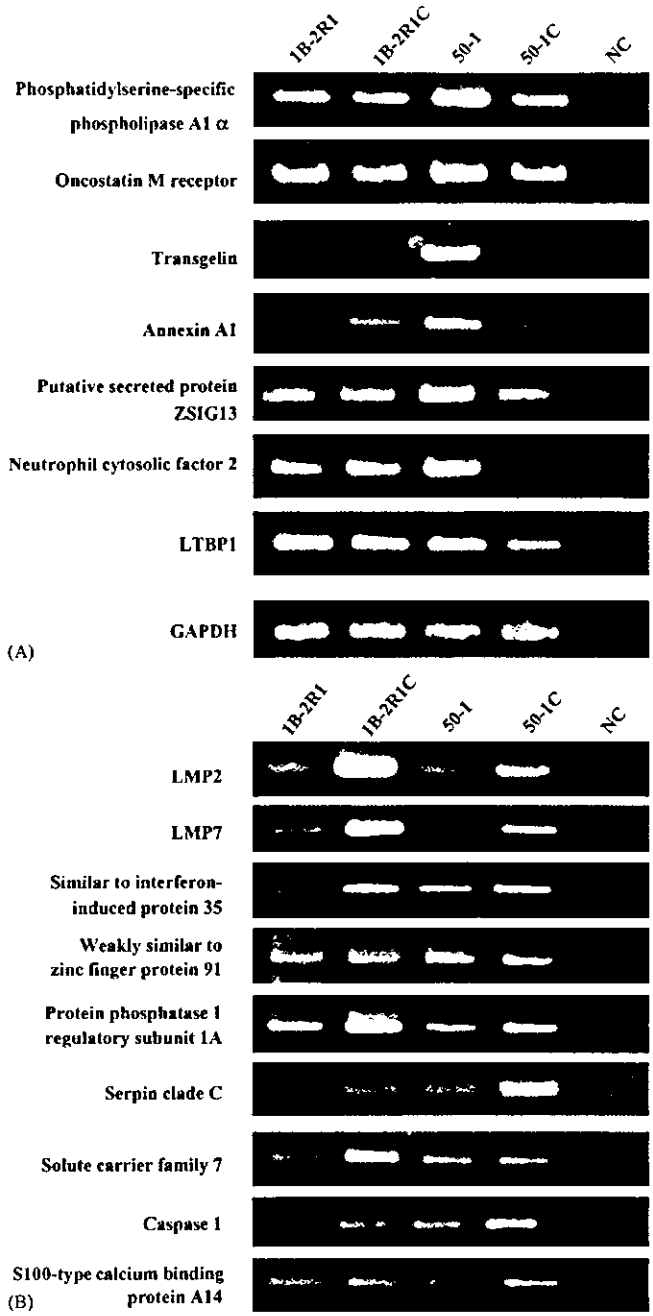

Fig. 2. RT-PCR analysis of mRNA expression of selected genes based on the microarray results. The total RNAs extracted from 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells were subjected to RT-PCR, and the mRNA levels of target genes were monitored as described in Section 2. The primers used, PCR product lengths, and PCR cycle numbers are listed in Table 1. GAPDH was used as a control. (A) Up-regulated genes. (B) Down-regulated genes.

eliminate replicon RNA. However, this possibility is quite low because most of the genes regulated by IFN-α were not transcriptionally altered according to our microarray analysis, and it is unlikely that IFN-α treatment irreversibly altered the expression of genes that it either induced or suppressed. In actuality, most genes selected by our cDNA microarray analysis were not related to the genes regulated by IFN-α, although the selected genes in this study did not show any common characteristics involved in the progression of hepatic diseases. Very recently, after most of the present study was finished, a cDNA microarray analysis using full-length HCV RNA replicating cells and their cured cells was reported by Scholle et al. (2004). Although those authors found dozens of genes whose expression levels were altered by the replication of HCV RNA derived from HCV strain N, we had no information on the genes they had selected and therefore we could not compare them with ours. Therefore, we are currently establishing full-length HCV RNA replicating cells derived from 1B-1 and 1B-2 HCV strains. Further cDNA microarray analysis using such full-length HCV RNA replicating cells will help to identify HCV's target genes.

Among the genes selected in this study, LMP2 and LMP7 are quite interesting. Both are known as important catalytic subunits in immunoproteasome induced by IFN-γ (Akiyama et al., 1994; Tanaka and Kasahara, 1998). In the presence of IFN-γ, the three catalytic subunits of vertebrate proteasomes are replaced by their homologous subunits, LMP2, LMP7, and MECL1, to form immunoproteasome, which increased the ability to produce peptides with a proper motif for efficient MHC binding (Fehling et al., 1994; Van Kaer et al., 1994). A number of peptides that were poorly processed by the standard proteasome were recently found to be more effectively produced by the immunoproteasome (Van den Eynde and Morel, 2001). Therefore, down-regulation of LMP2 and LMP7 expressions in HCV subgenomic replicon cells will reduce the production efficiency of viral antigenic peptides presented to CD8+ T cells (Van den Eynde and Morel, 2001), and may subsequently help to cause the persistent viral infection. In contrast with the expression of LMP2 and LMP7, that of the MECL1 gene was not altered regardless of the presence of HCV replicon. The molecular mechanism by which HCV replicon cells suppress LMP2 and LMP7 remains unknown. This phenomenon is considered to be caused by one of the HCV NS proteins in the replicon cells. As a first step toward identifying the responsible NS protein, we carried out a preliminary experiment using 1B-2R1C cells that stably expressed NS3, NS4A, NS4B, NS5A, NS5B, or NS3-NS5B protein by retrovirus-mediated gene transfer. Unfortunately, however, this experiment failed to identify the responsible NS protein. This result suggests that either the replication of replicon RNA or replicon RNA itself is necessary to suppress LMP2 and LMP7 gene expression. To clarify this point, further analysis will be necessary, using HCV subgenomic replicon cells derived from the other HCV strains or HCV subgenomic replicon cells re-established by the transfection of 50-1 or 1B-2R1 subgenomic replicon RNA.

A third interesting gene obtained in this study was serpin clade C. Although the expression of the serpin clade C gene was down-regulated to approximately one-third in HCV subgenomic replicon cells, those of the other eight clades of the serpin family were not quite altered. Since serpins are a unique class of proteinase inhibitors that irreversibly neu-

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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Original article

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

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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.

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

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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

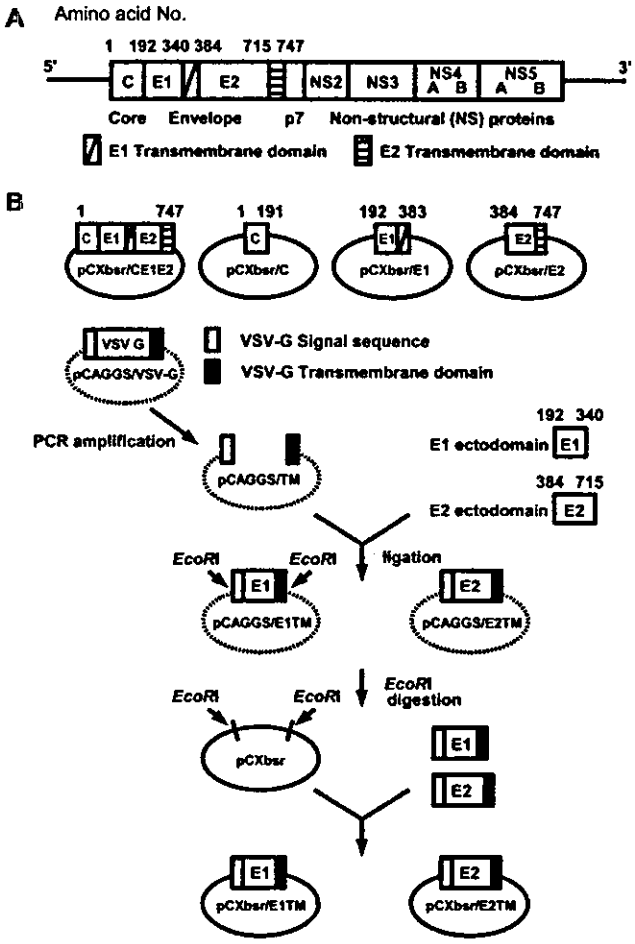

Fig. 1. Preparation of plasmids expressing HCV envelope proteins. (a) Structure of HCV genome. (b) Schematic representation of the plasmids and the chimeric gene constructs for the expression of HCV envelope glycoproteins. TM represents the signal sequence and the transmembrane domain of VSV G protein.

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 Ib [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′-AAAAGCTCTATTGCCTCTTTTT-TCTTTATC;
- antisense primer, 5′-GCAATTCACCCCAATGAATA-AAAAGGCTAA.

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′-TATGAAGTGCGCAACGTGTCCGG-GGTGTAC;
- antisense primer, 5′-GATCCGGAGCAACTGCGA-TACCACCAGGGC.

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′-GCTACCTACACGTCAGGGGGGAC-GGTAGGC;
- antisense primer, 5′-TCTGATTACAACGGAGACAAC-CACTGACCC.

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

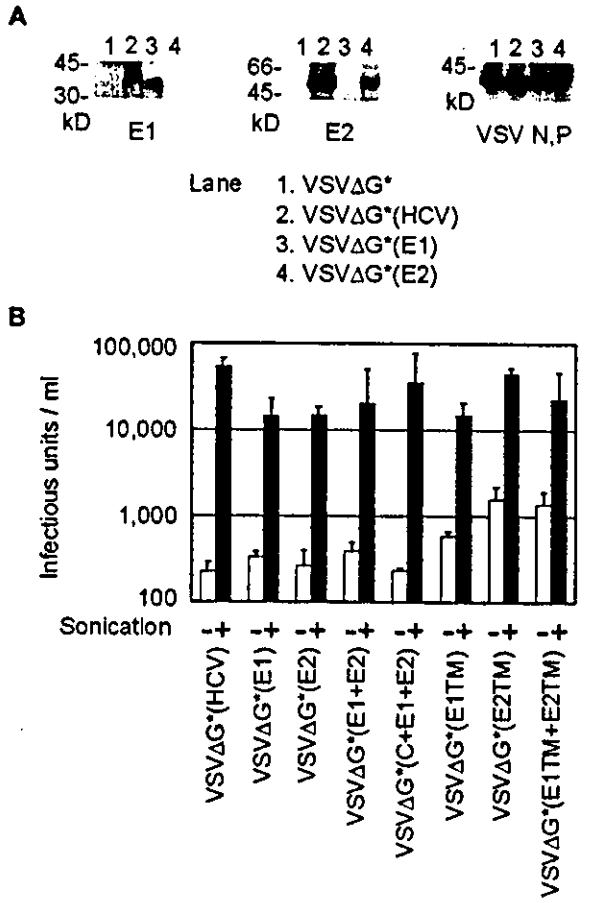

Fig. 2. (a) Western blot analyses of the pseudotype VSVs bearing HCV envelope proteins. Immunoblots of pseudotype virus samples through 20% sucrose cushions are shown. The preparation of each pseudotype sample is described in Table 2. E1 and E2 glycoproteins were detected with E1-384 and Mo-12 monoclonal 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].

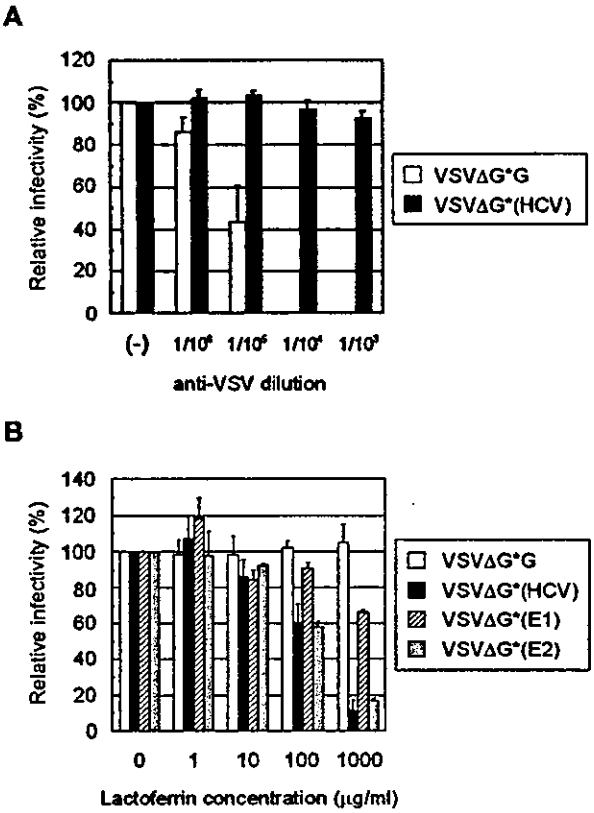

Fig. 3. (a) Neutralization of the pseudotype viruses. Two hundred IU of VSVΔG*(HCV) and VSVΔG*G was preincubated with the indicated dilutions of polyclonal antibody against VSV for 30 min and then inoculated to HepG2 cells. (b) Effect of bovine lactoferrin on the infectivity of pseudotype viruses. Each 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.

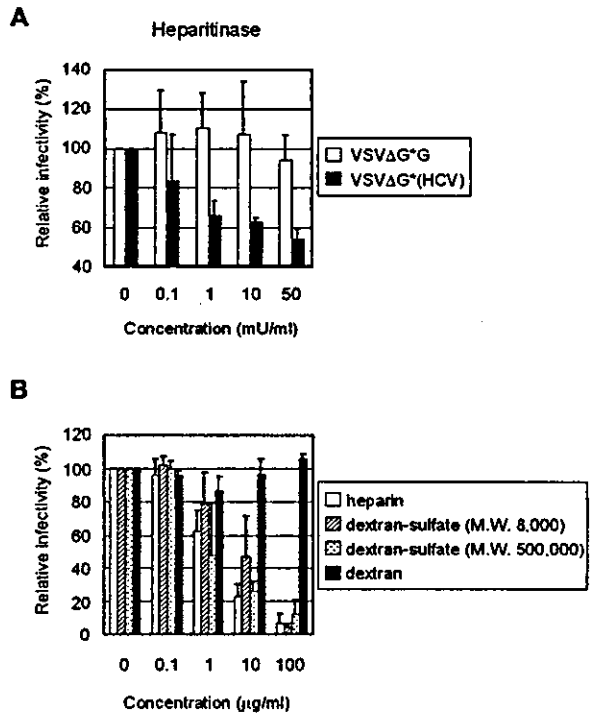

Fig. 4. Effects of sulfated polysaccharides on pseudotype infection. (a) Effect of heparitinase on infectivity of VSVΔG*(HCV). HepG2 cells were treated with various concentrations of heparitinase. The treated cells were infected with 200 IU of VSVΔG*(HCV) or VSVΔG*G. (b) Effect of sulfated polysaccharides 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

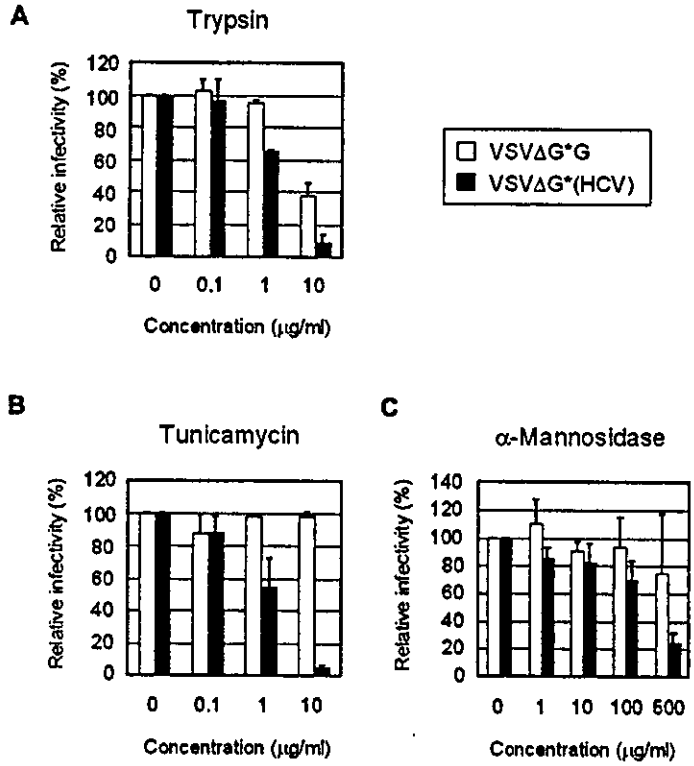

Fig. 5. Infectivity of pseudotype viruses in chemically modified cells. (a) HepG2 cells were preincubated with various concentrations of trypsin for 5 min. Subsequently, an equal volume of complete medium was added to stop the enzyme. Then, the cells were washed and infected with 200 IU of each virus. (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 post-mortem 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.

Acknowledgments

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Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture

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Hepatitis C virus (HCV) genomic sequences are known to vary widely among HCV strains, but to date there have been few reports on the genetic variations and dynamics of HCV in an experimental system of HCV replication. In this study, a genetic analysis of HCV replicons obtained in long-term culture of two HCV replicon cells (50-1 and 1B-2R1), which were established from two HCV strains, 1B-1 and 1B-2, respectively, was performed. One person cultured 50-1 cells for 18 months, and two people independently cultured 50-1 cells for 12 months. 1B-2R1 cells were also cultured for 12 months. The whole nucleotide sequences of the three independent replicon RNA clones obtained at several time points were determined. It was observed that genetic mutations in both replicons accumulated in a time-dependent manner, and that the mutation rates of both replicons were approximately $3{\cdot}0 \times 10^{-3}$ base substitutions/site/year. The genetic diversity of both replicons was also enlarged in a time-dependent manner. The colony formation assay by transfection of total RNAs isolated from both replicon cells at different time points into naïve HuH-7 cells revealed that the genetic mutations accumulating with time in both replicons apparently improved colony formation efficiency. Taken together, these results suggest that the HCV replicon system is useful for the analysis of evolutionary dynamics and variations of HCV. Using this replicon cell culture system, it was demonstrated further that neither ribavirin nor its derivative mizoribine accelerated the mutation rate or the increase in the genetic diversity of HCV replicon.

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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 9·6 kb and encodes a large polyprotein precursor of about 3000 aa residues (Kato *et al.*, 1990a; 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, and non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Grakoui *et al.*, 1993; Hijikata *et al.*, 1991, 1993; Mizushima *et al.*, 1994). These HCV proteins not only function in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction and apoptosis (Bartenschlager & Lohmann, 2000; Kato, 2001).

The most characteristic feature of the HCV genome is its remarkable genetic diversity and variation. To date, more than 50 HCV genotypes have been identified worldwide (Bukh *et al.*, 1995; Simmonds, 1995; Tokita *et al.*, 1996). Each of these genotypes shows more than 20 % difference at the nucleotide level and more than 15 % difference at the amino acid level compared with any of the other genotypes, although the 5′ untranslated regions (5′ UTRs) and core protein-encoding regions are highly homologous among the 50 genotypes (homology of >90 %). Comparisons of HCV genomes that belong to a single genotype have revealed 5–8 % diversity in nucleotide sequences and 4–5 % diversity in amino acid sequences (Kato *et al.*, 1990b; Kato, 2001). An analysis of the genetic diversity among the HCV genomes in an individual revealed that the diversity in nucleotide sequences averaged 0·9 %, and distributed throughout

Supplementary material is available in JGV Online.

the genome except in the 5′ UTR (Tanaka *et al.*, 1992). This so-called 'quasispecies' nature of the HCV genome has generally been observed in a single patient with chronic hepatitis C (Kato *et al.*, 1992; Martell *et al.*, 1992). This remarkable genetic diversity of the HCV genome suggests that HCV frequently causes mutations of the viral genome.

To date, two groups have estimated the mutation rate of the HCV genome using specimens from a chimpanzee (interval of 8 years) and a patient (interval of 13 years) infected with HCV (Ogata *et al.*, 1991; Okamoto *et al.*, 1992). They estimated that the mutation rate of the HCV genome was $1{\cdot}4–1{\cdot}9 \times 10^{-3}$ base substitutions/site/year; however, it is not clear whether this value indicates the actual mutation rate of the HCV genome, because complicated quasispecies are generally observed in patients or chimpanzees infected with HCV *in vivo*. On the other hand, Major *et al.* (1999) used chimpanzees that received intrahepatic inoculation with a full-length HCV RNA, and they estimated that the mutation rate of the HCV genome was $1{\cdot}5 \times 10^{-3}$ base substitutions/site/year. However, such experiments on HCV replication in humans are ethically problematic. Thus, there have been few reports on the genetic variations of HCV in an experimental system of HCV replication because of the lack of reproducible and efficient HCV proliferation in cell culture (Kato & Shimotohno, 2000).

In 1999, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions derived from the strain Con-1 was first established by using a human hepatoma cell line, HuH-7 (Lohmann *et al.*, 1999). Since then, several additional replicon systems have been established (Ali *et al.*, 2004; Blight *et al.*, 2000, 2003; Ikeda *et al.*, 2002; Kato *et al.*, 2003a; Pietschmann *et al.*, 2002; Zhu *et al.*, 2003). In these systems, replicated HCV RNAs were detected by Northern blot analysis and the HCV proteins, which were produced, were detected by Western blot analysis. Therefore, HCV replicon systems are thought to be useful for the analysis of genetic variations and dynamics of HCV.

Recently, we also established two HCV replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using HuH-7 cells (Kato *et al.*, 2003b; Kishine *et al.*, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 replicon showed differences of 8·1% from those in the 1B-2R1 replicon (Kato *et al.*, 2003b), although both HCV strains belonged to genotype 1b. In order to understand the genetic variations and dynamics of HCV, we performed genetic analysis of HCV replicons obtained in long-term culture of 50-1 and 1B-2R1 replicon cells (termed 50-1 and 1B-2R1 cells, respectively). Here, we show that the accumulation of genetic mutations and the acquisition of the genetic diversity among HCV replicons are time dependent. In addition, we evaluated the effect of ribavirin and mizoribine on the genetic variations and dynamics of HCV replicons.

METHODS

Cell cultures. 50-1 and 1B-2R1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 300 μg G418 (Geneticine; Invitrogen) ml^{-1}. The HCV replicon cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo^R) was produced by the efficient replication of HCV replicon in the cells. Therefore, when an HCV replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. 50-1 cells were also cultured in the presence of 5 or 25 μM ribavirin (Sigma) or 25 μM mizoribine (Sigma). In general, these replicon cells were passaged every 4 days.

Northern blot analysis. Total RNA from the cultured cells were prepared using an RNeasy extraction kit (Qiagen). Total RNA (3 μg) was used to detect the HCV replicon RNA and β-actin mRNA. Northern blotting and hybridization were performed as described previously (Ikeda *et al.*, 2002; Kato *et al.*, 2003b). A digoxigenin-labelled, negative-sense RNA probe complementary to the NS5B region (positions 8935–9374 of the HCV genome) was used for the detection of the replicon RNA. A β-actin specific digoxigenin-labelled antisense RNA probe was used to check the amount of RNA. The synthetic RNA transcribed from pNSS1RZ2RU (Kato *et al.*, 2003b) (10^8 and 10^7 genome equivalents spiked into normal cellular RNA) was used to compare the level of replicon RNA. An RNA ladder (Invitrogen) was also used to mark the molecular length.

Western blot analysis. The preparation of cell lysates, SDS-PAGE and immunoblotting analysis with a PVDF membrane were performed as described previously (Hijikata *et al.*, 1993; Naganuma *et al.*, 2000). The antibodies used to examine the expression levels of HCV proteins were those against NS3 (Novocastra Laboratories) and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-β-actin antibody (AC-15; Sigma) was also used to detect β-actin as an internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

RT-PCR. To amplify HCV RNA RT-PCR was performed as described previously (Kato *et al.*, 2003b). Briefly, the total RNA (2 μg) obtained from the replicon cells was used as a template for reverse transcriptase using SuperScript II (Invitrogen). PCR using proofreading KOD-plus DNA polymerase (Toyobo) was performed separately in two parts; one part covered the 5′ UTR to the amino terminal of the NS3 region, and the other part covered the NS3 region to the NS5B region. The PCR yielded a 2033 bp fragment for the former part and a 6107 bp fragment for the latter part.

cDNA cloning and sequencing. The PCR products were subcloned into the *Xba*I site of pBR322MC (Kishine *et al.*, 2002), which was derived from pBR322 and contained the multiple cloning site of pUC19, as described previously (Kato *et al.*, 2003b). Plasmid inserts were sequenced in both the sense and antisense directions by using Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyser (Applied Biosystems).

Molecular evolutionary analysis. Nucleotide sequences of the clones obtained by RT-PCRs from 50-1 and 1B-2R1 cells were analysed by the neighbour-joining analysis using the program GENETYX-MAC (Software Development).

RNA transfection and selection of G418-resistant cells. RNA transfection into Huh-7 cells was performed by electroporation as described previously (Lohmann *et al.*, 1999). Briefly, total RNA (80 μg) isolated from the replicon cells was electroporated into 5×10^6 HuH-7 cells, and then 1×10^5 or 3×10^5 cells were seeded into a 10 cm diameter dish. After 48 h, G418 was added to

0·3 mg ml^{-1}, and the medium was changed twice per week. After 3 weeks, the colonies obtained on the culture dish were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004).

RESULTS

Efficient replication of HCV replicons is maintained in long-term cell culture

In order to prepare the specimens for the genetic analysis of 50-1 and 1B-2R1 replicons, three people independently cultured 50-1 cells; one person cultured for 18 months (M) (K cell culture line; MK) and the two people cultured for 12 months (D and N cell culture lines; MD and MN), and one person cultured 1B-2R1 cells for 12 months. Using the specimens obtained at several time points (after 0, 4, 6, 12 and 18 months in culture), the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 1(a), replicon RNAs approximately 8 kb long were detected in all specimens except those from the cured cells, from which the replicons had been eliminated from the replicon cells by treatment with interferon-α. The number of copies of replicon RNAs in total RNA (each 3 μg) extracted from the replicon cells was estimated to be in the range of 10^7 to 10^8 by comparing these replicon RNAs with replicon RNA synthesized *in vitro*. The NS3 and NS5B were also detected in all specimens except those from the cured cells (Fig. 1b). The expression levels of replicon RNAs and HCV proteins differed somewhat among these specimens, and no strong quantitative relationship between replicon RNA and HCV proteins was observed (Fig. 1). These results suggest that the stability of replicon RNA or HCV proteins produced from the replicon RNA, or the efficiency of translation, changes during the periods of cell culture. In summary, we demonstrated that the replication efficiencies of the 50-1 and 1B-2R1 replicons remained high under the G418 selection pressure.

Sequence analysis of the 50-1 and 1B-2R1 replicon RNAs

To clarify the genetic variations and diversities of the replicons during the period of cell culture, we carried out sequence analysis of 50-1 and 1B-2R1 replicon RNAs obtained at several time points in the cultures of both replicon cells. Two separate RNA fragments (one was 2·0 kb in length, containing the 5′ UTR to the amino-terminal of the NS3 region; the other was 6·1 kb in length, containing the NS3 to NS5B regions) were amplified by RT-PCR, and three independent clones of each were sequenced after subcloning into pBR322MC, as described previously (Kato *et al.*, 2003b).

Genetic variations of 50-1 and 1B-2R1 replicons during long-term cell culture

The determined nucleotide sequences of the 50-1 and 1B-2R1 replicon RNAs were compared with those of the

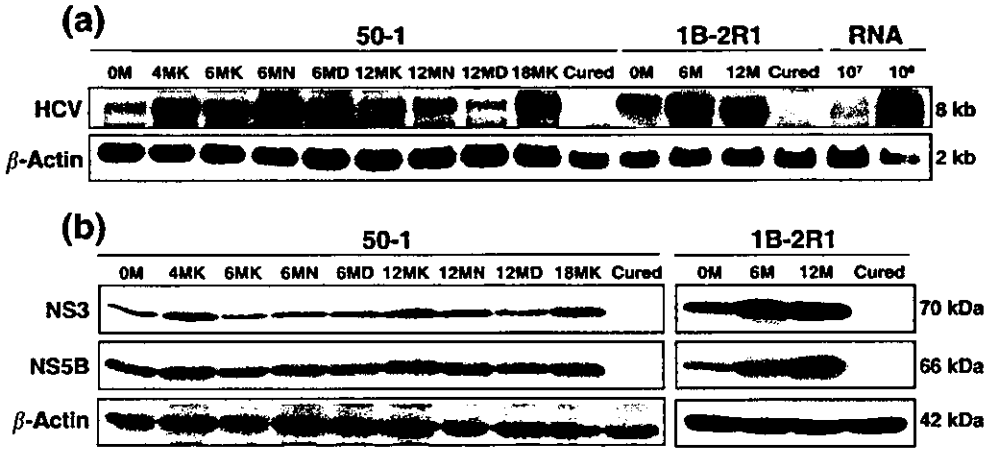

Fig. 1. Characterization of replicon cells in long-term cell culture. (a) Northern blot analysis. Total RNAs from 50-1 cells after 4 months (4MK), 6 months (6MK, 6MN and 6MD), 12 months (12MK, 12MN and 12MD) and 18 months (18MK) in culture, as well as total RNA from the parental 50-1 cells (0M) were used for the analysis. Total RNAs from 1B-2R1 cells after 6 months (6M) and 12 months (12M) in culture, as well as total RNA from the parental 1B-2R1 cells (0M) were used for the analysis. Total RNAs from each cured cells obtained from 50-1 and 1B-2R1 cells by interferon treatment were also used as a negative control. Northern blot analysis was performed using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific probe (lower panel). Synthetic RNA transcribed from pNSS1RZ2RU (10^8 and 10^7 genome equivalents spiked into normal cellular RNA) was used for the comparison of the expression level. (b) Western blot analysis. The orders of specimens were the same as in (a). Productions of NS3 and NS5B in 50-1 and 1B-2R1 cells were analysed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane.

original 50-1 (Kishine *et al.*, 2002; GenBank accession no. AB041927) and 1B-2R1 replicons (Kato *et al.*, 2003b; AB109543), respectively. The results revealed that the numbers of base substitutions in the first 2·0 kb region and in the NS region (6·1 kb) of both replicon RNAs were time-dependently increased with linearity (Fig. 2). These substitutions were considered to be mutations that occurred during the intracellular replication of replicon RNA. Based on the results after 12 months in culture, the apparent mutation rates in 50-1 replicon RNA were calculated to be $3{\cdot}1 \times 10^{-3}$ and $3{\cdot}0 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region and NS region, respectively, indicating that there was no difference in mutation rate between the two regions of 50-1 replicon RNA. Interestingly, almost the same mutation rates ($3{\cdot}0 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region; $3{\cdot}1 \times 10^{-3}$ base substitutions/site/year in NS region) were obtained for the 1B-2R1 replicon RNA, suggesting that the replication efficiency of the 1B-2R1 replicon was almost equal to that of the 50-1 replicon.

Fig. 3(a) shows the schematic presentation of mutations detected in the first 2 kb region by comparison with the original sequences (NNRZ2RU) of 50-1 and 1B-2R1 replicon RNAs (Kato *et al.*, 2003b; Kishine *et al.*, 2002). The results revealed that there were no common mutations among the four cell culture lines (three for 50-1 and one for 1B-2R1) over at least 12 months of cell culture. However, genetic mutations in both replicons were time-dependently increased and accumulated, and several mutations became abundant during the subsequent cell culture (Fig. 3a).

The NS regions (6·1 kb) of the 50-1 and 1B-2R1 replicon RNAs were also analysed in addition to the first 2 kb region. The mutation sites that showed amino acid substitutions are schematically presented in Fig. 3(b). Regarding the 50-1 replicon, 2 aa substitutions (P1115L and E1966A) were newly detected after 6 months in culture in all three cell culture lines, in addition to 2 aa substitutions (K1609E and V1896F) already observed when the replicon was first established. These four substituted amino acids were stably maintained over at least 12 months of cell culture. However, such amino acid substitutions were not observed in the 1B-2R1 replicon even after 12 months of culture. After more than 12 months in culture, several culture line-specific amino acid substitutions (*1–5 for the K culture line; *6–8 for the D culture line; and *9–12 for the N culture line in Fig. 3b) were observed in the 50-1 replicon. Also in the 1B-2R1 replicon, 1 aa substitution (*13 in Fig. 3b) was detected after 12 months in culture; however, no common amino acid substitutions were observed between the 50-1 and 1B-2R1 replicons. The mean numbers of amino acid substitutions occurring after 6 and 12 months in culture were 4·2 and 8·9, respectively, for the 50-1 replicon, and 4·7 and 10·0, respectively, for the 1B-2R1 replicon. These values indicate a steady genetic evolution of 50-1 and 1B-2R1 replicons during the cell culture.

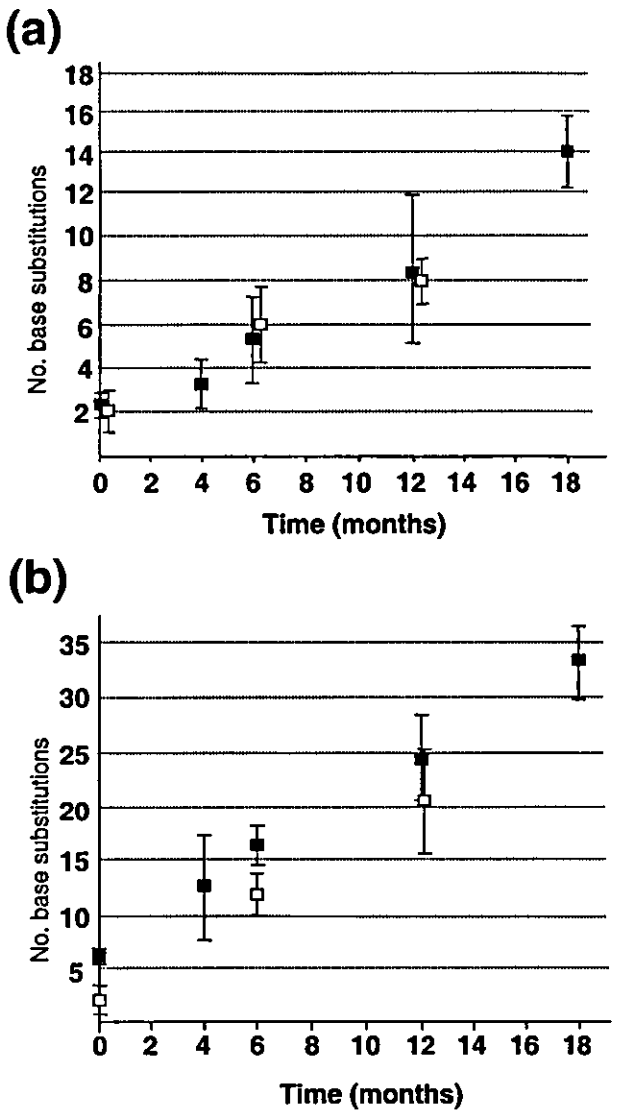

Fig. 2. Genetic variations of 50-1 and 1B-2R1 replicon RNAs. (a) First 2·0 kb region of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in nine (after 0, 6 and 12 months in culture) or three (after 4 and 18 months in culture) clones containing the first 2·0 kb region of 50-1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine *et al.*, 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the first 2·0 kb region of 1B-2R1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine *et al.*, 2002). (b) NS region (6·1 kb) of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in nine (after 0, 6 and 12 months in culture) or three (after 4 and 18 months in culture) clones containing the NS region of 50-1 replicon RNA, by comparison with its original sequences (Kishine *et al.*, 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the NS region of 1B-2R1 replicon RNA, by comparison with its original sequences (Kato *et al.*, 2003b).

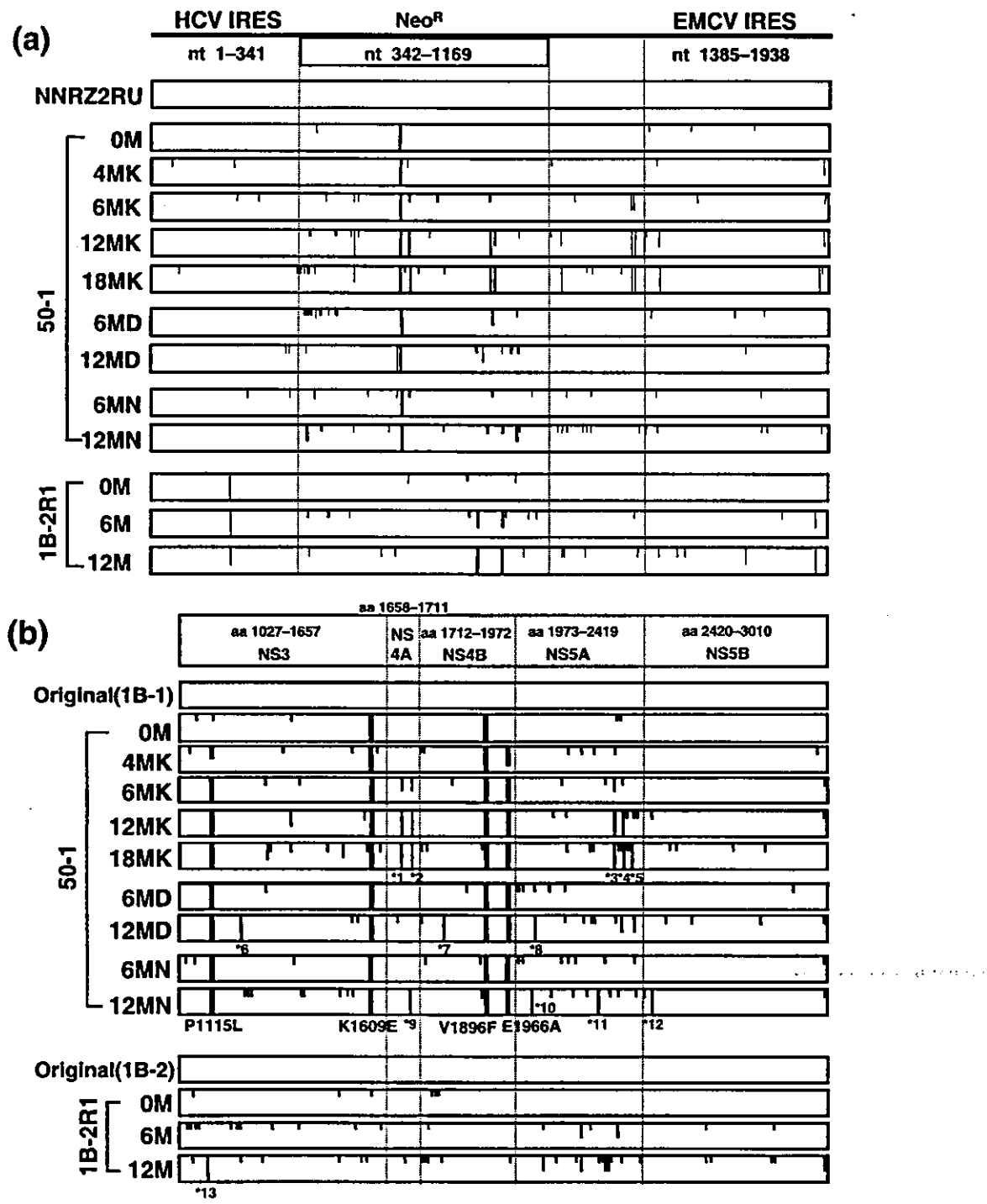

Fig. 3. Genetic variations of 50-1 and 1B-2R1 replicons in long-term cell culture. (a) Schematic presentation of mutations detected in first 2·0 kb regions of the replicon RNAs. Compared with the nucleotide sequences of the first 2·0 kb region of the original replicon RNA (NNRZ2RU), nucleotide positions mutated in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Non-synonymous substitutions in the Neo^R region are indicated by heavy vertical lines. (b) Schematic presentation of amino acid substitutions detected in the NS regions of the replicons. Compared with the amino acid sequences of NS region of the original 50-1 (Kishine *et al.*, 2002) and 1B-2R1 replicons (Kato *et al.*, 2003b), amino acid positions substituted in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Four amino acid substitutions (P1115L, K1609E, V1896F and E1966A) are indicated by heavy vertical lines. Culture line-specific amino acid substitutions (indicated by the numbers with asterisks) are as follows: *1, I1686V; *2, L1701R; *3, T2332A; *4, G2336E; *5, A2372T; *6, A1243G; *7, I1797V; *8, S2053G; *9, L1701R; *10, T2051N; *11, R2279G; *12, L2476M; *13, I1097V.