearing the native forms of HCV envelopes.

In this study, we developed a system to prepare VSV pseudotypes expected to bear the native HCV envelope proteins, E1E2, E1 or E2. That is, cells were transfected with the native structural protein genes, and then infected with a recombinant VSV, VSVΔG*G, containing the green fluorescent protein (GFP) gene as a reporter instead of VSV G [18]. Unlike previous reports [15–17], we could detect pseudotype virus activities after transfection of not only the native structural protein gene C–E1–E2, but also the unmodified E1 or E2 gene. These pseudotype virus-like activities were not neutralized by any sera from chronic hepatitis C patients, as previously reported using pseudotypes with chimeric HCV envelopes [17,19]. These infectious activities were inhibited by treatment with bovine lactoferrin, as we reported using PCR for detection of HCV infection [20,21]. Using these new VSV pseudotypes, we further examined the mechanism involved in HCV infection. That is, the infectivity of pseudotype viruses was studied in various cell lines, and the effects of chemical reagents on the infection were tested.

2. Materials and methods

2.1. Cells

293T is derived from the human embryonic kidney cell line 293 and contains the SV40 large T-antigen [22]. The other cell lines used in this study and their derivations are listed in Table 3. BALL-1, C8166, C91/PL, Daudi, HEL, HL-60, K562, Molt-4, MT-2, Raji, TALL-1, U937 and Wil2NS were cultured in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS). 293T, A172, HepG2, HOS and Huh7 were maintained in Dulbecco's modi-

fied Eagle's medium (DMEM: Nissui) supplemented with 10% FCS. U87MG was maintained in DMEM supplemented with 20% FCS. HUK-1, NP2 and U251 were maintained in Eagle's medium (Nissui) supplemented with 10% FCS. The maintenance of PH5CH8 cells was previously described in detail [23]. Human brain microvascular endothelial cells (HBMECs, Applied Cell Biology Research Institute, WA) were maintained in endothelial cell basal medium 2 supplemented with EGM-2 additives (Clontecs, CA). Human brain pericytes (HBP) were derived from surgically dissected human brain tissue. HBP cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 10 μg/ml of endothelial cell growth supplement, and 10 ng/ml of epidermal growth factor. All culture media were supplemented with 50 μg/ml of kanamycin before use.

2.2. Plasmids

Fragments of the HCV C, E1 and E2 genes were obtained from a subclone of an infectious clone of HCV, subtype 1b [6], the predominant subtype in Japan, and cloned into pCXbsr, a Moloney murine leukemia virus-based retroviral vector plasmid [24]. Mammalian expression plasmids encoding HCV core protein (pCXbsr/C), HCV E1 protein (pCXbsr/E1), HCV E2 protein (pCXbsr/E2) and all HCV structural proteins, core-E1–E2 (pCXbsr/CE1E2), were made as shown in Fig. 1b. To construct retroviral expression plasmids encoding chimeric HCV envelope proteins, we generated the plasmid pCAGGS/TM, encoding the signal sequence, the transmembrane domain, and the cytoplasmic tail of the VSV G protein, as described below. pCAGGS/TM was amplified by PCR using pCAGGS/VSV-G [25] as a template, in which VSV (Indiana serotype) G protein was placed under control of the CAG promoter, and the following primers:

- sense primer, 5′-AAAAGCTCTATTGCCTCTTTTTTCTTTATC;
- antisense primer, 5′-GCAATTCACCCCAATGAATAAAAAGGCTAA.

The coding sequence for the ectodomain of HCV E1 (aa 192–340) was amplified by PCR using pCXbsr/CE1E2 as a template and the following primers:

- sense primer, 5′-TATGAAGTGCGCAACGTGTCCGGGGTGTAC;
- antisense primer, 5′-GATCCGGAGCAACTGCGATACCACCAGGGC.

The ectodomain of the HCV E2 (aa 384–715) genomic region was amplified by PCR using pCXbsr/CE1E2 as a template and the following primers:

- sense primer, 5′-GCTACCTACACGTCAGGGGGGACGGTAGGC;
- antisense primer, 5′-TCTGATTACAACGGAGACAACCACTGACCC.

The ectodomains of E1 and E2 sequences were subcloned into pCAGGS/TM, using a Blunting High kit (Toyobo, Tokyo, Japan), and the plasmids pCAGGS/E1TM and pCAGGS/E2TM were isolated. pCAGGS/E1TM and pCAGGS/E2TM digested with *Eco*RI (Takara, Siga, Japan) were subcloned into pCXbsr, resulting in the formation of pCXbsr/E1TM and pCXbsr/E2TM, respectively (Fig. 1b).

2.3. Immunofluorescence staining of E1 and E2

293T cells were seeded onto slide glasses and the next day transfected with the expression plasmid vectors for HCV envelope proteins using FuGENE6 (Roche, Basel, Switzerland). After 32 h, the cells were tested for the expression of the viral envelope proteins by indirect immunofluorescence. Namely, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. Half the fixed cell samples were then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. A mouse monoclonal antibody (MAb) to E1, E1-384 [26], and a rat MAb to E2, Mo-12 [27], were used as follows. The MAb diluted to 1:1000 in PBS was added as the primary antibody and incubated for 60 min at 37 °C. After a wash with PBS, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG or FITC-conjugated rabbit anti-rat IgG (Dako, Glostrup, Denmark) diluted 1:50 in PBS was added, and the cells were incubated for 60 min at 37 °C. After three washes in PBS, the cells seeded on slide glasses were embedded with a solution of glycerol in PBS and examined with a fluorescence microscope for the expression of HCV glycoproteins.

2.4. Preparation of pseudotype viruses

VSVΔG*G is the recombinant VSV, generated by reverse genetics, as described previously [18] and kindly provided by Dr. M.A. Whitt. To generate VSV pseudotype viruses, 2×10^6 293T cells were grown in a poly-L-lysine-coated 60-mm dish and transfected with plasmids. Thirty-two hours after transfection, the cells were infected with VSVΔG*G at an MOI of 2 for 2.5 h at 37 °C in a 5% CO_2 incubator. Virus-infected cells were washed with serum-free DMEM and incubated in 1 ml of goat anti-VSV polyclonal antibody (diluted at 1:10) for 40 min at 37 °C to neutralize unabsorbed virus. This concentration was enough to completely neutralize the undiluted VSVΔG*G. Then, they were again washed with serum-free DMEM four times, and culture medium was added. After 15 h of incubation at 37 °C, the culture supernatants + adherent cells were harvested and centrifuged at 350 × *g* for 3 min at room temperature. Then, cell pellets were either sonicated or left untreated, and the virus samples were clarified by centrifugation at 350 × *g* for 5 min at room temperature to remove cell debris. Virus samples were stock frozen at −80 °C. These samples were found to show compatible properties with those of HCV virions, as described in Table 2. As control pseudotypes, VSVΔG*G and VSVΔG* were used. VSVΔG*G was prepared by infecting 293T cells that had been transfected with pCAGGS/VSV-G, while the VSVΔG* sample was prepared by infecting 293T cells that had been transfected with pCXbsr plasmid containing no envelope protein.

2.5. Detection of HCV envelope proteins and VSV structural proteins in pseudotype virus samples by Western blotting

We prepared 293T cells transfected with the expression plasmid vectors for HCV envelope proteins described in Table 2. These cells were infected with VSVΔG*G. We also prepared the 293T cells transfected with the expression plasmid vectors for HCV envelopes that were not infected with VSVΔG*G. These samples were sonicated and centrifuged at 350 × *g* for 5 min. Each 3-ml supernatant was subjected to ultracentrifugation (27,000 rpm for 3 h at 4 °C) through 2 ml of a 20% sucrose layer using an SCP70H HITACHI. Pellets were suspended in 30 μl of sample buffer (1% SDS, 1% 2-mercaptomethanol, 50 mM Tris–HCl [pH 6.8], and 20% glycerol). The samples were loaded onto 10% SDS-PAGE gel. E1 proteins were detected using an anti-E1 mouse MAb, E1-384 [26] (diluted at 1:1000), and then HRP-conjugated anti-mouse IgG (Dako; diluted at 1:1000). E2 proteins were detected using an anti-E2 rat MAb, Mo-12 [27] (diluted at 1:1000), and HRP-conjugated anti-rat IgG (Dako). VSV structural proteins were detected using goat anti-VSV polyclonal antibody (diluted at 1:4000), and HRP-conjugated anti-goat IgG (Dako). HRP-conjugated antibodies bound to filters were detected using enhanced chemiluminescence.

2.6. Titration of pseudotype viruses using various cell lines

Cells (2×10^4) were seeded into wells of 96-well flat-bottom plates. After 36 h of incubation, the cells were infected with the virus samples defined in Table 2 and incubated at 37 °C for 24 h. The HepG2 cell line was incubated at 33 °C, because a lower temperature had a better effect on infection in this cell line. Infectious units (IU) of the samples were determined by counting the number of GFP-expressing cells under a fluorescence microscope.

2.7. Sonication for preparation of pseudotype virus samples

As the native form of the HCV envelope protein was reported not to be expressed on the cell surface [28,29], we tested a sonication step to efficiently recover HCV pseudotypes. For this, the VSV pseudotype samples harvested as described above were sonicated with a SONIFIER 250 (Branson, CT) for 0.2 s five times on ice. The samples were centrifuged at 350 × *g* for 5 min, and supernatants were aliquoted and stocked frozen at −80 °C. HepG2 cells were used for titration of the pseudotype samples prepared with or without sonication. After 24 h of infection, IU were determined as described above.

2.8. Neutralization of the pseudotype virus samples

To judge whether the infectivity of each virus sample was HCV- or VSV-specific, the pseudotype virus samples were incubated with serially diluted polyclonal antibody against VSV in the presence or absence of human sera from patients with chronic HCV infection (final concentration up to 20%) for 30 min at 37 °C, and HepG2 cells were infected with these samples. After 24 h of incubation, the amount of remaining infectious titer was determined as described above. All previous reports have, however, shown that sera from patients with chronic HCV infection hardly neutralized chimeric E1 or E2 pseudotypes [17,19].

2.9. Treatment of the pseudotype viruses with chemicals

Pseudotype virus samples expected to bear E1, E2 or E1E2 protein were preincubated with various concentrations of bovine lactoferrin (Wako, Tokyo, Japan) at 37 °C for 1 h and inoculated onto HepG2 cells. After 1.5 h of incubation, the cells were washed with DMEM three times and incubated with fresh culture medium. The VSVΔG*(HCV) pseudotype was preincubated with heparin (Wako), dextran sulfate (molecular weight (MW) 8000 or 500,000) or dextran (MW 7000; Sigma, MO) at 37 °C for 1 h, and HepG2 cells in a 96-well plate were infected with these samples. VSVΔG*G was used as a control in most experiments. After 24 h of incubation, each infectious titer was determined as described above.

2.10. Enzymatic and chemical modification of target cells

HepG2 cells in a 96-well plate were washed with PBS and treated with 50 μl of heparitinase (Sigma) for 1 hr, 50 μl of trypsin (Sigma) for 5 min, or 50 μl of α-mannosidase (Sigma) for 1 hr at 37 °C. Subsequently, an equal volume of complete medium was added to stop the enzyme, and then, the cells were washed with PBS and infected with each pseudotype virus sample. HepG2 cells in a 96-well plate were also preincubated in DMEM containing tunicamycin (Sigma) overnight. Then, they were infected with each virus. After 24 h of incubation, the infectious titer was determined.

All the virus titration experiments were done in triplicate. In each figure, the results shown are means, with error bars representing standard deviations (S.D.).

3. Results

3.1. Localization of HCV envelope proteins expressed in 293T cells

We expressed the HCV envelope proteins by transfection with plasmid vectors encoding HCV envelope proteins shown in Table 1. The carboxyl-terminal domains of HCV envelope proteins, E1 and E2, contain ER retention signals [28–30]. To incorporate HCV envelope proteins into VSV particles, it has been reported to be necessary to express these proteins on the cell surface. Thus, to generate HCV pseudotype viruses, chimeric proteins of the ectodomain of HCV E1 or E2, and

Table 1
Detection of HCV envelope proteins by indirect immunofluorescence

Transfected plasmids	Positively stained cells (%)[a]			
	anti-E1		anti-E2	
	(–)[b]	(+)	(–)	(+)
pCXbsr	0	0	0	0
pCXbsr/CE1E2	0	30	0	30
pCXbsr/E1	0	40	0	0
pCXbsr/E2	0	0	0	40
pCXbsr/E1 and pCXbsr/E2	0	40	0	30
pCXbsr/E1, pCXbsr/E2 and pCXbsr/C	0	20	0	30
pCXbsr/E1TM	40	40	0	0
pCXbsr/E2TM	0	0	50	50
pCXbsr/E1TM and pCXbsr/E2TM	30	30	40	40

[a] A mouse monoclonal antibody to E1, E1-384, and a rat monoclonal antibody to E2, Mo-12, were used at a 1/1000 dilution. Percentage of positively stained cells is the mean value from at least three different experiments.

[b] 293T cells were transfected with the indicated plasmid DNA and cultivated for 2 days. The cells were fixed with 4% paraformaldehyde and permeabilized (+), or not (–), with Triton X-100, before immunofluorescence.

the transmembrane domain of VSV G have been used [15–17]. We also made pCXbsr/E1TM and pCXbsr/E2TM encoding the ectodomains of E1 and E2, respectively, joined to the signal sequence, transmembrane and cytoplasmic tail of VSV G protein. In addition, we made plasmid vectors, pCXbsr/CE1E2 coding for the entire HCV structural protein, pCXbsr/C, pCXbsr/E1 and pCXbsr/E2. The structural protein, CE1E2, will be cleaved by cellular signal peptidases [7,8]. Then, we examined the localization of the HCV envelope proteins by indirect immunofluorescence after the fixation of cells with paraformaldehyde (Table 1). Triton X-100-permeabilized cells and non-permeabilized cells were analyzed in parallel. The native forms of the HCV envelope proteins were apparently detected in the transduced cells only after permeabilization. In contrast, the chimeric proteins E1TM and E2TM were observed in both non-permeabilized and permeabilized cells, as reported [16,17].

3.2. Preparation of VSV pseudotypes bearing HCV envelope proteins

3.2.1. Western blotting for HCV envelope proteins

First, to examine whether the native forms of HCV envelope proteins expressed in the cytoplasm in 293T cells could be incorporated into VSV lacking G protein but expressing GFP, VSVΔG* pseudotype virus samples were analyzed by Western blotting (Fig. 2a). E1 was detected as a broad band in a MW range of 30–40 kDa, as previously reported [31,32]. E1 protein in VSVΔG*(HCV) preparation migrated more slowly than E1 protein in VSVΔG*(E1) preparation upon SDS-PAGE. This observation may be explained by different glycosylation of E1 proteins: the glycosylation of E1 has been reported to be enhanced when E1 and E2 are expressed in *cis* [33]. E2 was detected as a discrete band in a MW range of 50–60 kDa, as previously reported [31]. E2 migrated slightly

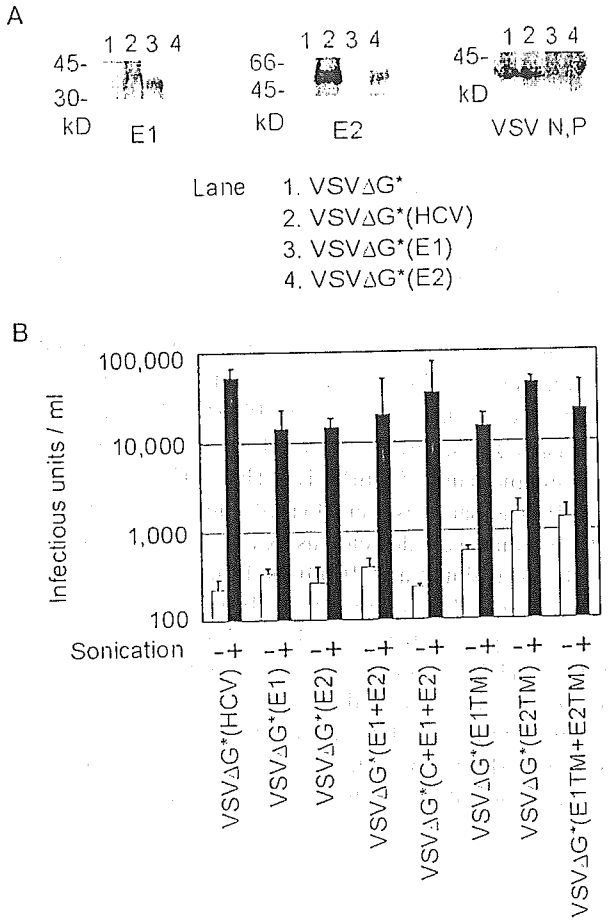

Fig. 2. (a) Western blot analyses of the pseudotype VSVs bearing HCV envelope proteins. Immunoblots of pseudotype virus samples through 20% sucrose cushions are shown. The preparation of each pseudotype sample is described in Table 2. E1 and E2 glycoproteins were detected with E1-384 and Mo-12 monoclonal antibodies against E1 [26] and E2 [27], respectively. VSV proteins were detected by polyclonal goat antibody. The positions of the molecular mass markers (kDa) are shown. (b) Effect of sonication on HCV pseudotype detection. VSV pseudotypes complemented with HCV envelope proteins were prepared after sonication (+, filled column) or without sonication (–, open column). HepG2 cells were infected with the indicated pseudotype viruses, and the IU were determined using the number of GFP-positive cells detected after 24 h of incubation.

faster than E2 reported in other studies [7,16]; this may be due to a variation in glycosylation of E2 among different HCV strains [31,34]. E1 and E2 bands were also detected in VSVΔG*(E1 + E2) or VSVΔG*(C + E1 + E2) samples (data not shown). Bands for the VSV structural proteins N and P with similar intensities were detected in all the four purified pseudotype samples by Western blotting, indicating that similar amounts of VSV were present there. As a control, 293T cells were transfected with E1 and/or E2 vectors but were not infected with VSVΔG*G later. Culture supernatants and cells were harvested, sonicated and subjected to ultracentrifugation, as described above. This sample was also analyzed by Western blotting, and neither E1 nor E2 was detected (data not shown). All these findings suggested the incorporation of the native forms of E1 and/or E2 into VSVΔG* viral particles.

Table 2
Designation of VSV pseudotype samples complemented with HCV glycoproteins

Pseudotype sample[a]	Plasmids
VSVΔG*G	pCAGGS/VSV-G
VSVΔG*(HCV)	pCXbsr/CE1E2
VSVΔG*(E1)	pCXbsr/E1
VSVΔG*(E2)	pCXbsr/E2
VSVΔG*(E1 + E2)	pCXbsr/E1 and pCXbsr/E2
VSVΔG*(C + E1 + E2)	pCXbsr/E1, pCXbsr/E2 and pCXbsr/C
VSVΔG*(E1TM)	pCXbsr/E1TM
VSVΔG*(E2TM)	pCXbsr/E2TM
VSVΔG*(E1TM + E2TM)	pCXbsr/E1TM and pCXbsr/E2TM
VSVΔG*[b]	PCXbsr

[a] VSV pseudotype samples were generated by transfection of cells with the indicated plasmids (total amount of DNA 2 μg per dish) and then by infection of the cells with VSVΔG*G 2 days later. Culture supernatants and the cells were harvested on the following day to prepare pseudotype samples, and stocked at −80 °C after sonication.

[b] VSVΔG* was recovered from cells transfected with pCXbsr plasmid containing no envelope glycoprotein.

3.2.2. *Effect of sonication on pseudotype virus preparation*

Next, we infected HepG2 cells with pseudotype samples that had been prepared with or without sonication. A large number of cells expressed GFP when the cells had been infected with the sonicated sample designated VSVΔG*(HCV), although much fewer cells expressed GFP when infected with the non-sonicated VSVΔG*(HCV) sample (Fig. 2b). The infectivities of other samples, i.e. VSVΔG*(E1), VSVΔG*(E2), VSVΔG*(E1 + E2), VSVΔG*(C + E1 + E2), VSVΔG*(E1TM), VSVΔG*(E2TM) and VSVΔG*(E1TM + E2TM), shown in Table 2, were also examined. The sonication procedure also enhanced their infectivities, as shown in Fig. 2b. In general, the infectivities of the virus samples that could bear the native forms of HCV envelope proteins were enhanced about 100-fold by sonication. With regard to VSVΔG*(E1TM), VSVΔG*(E2TM) and VSVΔG*(E1TM + E2TM), sonication enhanced these pseudotype titers about 10-fold (Fig. 2b).

3.3. *Neutralization of the pseudotype viruses*

To ascertain whether the infectivity of the VSV samples that could contain VSV pseudotypes was specific for the HCV envelope proteins, we examined whether the pseudotype virus activities could be inhibited by treatment with human sera as well as with bovine lactoferrin. While the anti-VSV antibody neutralized VSVΔG*G completely, it did not affect infection with VSVΔG*(HCV) at all (Fig. 3a). Infections of the other HCV pseudotype viruses were not affected by anti-VSV either (data not shown). These results suggested that the HCV envelope proteins conferred envelopes for VSVΔG*. None of the serum samples from 20 chronically HCV-infected Japanese patients, however, exhibited significant neutralization of HCV (E1E2), E1, or E2 pseudotype virus (data not shown). Previously, it was reported that serum samples from a majority of patients with chronic HCV infection failed to show detectable neutralization activity [19].

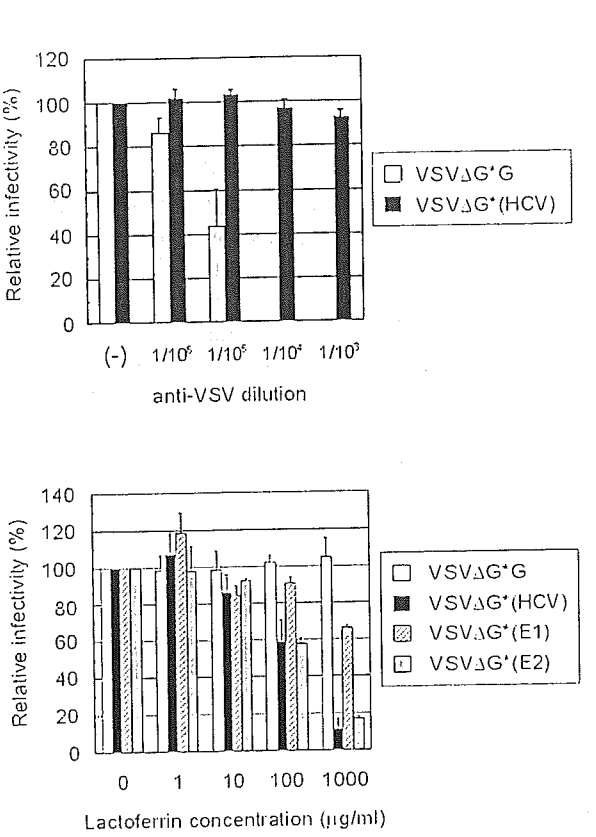

Fig. 3. (a) Neutralization of the pseudotype viruses. Two hundred IU of VSVΔG*(HCV) and VSVΔG*G was preincubated with the indicated dilutions of polyclonal antibody against VSV for 30 min and then inoculated to HepG2 cells. (b) Effect of bovine lactoferrin on the infectivity of pseudotype viruses. Each pseudotype virus (400 IU) was preincubated with various concentrations of bovine lactoferrin for 1 h and then inoculated to HepG2 cells for 1.5 h. Subsequently, the cells were washed with DMEM three times and maintained in culture medium. After 24 h of incubation, relative infectivity (%) was calculated by counting GFP-positive cells. The experiment was done in triplicate, and mean ± S.D. are shown.

3.4. *Effect of bovine lactoferrin on the infectivity of the pseudotype viruses*

We have reported, using PCR, that bovine lactoferrin prevents HCV infection in vitro [20,21]. As HCV-positive human sera were ineffective in inhibiting infection, to support the notion that HCV envelope-specific pseudotypes were formed, we examined whether we could show a specific interaction between lactoferrin and HCV pseudotype samples. Namely, each pseudotype sample was preincubated with various concentrations of lactoferrin, and then HepG2 cells were infected with them. The infectivities of the VSVΔG*(HCV) and VSVΔG*(E2) samples were reduced by preincubation with bovine lactoferrin in a dose-dependent manner, whereas VSVΔG*G was not inhibited (Fig. 3b). VSVΔG*(E1) was only slightly inhibited. This finding is consistent with the report that lactoferrin binds more specifically to E2 than E1 [35].

3.5. Susceptibility of various human cell lines to HCV pseudotypes

Next, we examined the susceptibility of various cell lines to the VSVΔG*(HCV) sample, using VSVΔG*G or VSVΔG* as a control (Table 3). VSVΔG* was prepared without supplying any envelope proteins and showed hardly any infectious titers. Hepatic cell lines, such as HepG2 and Huh7 cells, as well as 293T cells, showed a high susceptibility, and PH5CH8 cells showed a moderate susceptibility to VSVΔG*(HCV). Brain tumor-derived cell lines and primary brain-derived cells were moderately susceptible. Most hematopoietic cell lines were completely resistant to the pseudotype, while MT-2, a human T-cell leukemia virus type I (HTLV-I)-infected T cell line, and HEL, a human erythroleukemia cell line, showed a marginal susceptibility. MT-2 cells as well as HepG2 and PH5CH8 cells that have been reported to be susceptible to HCV infection [36] were susceptible to the VSVΔG*(HCV) sample, suggesting that VSV pseudotypes bearing HCV envelopes were formed.

The susceptibility of various types of cells shown in Table 3 to VSVΔG*(C + E1 + E2) or VSVΔG*(E1TM + E2TM) was also examined comparatively (Table 4). In hepatic cell lines, VSVΔG*(C + E1 + E2) and VSVΔG*(E1TM + E2TM) were nearly as infectious as VSVΔG*(HCV). Onto non-hepatic cells such as 293T, HBMEC or MT-2, VSVΔG*(HCV) plated

Table 3
Infectivity of pseudotype viruses in various human cells

Target	Origin	Pseudotype virus[a]			
		VSVΔG*(HCV)		VSVΔG*G	VSVΔG*
		IU/ml[b]	Ratio[c]	IU/ml	IU/ml
HepG2	Hepatoblastoma	53,000	1	3.4×10^8	280
Huh7	Hepatoma	25,000	0.47	1.2×10^9	1200
PH5CH8	Liver	4700	0.09	2.8×10^8	<10
293T	Embryonal kidney	50,000	0.94	6.8×10^8	300
HUK-1	Kidney	800	0.02	7.2×10^{10}	40
A172	Glioma	19,000	0.36	1.3×10^8	80
NP2	Glioma	15,000	0.28	2.6×10^8	<10
U251	Glioma	2200	0.04	1.1×10^9	<10
U87MG	Glioblastoma	1100	0.02	9.8×10^7	120
HBMEC	Brain microvascular endothelial cell	3600	0.07	3.1×10^8	100
HBP	Brain pericyte	520	0.01	2.8×10^7	60
HOS	Osteosarcoma	1500	0.03	1.0×10^7	20
Molt-4	T-cell acute lymphocytic leukemia	<10		1.2×10^9	<10
TALL-1	T-cell acute lymphocytic leukemia	40		3.2×10^8	<10
C8166	HTLV-1 (+) T cells	<10		8.0×10^8	<10
C91/PL	HTLV-1 (+) T cells	<10		2.0×10^8	100
MT-2	HTLV-1 (+) T cells	260	0.005	8.0×10^8	<10
BALL-1	B-cell acute lymphocytic leukemia	<10		1.7×10^8	<10
Daudi	Burkitt's lymphoma	<10		4.2×10^8	<10
Raji	Burkitt's lymphoma	<10		3.6×10^8	<10
Wil2NS	Plasmacytoma	20		5.2×10^8	<10
HEL	Erythroleukemia	240	0.005	1.4×10^9	<10
K562	Chronic myelogenous leukemia	40		2.8×10^8	20
HL-60	Acute promyelocytic leukemia	<10		4.2×10^7	20
U937	Histiocytic leukemia	<10		6.1×10^8	<10

[a] Pseudotype virus samples described in Table 2 were diluted and inoculated onto the indicated cells.
[b] Infectious units/ml (IU/ml) were determined by counting the number of GFP-expressing cells under a fluorescence microscope after 24 h infection. The experiments were done in triplicate, and means are shown.
[c] The relative ratio of infectious titers compared to HepG2 cells are shown.

Table 4
Infectivity of various HCV pseudotype viruses in human cells

Pseudotype virus[a]	IU/ml	Relative infectivity							
	HepG2	HepG2	Huh7	PH5CH8	293T	A172	NP2	HBMEC	MT-2
VSVΔG*(HCV)	53,000[b]	1[c]	0.47	0.09	0.94	0.36	0.28	0.07	0.005
VSVΔG*(C + E1 + E2)	35,000	1	0.40	0.05	0.45	0.19	0.10	0.01	0.001
VSVΔG*(E1TM + E2TM)	22,000	1	0.50	0.09	0.68	0.30	0.20	0.01	<0.001

The experiments were done in triplicate, and means are shown.
[a] Pseudotype virus samples described in Table 2 were diluted and inoculated onto the indicated cells.
[b] Infectious units/ml (IU/ml) were determined by counting the number of GFP-expressing cells under a fluorescence microscope after 24 h infection.
[c] The relative ratios of infectious titers to HepG2 cells are shown.

much more efficiently than VSVΔG*(C + E1 + E2) and VSVΔG*(E1TM + E2TM): these two latter pseudotypes were prepared with E1 and E2 expressed in *trans*.

3.6. Effect of sulfated polysaccharides on pseudotype virus infection

The infection of several flaviviruses, such as Japanese encephalitis virus and dengue virus serotype 2, has been reported to be inhibited by sulfated polysaccharides, especially heparan sulfate [37,38]. To investigate whether proteoglycans are involved in HCV infection, we examined the plating of VSVΔG*(HCV) and VSVΔG*G on HepG2 cells treated with heparitinase. Fig. 4a shows that heparitinase treatment of the cells reduced the plating of VSVΔG*(HCV). Next, we examined effects of highly sulfated polysaccharides, heparin, dextran sulfate (MW 8000 or 500,000), and unsulfated dextran (MW 7000) on VSVΔG*(HCV) infection (Fig. 4b). Heparin and sulfated dextrans effectively blocked VSVΔG*(HCV) infection, while unsulfated dextran was completely inactive in inhibiting VSVΔG*(HCV) infection. In contrast, the infectivity of VSVΔG*G was hardly affected by sulfated polysaccharides (data not shown).

To examine whether the sulfation level affected VSVΔG*(HCV) infection, sodium chlorate-treated HepG2 cells were infected with VSVΔG*(HCV), because sodium chlorate acts as a sulfate analog and reduces the sulfation level of cellular proteins and glycosaminoglycans (GAGs) [39,40]. Treatment of HepG2 cells with sodium chlorate reduced the VSVΔG*(HCV) titer by about 50% (data not shown). These results suggested that highly sulfated forms of the cell surface GAGs play roles in VSVΔG*(HCV) infection.

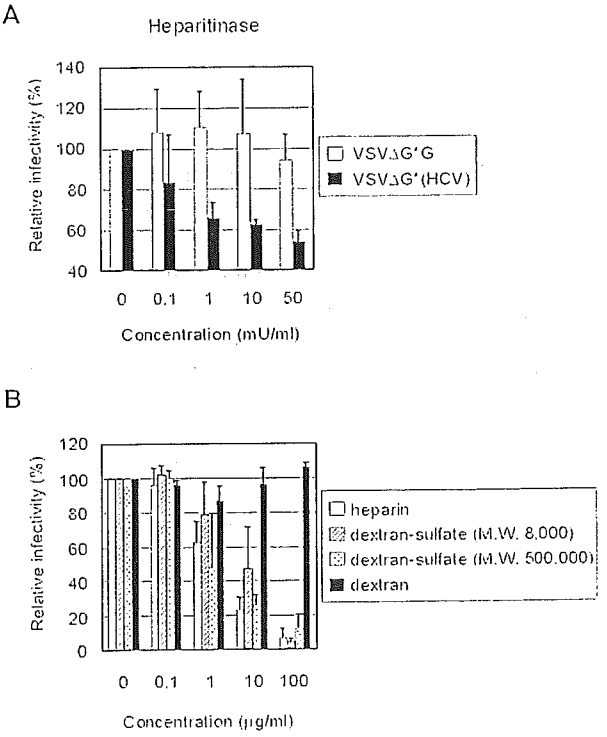

Fig. 4. Effects of sulfated polysaccharides on pseudotype infection. (a) Effect of heparitinase on infectivity of VSVΔG*(HCV). HepG2 cells were treated with various concentrations of heparitinase. The treated cells were infected with 200 IU of VSVΔG*(HCV) or VSVΔG*G. (b) Effect of sulfated polysaccharides on the infectivity of VSVΔG*(HCV). Two hundred IU of VSVΔG*(HCV) was preincubated with heparin, dextran sulfate or dextran at various concentrations for 1 h and then inoculated to HepG2 cells. After 24 h of incubation, the infectivity of the viruses was evaluated. The experiment was done in triplicate, and mean ± S.D. are shown.

3.7. Effects of enzymatic or chemical modification of the target cells on the plating of the HCV pseudotype

To characterize cellular factors necessary for HCV entry, we examined the plating of VSVΔG*(HCV) on HepG2 cells treated with various chemicals. Trypsin treatment of cells markedly reduced the infectivity of VSVΔG*(HCV), while the infectivity of VSVΔG*G was weakly affected (Fig. 5a). Either phospholipase C or sodium periodate marginally reduced the infectivity of VSVΔG*(HCV) (data not shown). Similar results were obtained with 293T cells (data not shown).

Next, the infection of VSVΔG*(HCV) was assessed with inhibitors of protein glycosylation. As shown in Fig. 5b, tunicamycin reduced the plating of VSVΔG*(HCV) by about 90%, whereas castanospermine reduced the infectivity by 20–30%, and neither deoxymannojirimycin nor swainsonine inhibited the plating of VSVΔG*(HCV) (data not shown). α-Mannosidase treatment of cells before infection reduced the infectivity of VSVΔG*(HCV) by about 70% at 500 μg/ml (Fig. 5c). These findings suggested that N-linked glycosylation of a protein(s) on the cell surface might have a role in HCV entry.

4. Discussion

We tried to develop a system to detect the infectivity of recombinant VSV pseudotypes bearing the native forms of HCV envelopes. The co-expression of E1 and E2, or expression of E1 or E2 alone, efficiently complemented the infectivity of VSV lacking the envelope G protein. We used the native forms of the HCV envelope proteins, because we considered that it might be more relevant to examine functions of the HCV envelopes. This system would enable us to study the early stages of HCV infection easily.

There has been no assay system in which infection of HCV has been detected readily and rapidly. For this, VSV pseudotype systems for HCV have been developed by several groups. Because both HCV E1 and E2 have ER retention signals in their C-terminal transmembrane domains, these proteins have been found to be retained in the ER [28–30]. This finding was confirmed by us (Table 1). Therefore, to prepare VSV pseudotypes bearing HCV envelopes, chimeric proteins consisting of carboxy-terminal-truncated HCV envelopes fused to the transmembrane and cytoplasmic tail of VSV G glycoprotein have been used to localize them on the cell surface [15–17]. Baumert et al. [41] reported that HCV-like particle

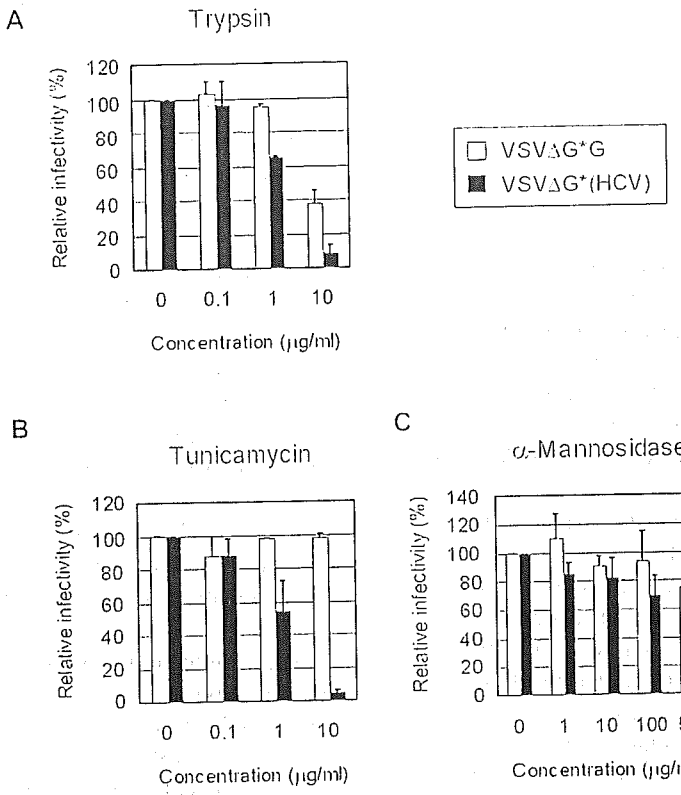

Fig. 5. Infectivity of pseudotype viruses in chemically modified cells. (a) HepG2 cells were preincubated with various concentrations of trypsin for 5 min. Subsequently, an equal volume of complete medium was added to stop the enzyme. Then, the cells were washed and infected with 200 IU of each virus. (b) Effect of glycosylation of cell surface components on the infectivity of VSVΔG*(HCV). HepG2 cells were preincubated with the indicated concentrations of tunicamycin for 24 h. Then, the cells were infected with 200 IU of each virus. (c) Effect of α-mannosidase treatment of cells on the entry of VSVΔG*(HCV). HepG2 cells were preincubated with α-mannosidase at various concentrations for 1 h. Then, the cells were washed and infected with 200 IU of each virus. After 24 h of incubation, the infectivity of the viruses was determined. The experiment was done in triplicate, and mean ± S.D. are shown.

assembly occurs in the cytoplasmic vesicles and that pseudoparticles bearing native HCV envelope proteins will not be released or secreted into culture medium, but accumulate in the ER, like parental HCV virions. There is, however, a possibility that these VSV pseudotype viruses might not exactly reflect the characteristics of the native envelope proteins of viral particles.

HCV virions bearing the native form of HCV envelope proteins should be released from cells in vivo, since plasma samples of HCV-infected humans have frequently shown high infectivity [1,5,42]. It is enigmatic for us how HCV virions have been produced in vivo; nevertheless, both E1 and E2 proteins harbor the ER retention signal. Very recently, Bartosch et al. [43] and Hsu et al. [44] reported the existence of pseudoparticles bearing unmodified HCV envelopes on retroviral core particles. They suggested that a small portion of E1 and E2 would be expressed on the cell surface when these proteins had been expressed abundantly in cells, and thus the retroviral pseudotype bearing E1E2 could be detected. In contrast, we showed that VSV pseudotypes bearing the native form of HCV envelope proteins with highly infectious titers, as compared with previous reports, could be produced by the cells expressing the HCV envelope proteins in the cytoplasm (Table 1 and Fig. 2).

Unlike previous studies [15–17], we thus successfully detected the infectious activities considered to be due to the formation of VSV pseudotype viruses when VSV was complemented with the native forms of HCV envelope proteins. Although only a small amount of pseudotype virus was initially detected in the culture medium, when the harvested pseudotype samples were sonicated for a short time, their titers were enhanced about 100-fold (Fig. 2b). In our assay system, the carryover of VSVΔG*G into HCV pseudotype samples would be minimized by treatment with polyclonal antibody to VSV. Probably due to the efficient decrease in the carryover and the release of pseudotype virions associated with the cell membrane by sonication, we could detect HCV pseudotypes with the native forms of envelopes.

We have reported that bovine and human lactoferrins prevent HCV infection in PH5CH8 human liver cells and MT-2 cells [20,21], and HCV E1 and E2 bind to lactoferrin [35]. We have also reported that the lactoferrin-binding activity of E2 contributes to inhibition of HCV infection [45]. In the present study, pretreatment of VSV pseudotypes with bovine lactoferrin reduced the infectivity of VSVΔG*(HCV) and VSVΔG*(E2) in a dose-dependent manner, whereas pretreatment with transferrin did not (data not shown). In contrast, lactoferrins partially inhibited the infectivity of VSVΔG*(E1) (Fig. 3b). Our results suggested that the interaction between lactoferrin and E2 plays a central role in the inhibition of HCV infection. Taken together, our findings

showed that properties of HCV pseudotypes are consistent with those of HCV virions determined by PCR.

Previously, several groups have demonstrated that not only human hepatic cell lines but also human T cell lines, Molt-4Ma, HPB-Ma, MT-2, and a human B cell line, Daudi, are susceptible to HCV infection [46–49]. In the present study, almost no hematopoietic cell lines were susceptible to any HCV pseudotypes. Only MT-2 and HEL cells showed a marginal susceptibility to the HCV pseudotypes. It is probable that the characteristics of the cell lines might change after long-term cell culture in different laboratories. Interestingly, our results demonstrated that several cell lines derived from the human brain were apparently susceptible to HCV pseudotypes. Encephalomyelitis or encephalitis associated with HCV and cerebral involvement of HCV infection have been reported [50–52]; HCV RNA has been detected in the postmortem brain and brainstem [53].

Weak immunity against HCV infection has been reported [54]. Recently, it has been documented that serum samples from a majority of patients with chronic HCV infection failed to show a detectable neutralization activity against VSV pseudotypes bearing chimeric HCV envelopes [19]. Also in our study, no significant neutralization of any HCV pseudotypes was observed with serum samples from 20 patients with chronic HCV infection. It should be determined whether neutralizing antibody against E1 or E2 alone can neutralize the VSVΔG*(HCV) pseudotype. If E1 and E2 can function independently and the neutralization of both E1 and E2 is necessary for marked inhibition of HCV infectivity, the development of an effective vaccine or an HCV entry-inhibiting agent will be quite difficult. For detection of neutralizing antibody, it may be necessary to examine patients at the different stages of HCV infection, e.g. acute stage of hepatitis.

Table 4 shows that the three different types of HCV pseudotypes prepared with two HCV envelopes showed only a small difference in infectivity in eight types of cells. As for the difference in infectivity between VSVΔG*(HCV) prepared with structural proteins in *cis* and VSVΔG*(C + E1 + E2) prepared with structural proteins in *trans*, it might be explained by the difference in E1–E2 interaction between them. There are reports that both E1 and E2 are necessary for the efficient formation of VSV or retroviral pseudotypes [17,43,44], while VSV pseudotypes complemented with either E1 or E2 alone have been developed [16]. Our findings also suggest that either E1 or E2 alone is enough to make HCV virions (Fig. 2). Recent study indicates that the presence of the complete HCV core sequence is crucial for the expression and/or post-translational processing of the complex-type glycosylated form of E2 [34], and the glycosylation of E1 is enhanced by coexpression of E2 in *cis* [33]. Our results indicate that the core protein might be required for maximal infectivity of pseudotypes (Fig. 2b). Further studies are needed to clarify the role of each envelope protein in the infection by HCV.

Many viruses including herpes viruses, human immunodeficiency virus, Sindbis virus, and in particular, flaviviruses such as dengue virus serotype 2 and Japanese encephalitis virus utilize proteoglycans, especially heparan sulfate, to mediate attachment to and infection of target cells [37,38,55–57]. Recently, Germi et al. [58] reported that cellular heparin-like GAGs might bind to HCV. Our results suggested that highly sulfated forms of GAGs play a role in the early stage of HCV infection (Fig. 4).

Assays of virus infectivity using chemically modified cells suggest that certain cell surface glycoproteins with N-linked oligosaccharides play an important role in VSVΔG*(HCV) infection (Fig. 5b). In addition, pre-treatment of cells with α-mannosidase suppressed the infectivity of VSVΔG*(HCV) by about 70% (Fig. 5c). Further studies on the surface sugar chain structures of cells will be needed to analyze their roles in the entry of HCV.

In conclusion, our system of producing VSV pseudotypes complemented with the native forms of HCV envelopes will be a useful tool with which to analyze the mechanism for HCV virion formation and the function of HCV envelope proteins. This system may also be an efficient tool for research on HCV entry and its inhibitors.

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References

[1] H.J. Alter, R.H. Purcell, J.W. Shih, J.C. Melpolder, M. Houghton, Q.L. Choo, et al., Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis, N. Engl. J. Med. 321 (1989) 1494–1500.

[2] M.E. Major, B. Rehermann, S.M. Feinstone, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, fourth ed. Lippincott, Philadelphia, PA, 2001, pp. 1127–1161.

[3] M.J. Alter, H.S. Margolis, K. Krawczynski, F.N. Judson, A. Mares, W.J. Alexander, et al., The natural history of community-acquired hepatitis C in the United States, N. Engl. J. Med. 327 (1992) 1899–1905.

[4] I. Saito, T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, et al., Hepatitis C virus infection is associated with the development of hepatocellular carcinoma, Proc. Natl. Acad. Sci. USA 87 (1990) 6547–6549.

[5] Q.L. Choo, G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, M. Houghton, Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome, Science 244 (1989) 359–362.

[6] N. Kato, M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, et al., Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis, Proc. Natl. Acad. Sci. USA 87 (1990) 9524–9528.

[7] A. Grakoui, C. Wychowski, C. Lin, S.M. Feinstone, C.M. Rice, Expression and identification of hepatitis C virus polyprotein cleavage products, J. Virol. 67 (1993) 1385–1395.

[8] M.J. Selby, Q.L. Choo, K. Berger, G. Kuo, E. Glazer, M. Eckart, et al., Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome, J. Gen. Virol. 74 (1993) 1103–1113.

[9] V. Deleersnyder, A. Pillez, C. Wychowski, K. Blight, J. Xu, Y.S. Hahn, et al., Formation of native hepatitis C virus glycoprotein complexes, J. Virol. 71 (1997) 697–704.

[10] P. Pileri, Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, et al., Binding of hepatitis C virus to CD81, Science 282 (1998) 938–941.

[11] E. Scarselli, H. Ansuini, R. Cerino, R.M. Roccaseccа, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, A. Vitelli, The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus, EMBO J. 21 (2002) 5017–5025.

[12] V. Agnello, G. Abel, M. Elfahal, G.B. Knight, Q.X. Zhang, Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor, Proc. Natl. Acad. Sci. USA 96 (1999) 12766–12771.

[13] S. Wunschmann, J.D. Medh, D. Klinzmann, W.N. Schmidt, J.T. Stapleton, Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor, J. Virol. 74 (2000) 10055–10062.

[14] F. Masciopinto, G. Freer, V.L. Burgio, S. Levy, L. Galli-Stampino, M. Bendinelli, M. Houghton, S. Abrignani, Y. Uematsu, Expression of human CD81 in transgenic mice does not confer susceptibility to hepatitis C virus infection, Virology 304 (2002) 187–196.

[15] L. Buonocore, K.J. Blight, C.M. Rice, J.K. Rose, Characterization of vesicular stomatitis virus recombinants that express and incorporate high levels of hepatitis C virus glycoproteins, J. Virol. 76 (2002) 6865–6872.

[16] L.M. Lagging, K. Meyer, R.J. Owens, R. Ray, Functional role of hepatitis C virus chimeric glycoproteins in the infectivity of pseudotyped virus, J. Virol. 72 (1998) 3539–3546.

[17] Y. Matsuura, H. Tani, K. Suzuki, T.S. Kimura, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C.S. Robison, M.A. Whitt, T. Miyamura, Characterization of pseudotype VSV possessing HCV envelope proteins, Virology 286 (2001) 263–275.

[18] A. Takada, C. Robison, H. Goto, A. Sanchez, K.G. Murti, M.A. Whitt, et al., A system for functional analysis of Ebola virus glycoprotein, Proc. Natl. Acad. Sci. USA 94 (1997) 14764–14769.

[19] L.M. Lagging, K. Meyer, J. Westin, R. Wejstal, G. Norkrans, M. Lindh, R. Ray, Neutralization of pseudotyped vesicular stomatitis virus expressing hepatitis C virus envelope glycoprotein 1 or 2 by serum from patients, J. Infect. Dis. 185 (2002) 1165–1169.

[20] M. Ikeda, K. Sugiyama, T. Tanaka, K. Tanaka, H. Sekihara, K. Shimotohno, et al., Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes, Biochem. Biophys. Res. Commun. 245 (1998) 549–553.

[21] M. Ikeda, A. Nozaki, K. Sugiyama, T. Tanaka, A. Naganuma, K. Tanaka, et al., Characterization of antiviral activity of lactoferrin against hepatitis C virus infection in human cultured cells, Virus Res. 66 (2000) 51–63.

[22] R.B. DuBridge, P. Tang, H.C. Hsia, P.M. Leong, J.H. Miller, M.P. Calos, Analysis of mutation in human cells by using an Epstein–Barr virus shuttle system, Mol. Cell. Biol. 7 (1987) 379–387.

[23] M. Ikeda, K. Sugiyama, T. Mizutani, T. Tanaka, K. Tanaka, H. Sekihara, et al., Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus, Virus Res. 56 (1998) 157–167.

[24] T. Akagi, T. Shishido, K. Murata, H. Hanafusa, v-Crk activates the phosphoinositide 3-kinase/AKT pathway in transformation, Proc. Natl. Acad. Sci. USA 97 (2000) 7290–7295.

[25] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.

[26] T. Wakita, C. Taya, A. Katsume, J. Kato, H. Yonekawa, Y. Kanegae, et al., Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the cre/loxP system, J. Biol. Chem. 273 (1998) 9001–9006.

[27] M. Inudoh, N. Kato, Y. Tanaka, New monoclonal antibodies against a recombinant second envelope protein of hepatitis C virus, Microbiol. Immunol. 42 (1998) 875–877.

[28] L. Cocquerel, J.C. Meunier, A. Pillez, C. Wychowski, J. Dubuisson, A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2, J. Virol. 72 (1998) 2183–2191.

[29] L. Cocquerel, S. Duvet, J.C. Meunier, A. Pillez, R. Cacan, C. Wychowski, et al., The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum, J. Virol. 73 (1999) 2641–2649.

[30] L. Cocquerel, C. Wychowski, F. Minner, F. Penin, J. Dubuisson, Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a major role in the processing, subcellular localization, and assembly of these envelope proteins, J. Virol. 74 (2000) 3623–3633.

[31] H.J. Ezelle, D. Markovic, G.N. Barber, Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector, J. Virol. 76 (2002) 12325–12334.

[32] B. Bartosch, A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, F.L. Cosset, Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-IB scavenger receptor, J. Biol. Chem. 278 (2003) 41624–41630.

[33] A. Goffard, J. Dubuisson, Glycosylation of hepatitis C virus envelope proteins, Biochimie 85 (2003) 295–301.

[34] L.X. Zhu, L. Liu, Y.C. Li, Y.Y. Kong, C. Staib, G. Sutter, Y. Wang, G.D. Li, Full-length core sequence dependent complex-type glycosylation of hepatitis C virus E2 glycoprotein, World J. Gastroenterol. 8 (2002) 499–504.

[35] M. Yi, S. Kaneko, D.Y. Yu, S. Murakami, Hepatitis C virus envelope proteins bind lactoferrin, J. Virol. 71 (1997) 5997–6002.

[36] N. Kato, M. Ikeda, K. Sugiyama, T. Mizutani, T. Tanaka, K. Shimotohno, Hepatitis C virus population dynamics in human lymphocytes and hepatocytes infected in vitro, J. Gen. Virol. 79 (1998) 1859–1869.

[37] Y. Chen, T. Maguire, R.E. Hileman, J.R. Fromm, J.D. Esko, R.J. Linhardt, et al., Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate, Nat. Med. 3 (1997) 866–871.

[38] C.M. Su, C.L. Liao, Y.L. Lee, Y.L. Lin, Highly sulfated forms of heparin sulfate are involved in Japanese encephalitis virus infection, Virology 286 (2001) 206–215.

[39] P.A. Baeuerle, W.B. Huttner, Chlorate—a potent inhibitor of protein sulfation in intact cells, Biochem. Biophys. Res. Commun. 141 (1986) 870–877.

[40] F. Safaiyan, S.O. Kolset, K. Prydz, E. Gottfridsson, U. Lindahl, M. Salmivirta, Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate, J. Biol. Chem. 274 (1999) 36267–36273.

[41] T.F. Baumert, S. Ito, D.T. Wong, T.J. Liang, Hepatitis C virus structural proteins assemble viruslike particles in insect cells, J. Virol. 72 (1998) 3827–3836.

[42] G. Kuo, Q.L. Choo, H.J. Alter, G.L. Gitnick, A.G. Redeker, R.H. Purcell, et al., An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis, Science 244 (1989) 362–364.

[43] B. Bartosch, J. Dubuisson, F.L. Cosset, Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes, J. Exp. Med. 197 (2003) 633–642.

[44] M. Hsu, J. Zhang, M. Flint, C. Logvinoff, C.C. Mayer, C.M. Rice, J.A. McKeating, Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles, Proc. Natl. Acad. Sci. USA 100 (2003) 7271–7276.

[45] A. Nozaki, M. Ikeda, A. Naganuma, T. Nakamura, M. Inudoh, K. Tanaka, N. Kato, Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein, J. Biol. Chem. 278 (2003) 10162–10173.

[46] T. Mizutani, N. Kato, S. Saito, M. Ikeda, K. Sugiyama, K. Shimotohno, Characterization of hepatitis C virus replication in cloned cells obtained from a human T-cell leukemia virus type I-infected cell line, MT-2, J. Virol. 70 (1996) 7219–7223.

[47] N. Nakajima, M. Hijikata, H. Yoshikura, Y.K. Shimizu, Characterization of long-term cultures of hepatitis C virus, J. Virol. 70 (1996) 3325–3329.

[48] Y.K. Shimizu, A. Iwamoto, M. Hijikata, R.H. Purcell, H. Yoshikura, Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line, Proc. Natl. Acad. Sci. USA 89 (1992) 5477–5481.

[49] Y.K. Shimizu, R.H. Purcell, H. Yoshikura, Correlation between the infectivity of hepatitis C virus in vivo and its infectivity in vitro, Proc. Natl. Acad. Sci. USA 90 (1993) 6037–6041.

[50] D.M. Forton, J.M. Allsop, J. Main, G.R. Foster, H.C. Thomas, S.D. Taylor-Robinson, Evidence for a cerebral effect of the hepatitis C virus, Lancet 358 (2001) 38–39.

[51] H. Fujita, Y. Chuganji, M. Yaginuma, M. Momoi, T. Tanaka, Case report: acute encephalitis immediately prior to acute onset of hepatitis C virus infection, J. Gastroenterol. Hepatol. 14 (1999) 1129–1131.

[52] S. Sacconi, S. Salviati, E. Merelli, Acute disseminated encephalomyelitis associated with hepatitis C virus infection, Arch. Neurol. 58 (2001) 1679–1681.

[53] H. Bolay, F. Soylemezoglu, G. Nurlu, S. Tuncer, K. Varli, PCR detected hepatitis C virus genome in the brain of a case with progressive encephalomyelitis with rigidity, Clin. Neurol. Neurosurg. 98 (1996) 305–308.

[54] A.M. Prince, B. Brotman, T. Huima, D. Pascual, M. Jaffery, G. Inchauspe, Immunity in hepatitis C infection, J. Infect. Dis. 165 (1992) 438–443.

[55] A.P. Byrnes, D.E. Griffin, Binding of sindbis virus to cell surface heparan sulfate, J. Virol. 72 (1998) 7349–7356.

[56] M. Moulard, H. Lortat-Jacob, I. Mondor, G. Roca, R. Wyatt, J. Sodroski, et al., Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type I gp120, J. Virol. 74 (2000) 1948–1960.

[57] D. Shukla, J. Liu, P. Blaiklock, N.W. Shworak, X. Bai, J.D. Esko, et al., A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry, Cell 99 (1999) 13–22.

[58] R. Germi, J.M. Crance, D. Garin, J. Guimet, H. Lortat-Jacob, R.W.H. Ruigrok, et al., Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption, J. Med. Virol. 68 (1999) 206–215.

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cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells

Ken-ichi Abe[a], Masanori Ikeda[a], Hiromichi Dansako[a], Kazuhito Naka[a], Kunitada Shimotohno[b], Nobuyuki Kato[a,*]

[a] *Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[b] *Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawara-cho Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan*

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Abstract

The hepatitis C virus (HCV) replicon system carrying autonomously replicating HCV subgenomic RNA in human hepatocyte cells is a potent tool for basic studies of HCV, such as viral replication and drug development. Recently, we developed two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively. Since the expression of HCV proteins is thought to affect the host cells' gene expression profiles, we attempted to identify target genes of HCV proteins using microarray analysis (9970 genes) by comparing 50-1 and 1B-2R1 replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with interferon-α. The results showed that HCV replicons could have a variety of expression profiles in human hepatocytes. The results also showed that 2 and 6 genes were commonly up-regulated (more than 2.0-fold) and down-regulated (less than 0.50-fold), respectively, in both 50-1 and 1B-2R1 replicon cells compared with their cured cells. The differential expression profiles of genes selected by the microarray analysis were confirmed with standard RT-PCR and real-time LightCycler PCR. It was noteworthy that the commonly down-regulated genes contained large multifunctional proteases 2 and 7, which are known as catalytic subunits of immunoproteasome, and serine proteinase inhibitor clade C. Our microarray analysis demonstrated that HCV subgenomic replicons can change the gene expression profiles of host cells, and it allowed us to compile the first list of genes that the replicons transcriptionally regulate.
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Keywords: Hepatitis C virus; HCV subgenomic replicon; Cured cells; cDNA microarray; Gene expression profile

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo et al., 1989; Kuo et al., 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi et al., 1990; Saito et al., 1990). HCV belongs to the family Flaviviridae, whose genome consists of a positive-stranded RNA molecule of about 9.6 kb and encodes a large polyprotein precursor of about 3000 amino acids (Kato et al., 1990; Tanaka et al., 1995). This precursor protein is cleaved by the host and viral proteases to generate at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. Although many hypotheses have been proposed over the past decade regarding the functions of the viral proteins (Bartenschlager and Lohmann, 2000; Kato, 2001), the lack of reproducible and efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000) has been a serious obstacle in understanding those proteins' actual functions.

However, in 1999, an HCV replicon system carring autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions was first established using a human hepatoma cell line, Huh-7 (Lohmann et al., 1999). Since then, several additional replicons have also been established (Blight et al., 2000, 2003; Ikeda et al., 2002; Kato et al., 2003b). In these systems, replicated HCV RNAs were detected by Northern blot analysis, and the HCV proteins pro-

* Corresponding author. Tel.: +81 86 235 7385; fax: +81 86 235 7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).

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duced were detected by Western blot analysis. Therefore, the system of HCV replicons has become a powerful tool for basic studies in HCV, such as viral replication and drug development (Bartenschlager, 2002).

Recently, we also established two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using Huh-7 cells (Kato et al., 2003a; Kishine et al., 2002). We demonstrated that the 50-1 and 1B-2R1 subgenomic replicons (Kato et al., 2003a) were sensitive to interferon (IFN)-α, IFN-β and IFN-γ as are the other replicons (Frese et al., 2001, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 subgenomic replicon showed differences of 8.1% from those in the1B-2R1 subgenomic replicon (Kato et al., 2003a), although both the 1B-1 and 1B-2 strains belong to genotype 1b. Although the efficient replication of an HCV subgenomic replicon expressing HCV proteins is considered to affect the gene expression profiles of host cells (Bartenschlager and Lohmann, 2000; Kato, 2001), few reports have demonstrated inclusive searches for HCV's target genes (Zhu et al., 2003). Therefore, we thought a comprehensive search for HCV subgenomic replicon-regulated cellular genes would be important in understanding the molecular interplay exerted by HCV in vivo.

In the present study, to obtain the candidates of HCV's target genes, we performed cDNA microarray analysis by comparing two types of HCV subgenomic replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with IFN-α. Here we report on the differential gene expression profiles in the replicon cells, and we first provide a list of genes that the replicons transcriptionally regulate.

2. Materials and methods

2.1. Cell cultures

50-1 and 1B-2R1 cells possessing 50-1 and 1B-2R1 subgenomic replicons, respectively, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and G418 (300 mg/ml; Geneticine, Invitrogen). The 50-1 and 1B-2R1 cells were known to possess the G418-resistant phenotype, because neomycin phophotransferase (NEOR) was produced by the efficient replication of HCV subgenomic replicon in the cells. When an HCV subgenomic replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. Therefore, the cured cells obtained from 50-1 and 1B-2R1 cells were maintained in the absence of G418.

2.2. IFN treatment

To prepare the cured cells, 50-1 and 1B-2R1 cells (each 1×10^6) were plated onto 10-cm plates and were cultured for 1 day immediately before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 3000 IU/ml as described previously (Kato et al., 2003a). The incubation in the absence of G418 was continued for 3 weeks with the addition of IFN-α (3000 IU/ml) at 4-day intervals. The cured cells obtained from 50-1 and 1B-2R1 cells were named 50-1C and 1B-2R1C cells, respectively.

2.3. Northern blot analysis

Total RNAs from the cultured cells were prepared using the RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used to detect the HCV replicon RNA and β-actin. Northern blotting and hybridization were performed as described previously (Ikeda et al., 2002; Kato et al., 2003a). As a molecular length marker, replicon RNA synthesized in vitro from replicon cassette plasmid pNSS1RZ2RU (Kato et al., 2003a) was also utilized.

2.4. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as previously described (Hijikata et al., 1993). The antibodies used in this study were those against NS3 (Novacastra Laboratories, UK), NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), and β-actin (Sigma). Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

2.5. cDNA microarray analysis

The 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells (each 1×10^6 cells) were plated onto 10 cm plates, and each plate was cultured for 5 days in the absence of G418. The confluent cells were harvested and total RNAs were prepared using the RNeasy extraction kit (Qiagen). Using the obtained total RNAs, cDNA microarray analysis (CodeLink™, Uniset human I containing 9970 spots of 30-mer oligonucleotides; Amersham Biosciences) was performed by Kurabo Industries Ltd. (Osaka, Japan) with the authorization of Amersham Biosciences.

2.6. Analysis of mRNA expression by RT-PCR

The total RNAs (each 2 μg) that were the same as those subjected to cDNA microarray analysis were reverse-transcribed with Superscript II using an oligo dT primer (Invitrogen). One-tenth of the synthesized cDNA was subjected to PCR. The PCR primers are listed in Table 1. After 10 min at 98 °C, PCR was performed with Taq DNA polymerase (TaKaRa, Japan). Each cycle consisted of annealing at 60 °C (64 °C for LMP2 and LMP7 only) for 45 s, primer extension at 72 °C for 1 min, and denaturation at 94 °C for 20 s. The cycle numbers and the size of PCR products were also

Table 1
The primers used for RT-PCR analysis of mRNA expression

Genes	Orientation	Nucleotide sequence	Product (bp)	Cycles
Large multifunctional protease2 (LMP2)	Forward	ATGGAACCCTGGGAGGAATGCTG	145	30
	Reverse	GCAATAGCGTCTGTGGTGAAGCG		
Large multifunctional protease 7 (LMP7)	Forward	CTGGGATAAGAAGGGTCCTGGAC	293	27
	Reverse	TACTGGTGCAGCAGGTCACTGGAC		
Serine proteinase inhibitor (serpin) clade C	Forward	TGGATGAATTGGAGGAGATGATGC	249	25
	Reverse	CAATCACAACAGCGGTACTTGCAG		
S100-type calcium binding protein A14	Forward	CAGAGGATGCTCAGGAATTCAGTG	256	27
	Reverse	CTCTTGGCCGCTTCTCCAATGAG		
Latent transforming growth factor β binding protein 1 (LTBP1)	Forward	GCCTTGGTTGACTTCAGTGAACAG	325	27
	Reverse	CAGAAGGCACGTAGCCTGGCAG		
Weakly similar to zinc finger protein 91	Forward	CCAGAACCACATCCAAACCATCC	299	33
	Reverse	CCATCCCTTCGAAGCTGTGCTC		
Transgelin	Forward	GATTCTGAGCAAGCTGGTGAACAG	254	25
	Reverse	AGTGCCCATCATTCTTGGTCACTG		
Annexin A1	Forward	GATGCCAGGGCCTTGTATGAAGC	264	25
	Reverse	AACACCTTTCATGGCTTGATGAAGC		
Solute carrier family 7	Forward	AGTCCTTCGCTGGAAGAAGCCTG	314	27
	Reverse	CCATGTCCTCATTAGCCTCCTCTG		
Protein phosphatase 1 regulatory subunit 1A	Forward	CCACGGCAACGGAAGAAGATGAC	302	27
	Reverse	GCTCCCTTGGAATCCAGTGGTGG		
Phosphatidylserine-specific phospholipase A1α	Forward	GAGAAACAAGGACACCAACATCGAG	288	28
	Reverse	GTCACACTTGCTTGTAAGTTCACTG		
Oncostatin M receptor	Forward	CAGAAAAGAGTCACTCTGGCCCTG	292	27
	Reverse	GGTGCCTCTACTGGGTTTGTTGG		
Similar to interferon-induced protein 35	Forward	CCGTATGTGAATGGGGAGATCCAG	222	27
	Reverse	GCCTGACTCAGAGGTGAAGACTG		
Caspase 1	Forward	AGAAACACTCTGAGCAAGTCCCAG	278	30
	Reverse	AACATTATCTGGTGTGGAAGAGCAG		
Neutrophil cytosolic factor 2	Forward	GACATGGTGTCTAAGAAACTGGAG	277	27
	Reverse	CTCATAACTGAAGAGTGCCTCCAC		
Putative secred protein ZSIG13	Forward	CTGGTTATGACAATGACCGACCAG	272	25
	Reverse	GCAGATCTGGGCATATTTGAGAGG		
GAPDH	Forward	GACTCATGACCACAGTCCATGC	334	22
	Reverse	GAGGAGACCACCTGGTGCTCAG		

listed in Table 1. The PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. RT-PCR was performed in duplicate experiments. The mRNA levels of target genes were monitored by a Chemilmager 4400 (Alpha Innotech), which measured the intensities of bands stained with ethidium bromide as described previously (Kato et al., 2003a). As an internal control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was amplified by RT-PCR, and the products were used to normalize the mRNA levels of the target genes.

2.7. LightCycler PCR

One-twentieth of the cDNA synthesized above was subjected to real-time LightCycler PCR as described previously (Nozaki and Kato, 2002; Nozaki et al., 2003). The primers listed in Table 1 were also used for LightCycler PCR. Temperature cycling conditions for each primer set consisted of 10 min at 95 °C followed by 35 cycles for 1 s at 94 °C, 5 s at 60 °C (64 °C for LMP2 and LMP7 only), and 6–14 s (25 bp per second) at 72 °C. All reactions were performed in a LightCycler™ Quick System 330 (Roche) using Fast-Start DNA Master SYBR Green I mix (Roche) according to the manufacturer's instructions. The experiments were performed in at least triplicate. The relative mRNA expression ratios of the target genes were calculated based on crossing-point analysis using a second derivative maximum method (LightCycler analysis software version 3.5). To correct for differences in RNA quality and quantity between the samples, data were normalized using the ratio of the target cDNA concentration to that of GAPDH. This ratio was assessed by a different reaction in the same experimental round.

Table 2
Genes whose expression levels were commonly altered in 1B-2R1 and 50-1 cells compared with their cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Up-regulation (more than 2-fold)			
Phosphatidylserine-specific phospholipase A1α[a]	2.2	2.9	NM_015900
Oncostatin M receptor[a]	2.1	2.2	NM_003999
Down-regulation (less than 0.50-fold)			
LMP2[a]	0.14	0.30	NM_002800
LMP7[a]	0.21	0.44	NM_004159
Similar to interferon-induced protein 35[a]	0.31	0.32	BC001356
Weakly similar to zinc finger protein 91[a]	0.36	0.42	AK027354
Protein phosphatase 1, regulatory subunit 1A[a]	0.40	0.32	NM_006741
Serpin clade C[a]	0.49	0.31	NM_000488

[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

3. Results

3.1. Preparation of the cured cells from 50-1 and 1B-2R1 cells

To obtain cured cells for the microarray analysis, 50-1 and 1B-2R1 cells were cultured with prolonged IFN-α treatment as described Section 2. After 3 weeks of this treatment, we demonstrated by Northern blot analysis that the replicon RNAs were not detected in the IFN-α-treated (50-1C and 1B-2R1C) cells, although approximately 10^8 copies of replicon RNA were detected in the total RNA (3 mg) extracted from 50-1 and 1B-2R1 cells (Fig. 1A). We further confirmed by RT-nested PCR (Mizutani et al., 1996) for the detection of the 5′-untranslated region that the replicon RNAs were absolutely excluded from the cells (data not shown). Western blot analysis also showed that the NS3 and NS5B proteins were no longer detected in 50-1C and 1B-2R1C cells, but were detected in 50-1 and 1B-2R1 cells, as shown in Fig. 1B.

3.2. cDNA microarray analysis

To examine the effects of HCV replicons on gene expression in host cells, cDNA microarray analyses (CodeLink™, Amersham Biosciences; 9970 human genes) were performed by comparing 1B-2R1 with 1B-2R1C cells and 50-1 with 50-1C cells. The majority of genes examined showed only small differences, with ratios ranging between 2.0 and 0.50 (data not shown). There were 55 and 101 up-regulated genes (those

Table 3
Genes whose expression levels were up-regulated (more than 3-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
AU62G04.X1	**8.5**	1.4	AI929792
Homeobox 1(HESX1)	**4.2**	0.50	NM_003865
Microsomal NAD+ dependent retinol dehydrogenase 4	**3.4**	0.92	NM_003708
Advillin	**3.3**	0.61	NM_006576
SSFV proviral integration oncogene Spi1	**3.1**	1.0	NM_003120
Napsin 2 precursor	**3.1**	0.94	AF098485
Transgelin[a]	0.85	**8.5**	NM_003186
Uncharacterized bone marrow protein BM040	0.81	**5.8**	AF217516
Annexin A1[a]	1.0	**4.2**	NM_000700
Putative secreted protein ZSIG13[a]	1.7	**3.9**	AF193611
Protease serine 23	1.2	**3.8**	NM_007173
Colon cancer antigen NY-CO-45	1.3	**3.7**	AF039442
HSPC157 protein	1.1	**3.5**	NM_014179
Uronyl-2-sulfotransferase	1.0	**3.5**	NM_005715
Cadherin, EGF lag seven-pass G-type receptor 2	0.68	**3.5**	NM_001408
Hypothetical protein (LOC51321)	1.1	**3.4**	NM_016627
Kidney-specific membrane protein (NX-17)	1.0	**3.3**	NM_020665
Neutrophil cytosolic factor 2[a]	1.8	**3.2**	NM_000433
Amphiregulin	1.4	**3.1**	NM_001657
Fibrillin 1	0.83	**3.1**	NM_000138
LTBP1[a]	1.6	**3.0**	NM_000627

The numbers of more than 3-fold were indicated by bold letters.
[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

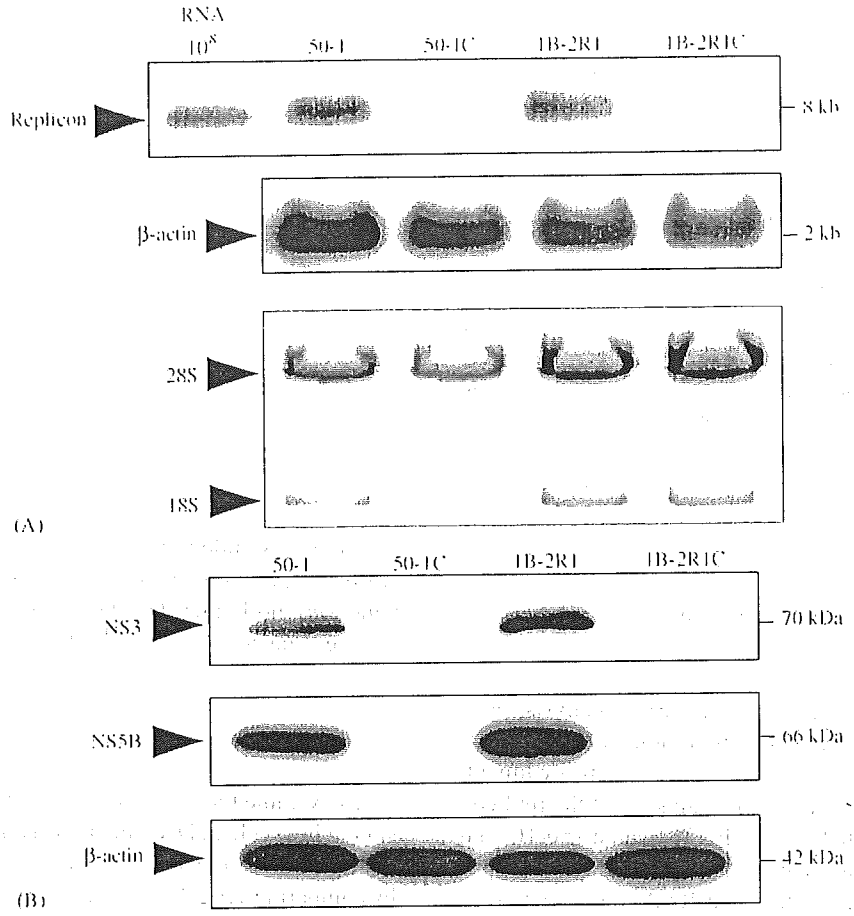

Fig. 1. Characterization of the replicon cells and their cured cells. (A) Northern blot analysis. Total RNAs from 50-1 and 1B-2R1 cells, as well as total RNAs from the cured cells, were analyzed by Northern blotting using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific RNA probe (middle panel). RNA samples were equalized for 28S and 18S ribosomal RNAs stained with ethidium bromide (lower panel). A synthetic RNA transcribed from pNSS1RZ2RU (10^8 genome equivalents spiked into normal cellular RNA) was used as a positive control. (B) Western blot analysis. Productions of NS3 and NS5B in 50-1 and 1B-2R1 cells were analyzed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. 50-1C and 1B-2R1C cells were also analyzed to confirm the lack of NS3 and NS5B proteins. β-actin was used as a control for the amount of protein loaded per lane.

with ratios of more than 2.0) in 1B-2R1 and 50-1 cells, respectively. Between the two types of replicon cells, only two genes were commonly up-regulated. There were 56 and 74 down-regulated genes (those with ratios of less than 0.50) in 1B-2R1 and 50-1 cells, respectively, of which 6 genes were commonly down-regulated in both types of replicon cells. Table 2 summarizes the genes that the replicons commonly affected. Among these genes, it is noteworthy that large multifunctional proteases 2 (LMP2) and LMP7, which have been known as catalytic subunits in immunoproteasome (Akiyama et al., 1994; Tanaka and Kasahara, 1998), and serine proteinase inhibitor (serpin) clade C (Gettins, 2002) were down-regulated in both types of replicon cells (discussed below). However, no common genes were directly linked to the transformation of the cells. Since the standard of selection seemed to be rather strict, we further selected the genes whose expression levels were up-regulated or down-regulated with ratios of more than 3.0 or less than 0.33, respectively, in either 1B-2R1 or 50-1 cells. By this method, we selected 6 and 15 genes as up-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 3); and 6 and 9 genes as down-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 4). These selections allowed us to find several additional genes, including latent transforming growth factor β binding protein 1 (LTBP1) and caspase 1, that were commonly regulated in both types of replicons.

3.3. RT-PCR confirmation of the alteration of gene expression by HCV replicons

To confirm the results of our microarray selection, we examined the levels of several mRNAs by RT-PCR in duplicate. As shown by the stars in Tables 2-4, 16 genes (7 up-regulated and 9 down-regulated) were subjected to RT-PCR analysis. As shown in Fig. 2, RT-PCR confirmed that the expressions of most of these genes changed. This result suggests that the relative mRNA expression ratio obtained by the microarray analysis reflects the differential expres-

Table 4
Genes whose expression levels were down-regulated (less than 0.33-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Hephaestin	**0.14**	1.7	NM_014799
Solute carrier family 7[a]	**0.15**	0.62	NM_003982
Caspase 1[a]	**0.18**	0.65	NM_033292
Protease inhibitor 3	**0.19**	1.1	NM_002638
Collagen type II α1	**0.31**	1.6	NM_033150
C-terminal binding protein 2	**0.31**	0.71	NM_022802
ATPase α polypeptide (ATP 12A)	0.57	**0.26**	NM_001676
Hypothetical protein FLJ20043	0.79	**0.27**	NM_017637
CM2-HT0948-070900-368-D08 cDNA	1.0	**0.28**	BF089733
S100-type calcium binding protein A14[a]	0.62	**0.30**	NM_020672
Hypothetical protein MGC2827	0.65	**0.31**	NM_023940
EGFL6	2.4	**0.32**	NM_015507
ISL1 transcription factor	0.94	**0.32**	NM_002202
Pre-α globulin inhibitor	1.2	**0.32**	NM_002217
Regulator of G-protein signalling 16	0.65	**0.33**	NM_002928

The numbers of less than 0.33-fold were indicated by bold letters.
[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

sion profiles of the replicon and its cured cells. Of the 16 genes, 9 (4 up-regulated and 5 down-regulated) were further subjected to real-time LightCycler PCR analysis in order to obtain the actual ratios of mRNA expression. As shown in Table 5, the resultant relative mRNA expression ratios actually correlated with those obtained by our microarray analysis. Regarding the selected genes in this study, we confirmed by RT-PCR the reproducibility of the relative mRNA ratios using different lots of RNA specimens derived from 1B-2R1 and 1B-2R1C cells (data not shown). Taken together, our results suggest that these altered mRNA expressions are caused by the multiplication of HCV subgenomic replicons.

4. Discussion

This study yielded evidence of alterations in gene expression by HCV subgenomic replicons in human hepatocytes, as observed through microarray analysis (9970 genes), and first provided a list of genes including LMP2, LMP7, and serpin clade C that the replicons transcriptionally regulate.

To date, only one report of cDNA microarray analysis (832 cytokine-related genes) has been conducted by comparing HCV subgenomic replicon cells with parental Huh-7 cells (Zhu et al., 2003). That analysis obtained 14 up-regulated genes (those with ratios of more than 2.0) in the replicon cells. However, the parental Huh-7 cells may not be appropriate for use as control cells in such microarray analyses, because the HCV subgenomic replicon cells used are derived from a single cloned cell. Therefore, it is very important to avoid the clone-based differences for microarray analysis. From this principal reason, we used two types of cured cells derived from 50-1 and 1B-2R1 cells as the control cells for our microarray analysis. The cured cells are considered to have the same background as the replicon cells. The possibility remains that the genes selected in this study were obtained by the effect of IFN-α that was used to

Table 5
LightCycler RT-PCR analysis of genes whose expression levels were altered by HCV replicons

Genes	Relative mRNA expression ratio (mean ± S.D.)	
	1B-2R1/1B-2R1C	50-1/50-1C
Up-regulation		
Phosphatidylserine-specific phospholipase A1α	2.03 ± 0.09	3.09 ± 0.74
Oncostatin M receptor	2.58 ± 0.20	2.46 ± 0.49
Transgelin	0.83 ± 0.11	13.72 ± 0.56
Annexin A1	1.19 ± 0.17	4.23 ± 0.72
Down-regulation		
LMP2	0.06 ± 0.00	0.40 ± 0.12
LMP7	0.09 ± 0.02	0.33 ± 0.08
Serpin clade C	0.39 ± 0.11	0.37 ± 0.11
Solute carrier family 7	0.13 ± 0.08	0.77 ± 0.18
S100-type calcium binding protein A14	0.37 ± 0.21	0.32 ± 0.17

tralize target proteinases by a mechanism that conformationally distorts the proteinase (Gettins, 2002), the relationship between serpin clade C and HCV serine proteinase is interesting. To clarify this relationship, further analysis, such as that of the compulsory expression of serpin clade C in the replicon cells, will be necessary.

In this study, we demonstrated that microarray analysis to compare HCV subgenomic replicon cells with their cured cells was useful for screening and selecting HCV's target genes. Also, we compiled the first list of genes transcriptionally regulated by the multiplication of HCV subgenomic replicons. Although we need to clarify the mechanisms underlying transcriptional regulation by HCV subgenomic replicons, we believe that the genes involved in viral replication and multiplication are among the genes listed in this study. Further analysis using new experimental systems, such as the full-length HCV RNA replicating system, will be useful to clarify this point.

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References

Akiyama, K., Kagawa, S., Tamura, T., Shimbara, N., Takashina, M., Kristensen, P., Hendil, K.B., Tanaka, K., Ichihara, A., 1994. Replacement of proteasome subunits X and Y by LMP7 and LMP2 induced by interferon-gamma for acquirement of the functional diversity responsible for antigen processing. FEBS Lett. 18, 85–88.

Bartenschlager, R., 2002. Hepatitis C virus replicons: potential role for drug development. Nat. Rev. Drug Discov. 1, 911–916.

Bartenschlager, R., Lohmann, V., 2000. Replication of hepatitis C virus. J. Gen. Virol. 81, 1631–1648.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974.

Blight, K.J., McKeating, J.A., Marcotrigiano, J., Rice, C.M., 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. 77, 3181–3190.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359–362.

Fehling, H.J., Swat, W., Laplace, C., Kuhn, R., Rajewsky, K., Muller, U., von Boehmer, H., 1994. MHC class I expression in mice lacking the proteasome subunit LMP-7. Science 265, 1234–1237.

Frese, M., Pietschmann, T., Moradpour, D., Haller, O., Bartenschlager, R., 2001. Interferon-a inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. J. Gen. Virol. 82, 723–733.

Frese, M., Schwarzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O., Bartenschlager, R., 2002. Interferon-g inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology 35, 694–703.

Gettins, P.G., 2002. Serpin structure, mechanism, and function. Chem. Rev. 102, 4751–4804.

Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Kimura, K., Shimotohno, K., 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. U.S.A. 90, 10773–10777.

Ikeda, M., Yi, M., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76, 2997–3006.

Kato, N., 2001. Molecular virology of hepatitis C virus. Acta Med. Okayama 55, 133–159.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., Shimotohno, K., 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U.S.A. 87, 9524–9528.

Kato, N., Shimotohno, K., 2000. Systems to culture hepatitis C virus. Curr. Top. Microbiol. Immunol. 242, 261–278.

Kato, N., Sugiyama, K., Namba, K., Dansako, H., Nakamura, T., Takami, M., Naka, K., Nozaki, A., Shimotohno, K., 2003a. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem. Biophys. Res. Commun. 306, 756–766.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003b. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125, 1808–1817.

Kishine, H., Sugiyama, K., Hijikata, M., Kato, N., Takahashi, H., Noshi, T., Nio, Y., Hosaka, M., Miyanari, Y., Shimotohno, K., 2002. Subgenomic replicon derived from a cell line infected with the hepatitis C virus. Biochem. Biophys. Res. Commun. 293, 993–999.

Kuo, G., Choo, Q.L., Alter, H.J., Gitnick, G.L., Redeker, A.G., Purcell, R.H., Miyamura, T., Dienstag, J.L., Alter, M.J., Stevens, C.E., Tegtmeier, G.E., Bonino, F., Colombo, W.S., Lee, W.S., Kuo, C., Berger, K., Shuster, J.R., Overby, L.R., Bradley, D.W., Houghton, M., 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244, 362–364.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Mizutani, T., Kato, N., Saito, S., Ikeda, M., Sugiyama, K., Shimotohno, K., 1996. Characterization of hepatitis C virus replication in cloned cells obtained from a human T-cell leukemia virus type I infected cell line MT-2. J. Virol. 70, 7219–7223.

Nozaki, A., Kato, N., 2002. Quantitative method of intracellular hepatitis C virus RNA using LightCycler PCR. Acta Med. Okayama 56, 107–110.

Nozaki, A., Ikeda, M., Naganuma, A., Nakamura, T., Inudoh, M., Tanaka, K., Kato, N., 2003. Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein. J. Biol. Chem. 278, 10162–10173.

Ohkoshi, S., Kojima, H., Tawaraya, H., Miyajima, T., Kamimura, T., Asakura, H., Satoh, A., Hirose, S., Hijikata, M., Kato, N., Shimotohno, K., 1990. Prevalence of antibody against non-A, non-B hepatitis virus in Japanese patients with hepatocellular carcinoma. Jpn. J. Cancer Res. 81, 550–553.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.L., Houghton, M., Kuo, G., 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. U.S.A. 87, 6547–6549.

Scholle, F., Li, K., Bodola, F., Ikeda, M., Luxon, B.A., Lemon, S.M., 2004. Virus-host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis. J. Virol. 78, 1513–1524.

Tanaka, K., Kasahara, M., 1998. The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-gamma-inducible proteasome activator PA28. Immunol. Rev. 163, 161–176.

Tanaka, T., Kato, N., Cho, M.-J., Shimotohno, K., 1995. A novel sequence found at the 3' terminus of hepatitis C virus genome. Biochem. Biophys. Res. Commun. 215, 744–749.

Van den Eynde, B.J., Morel, S., 2001. Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. Curr. Opin. Immunol. 13, 147–153.

Van Kaer, L., Ashton-Rickardt, P.G., Eichelberger, M., Gaczynska, M., Nagashima, K., Rock, K.L., Goldberg, A.L., Doherty, P.C., Tonegawa, S., 1994. Altered peptidase and viral-specific T cell response in LMP2 mutant mice. Immunity 1, 533–541.

Zhu, H., Zhao, H., Collins, C.D., Eckenrode, S.E., Run, Q., McIndoe, R.A., Crawford, J.M., Nelson, D.R., She, J.X., Liu, C., 2003. Gene expression associated with interferon alfa antiviral activity in an HCV replicon cell line. Hepatology 37, 1180–1188.