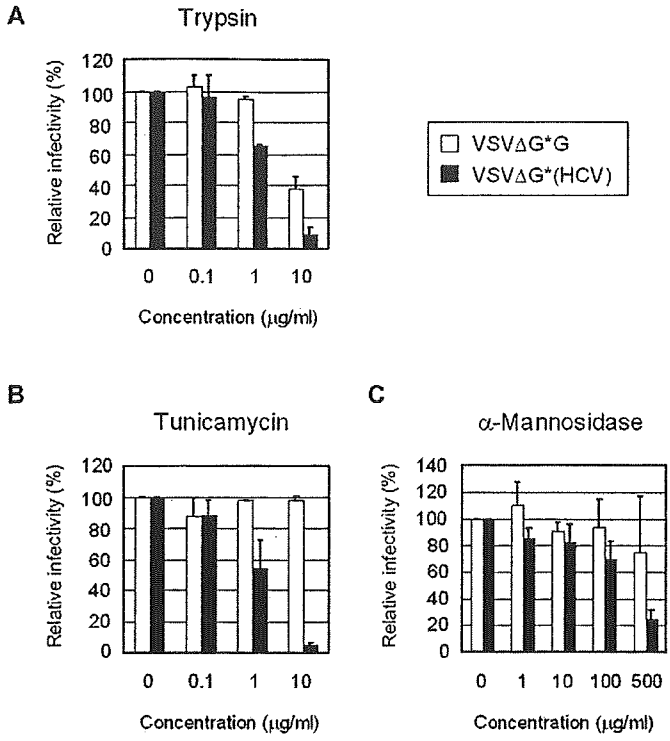


Fig. 5. Infectivity of pseudotype viruses in chemically modified cells. (a) HepG2 cells were preincubated with various concentrations of trypsin for 5 min. Subsequently, an equal volume of complete medium was added to stop the enzyme. Then, the cells were washed and infected with 200 IU of each virus.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

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

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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.


*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-

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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 $1 \times 10^6$) 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 $1 \times 10^6$ 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 ChemiImager 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_[illegible]173 |
| 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.

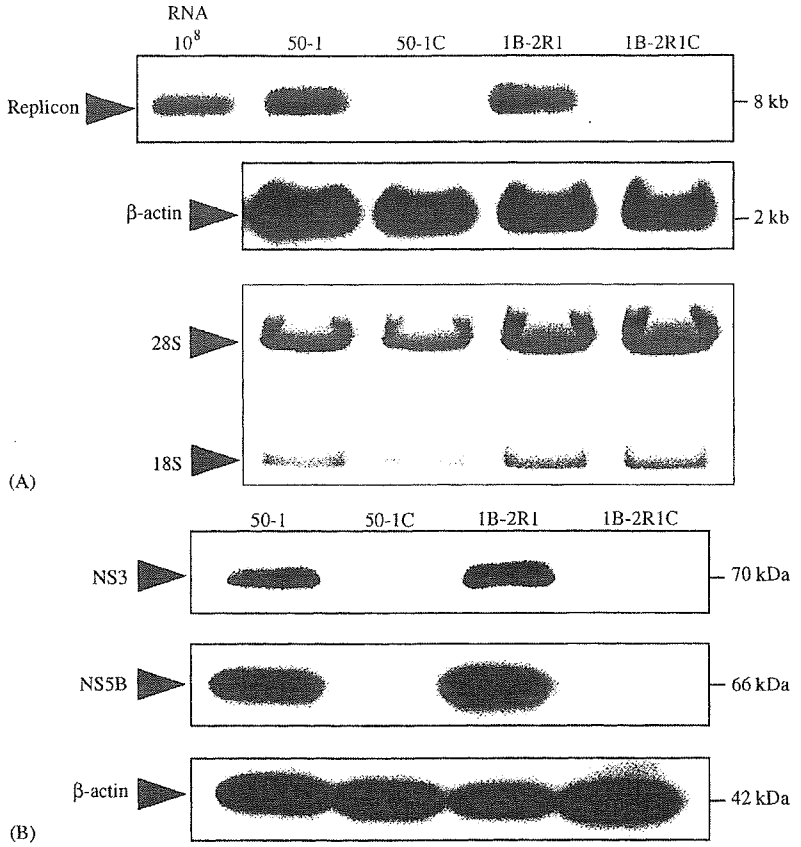


Fig. 1. Characterization of the replicon cells and their cured cells. (A) Northern blot analysis. Total RNAs from 50-1 and 1B-2R1 cells, as well as total RNAs from the cured cells, were analyzed by Northern blotting using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific 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 expression profiles of the replicon and its cured cells. Of the 16 genes, 9 (4 up-regulated and 5 down-regulated) were fur-

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.

ther 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 |

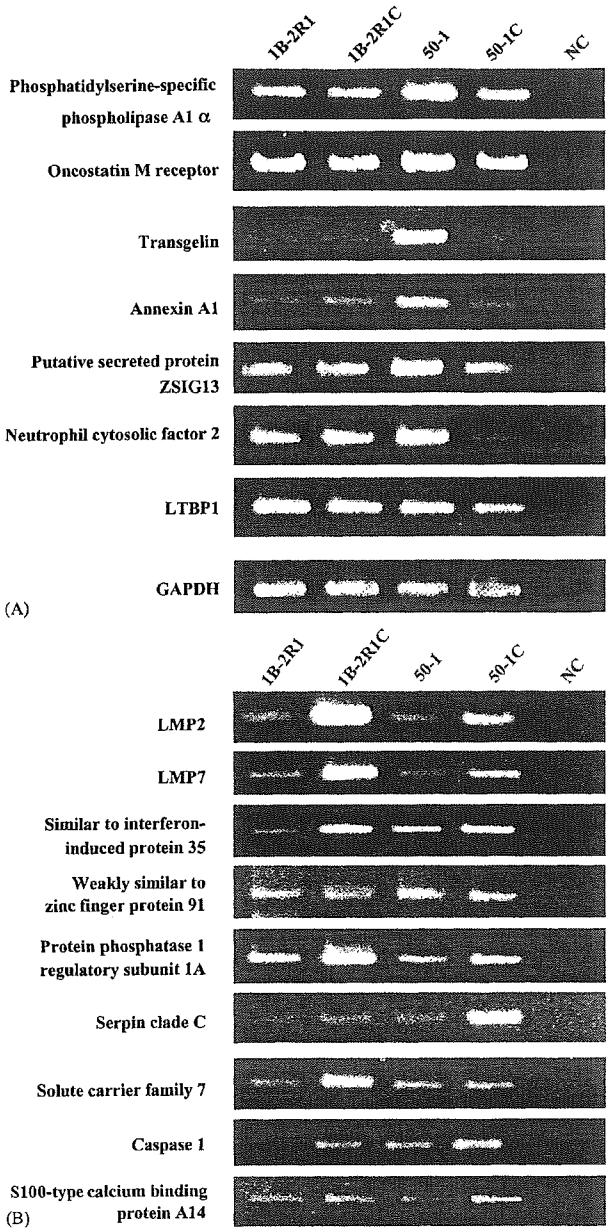


Fig. 2. RT-PCR analysis of mRNA expression of selected genes based on the microarray results. The total RNAs extracted from 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells were subjected to RT-PCR, and the mRNA levels of target genes were monitored as described in Section 2. The primers used, PCR product lengths, 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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# Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B

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**The hepatitis C virus (HCV) nonstructural protein (NS) 5A is a phosphoprotein that associates with various cellular proteins and participates in the replication of the HCV genome. Human vesicle-associated membrane protein-associated protein (VAP) subtype A (VAP-A) is known to be a host factor essential for HCV replication by binding to both NS5A and NS5B. To obtain more information on the NS5A protein in HCV replication, we screened human brain and liver libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. Immunoprecipitation and mutation analyses revealed that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. VAP-A interacts with VAP-B through the transmembrane domain. NS5A interacts with the coiled-coil domain of VAP-B via 70 residues in the N-terminal and 341 to 344 amino acids in the C-terminal polyproline cluster region. NS5A was colocalized with VAP-B in the endoplasmic reticulum and Golgi apparatus. The specific antibody to VAP-B suppressed HCV RNA replication in a cell-free assay. Overexpression of VAP-B, but not of a mutant lacking its transmembrane domain, enhanced the expression of NS5A and NS5B and the replication of HCV RNA in Huh-7 cells harboring a subgenomic replicon. In the HCV replicon cells, the knockdown of endogenous VAP-B by small interfering RNA decreased expression of NS5B, but not of NS5A. These results suggest that VAP-B, in addition to VAP-A, plays an important role in the replication of the HCV genome.**

Hepatitis C virus (HCV) infects 170 million people worldwide and frequently leads to cirrhosis or hepatocellular carcinoma (6, 29). HCV is classified in the family *Flaviviridae* and possesses a single-stranded positive-sense RNA with a length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3,000 amino acids (aa) that is processed by cellular and viral proteases, resulting in at least 10 structural and nonstructural (NS) proteins (29). Details of HCV's replication cycle are unknown because of the low viral load in the sera of HCV-infected individuals and the lack of a reliable and robust cell culture system to support HCV infection and replication. The development of HCV RNA replicons in which a synthetic HCV genomic or subgenomic RNA replicates efficiently in the human hepatocarcinoma cell line Huh-7 has enabled the study of viral RNA replication in cell culture (4, 20, 24). The HCV RNA replication complex, composed of the viral NS proteins and host cellular proteins, replicates the viral RNA genome at the intracellular membrane. Thus far, the HCV replicon system has greatly contributed to the understanding of HCV replication and pathogenesis associated with the expression of viral NS proteins. Replication of positive-strand RNA viruses generally involves certain intracellular membrane structures, including the endoplasmic reticulum (ER), Golgi apparatus, endosome, and lysosome (39). Recently, several groups have succeeded in demonstrating cell-free replication activities of replication complexes in crude membrane fractions of HCV subgenomic replicon cells (2, 3, 14, 53). These cell-free systems provide semi-intact polymerase assays for biochemical dissection of HCV RNA replication and are a useful source for the isolation of HCV replication complexes. Replication complexes were detected in detergent-resistant membrane structures, most likely lipid raft structures (2, 14). Although HCV NS proteins presumably form a membrane-associated RNA replication complex with host proteins, the precise components and mechanisms for replication are poorly understood.

HCV NS5A is a phosphoprotein that appears to possess multiple and diverse functions in viral replication, interferon resistance, and pathogenesis (26, 35). Cell culture-adaptive mutations have been shown to cluster in the central portion of NS5A in subgenomic HCV replicons, indicating that NS5A is involved in the viral replication process either directly or by interacting with host cellular proteins (4, 55). This observation, together with the modulation of NS5A hyperphosphorylation by NS3, NS4A, and NS4B and physical interaction with other viral NS proteins, strongly supports the notion that NS5A is an essential component of the HCV replication complex (21, 30, 36). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as interferon-induced kinase PKR (11), growth factor receptor-binding protein 2 (Grb2) (45), p53 (27, 37), phosphoinositide-3-kinase p85 subunit (15), and proteins in protein trafficking and membrane morphology, such as karyopherin β3 (8),

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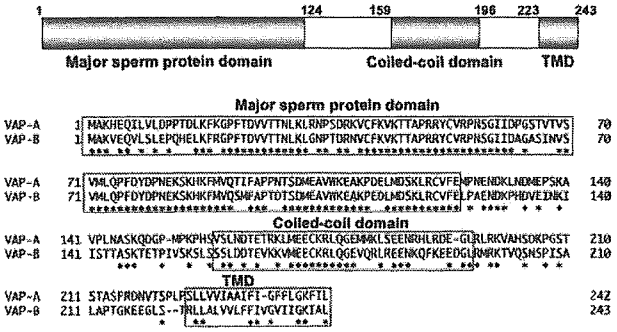


FIG. 1. Schematic representation of VAP-B and alignment of amino acid sequences of VAP-A and VAP-B. The major sperm protein domain, coiled-coil domain, and TMD are indicated. The asterisks indicate identical amino acid residues between VAP-A and VAP-B.

apolipoprotein A1 (40), amphiphysin II (56), and vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A), also called VAP-33 (48). Host fatty acids and geranylgeranylation appear to modulate the host and viral proteins involved in HCV RNA replication (19, 49, 54). Gao et al. showed that small interfering RNA (siRNA) or the dominant-negative mutant of VAP-A resulted in relocation of NS5B from detergent-resistant to detergent-sensitive membranes and reduced HCV RNA replication (12). In addition, Evans et al. suggested that NS5A hyperphosphorylation disrupts interaction with VAP-A and negatively regulates HCV RNA replication (9). Like many of the fusion proteins, VAP is a tail-anchored protein with a globular amino-terminal domain followed by a stalk region containing a coiled coil (Fig. 1), and it is ubiquitously expressed in human tissues (7). In humans, there are two isoforms of VAP, VAP-A and VAP-B, encoded by separate genes, and VAP-C is a splicing variant of VAP-B missing the C-terminal two-thirds (23, 32). VAP-B shows 63% amino acid identity to VAP-A (32, 51). The first proposed function for VAP arose from its initial identification as an interactor with the membrane fusion protein synaptobrevin/VAMP in *Aplysia* (43). Since then, it has been shown to be involved in vesicle transport, including the regulation of COP-I vesicle transport in the ER/Golgi pathway (13, 44), VAMP/synaptobrevin-mediated neurotransmitter release (38), and VAMP-2-mediated Glut-4 trafficking at the plasma membrane (10); it is also involved in the interaction between the microtubule network and tight junctions (22). Recently, VAP has been linked to the function of mammalian neurons, where VAP is enriched on microtubules (42), because a mutation in human VAP-B causes familial amyotrophic lateral sclerosis type 8 (32).

To gain a better understanding of the interactions between NS5A and host proteins involved in HCV replication, we screened human libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. In this study, we examined the biological significance of the interaction between VAP-B and NS proteins in HCV replication and found that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. Immunodepletion of VAP-B suppressed the replication of HCV RNA in a cell-free replication assay, and the knockdown of endogenous VAP-B by siRNA decreased the expression of NS5B but not that of NS5A. These results suggest that VAP-B plays an important role in HCV replication through interaction with NS5A and NS5B.

## MATERIALS AND METHODS

**Cells.** Human embryo kidney 293T, human cervical carcinoma HeLa, and human hepatoma Huh-7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Mo) containing 10% fetal calf serum (FCS), while the Huh-9-13 cell line, which possesses an HCV subgenomic replicon (4, 20, 23), was cultured in DMEM supplemented with 10% FCS and 1 mg/ml G418. All cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$.

**Antibodies.** Chicken anti-human VAP-B antibody was prepared by immunization using the synthetic peptides of residues from 188 to 203, KQFKEEDGL RMRKTVQ, of human VAP-B. A mouse monoclonal antibody to human VAP-A was purchased from BD Pharmingen (San Diego, CA). Mouse monoclonal antibodies to giantin, influenza virus hemagglutinin (HA), and GluGlu (EE) tag were from Covance (Richmond, CA). Mouse anti-FLAG antibody M2, horseradish peroxidase-conjugated antibody, and mouse monoclonal anti-beta-actin antibody were from Sigma. A mouse monoclonal antibody to protein disulfide isomerase (PDI) was from Affinity Bioreagents (Golden, CO). Rabbit polyclonal antibody to NS5A was prepared by immunization using peptides of residues from 409 to 422, DVESYSSMPPLEGE. Mouse monoclonal antibody to NS5B was described previously (41).

**Plasmids.** For expression in mammalian cells, a DNA fragment encoding NS5A was generated from HCV genotype 1b strain J1 (1) (GenBank database accession number D89815), and another was generated from genotype 1a strain H77 (52) (GenBank database accession number AF009606) by PCR using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were then cloned into the appropriate sites in pEF-FLAG pGBK puro (18) and pEGFP-C3 (Clontech, Palo Alto, CA). The mutations of the NS5A gene were generated by a method known as "splicing by overlapping extension" (16, 17) and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding NS5B of the J1 strain was generated by PCR and cloned into pCAGGs-PUR (33). The DNA fragment encoding human VAP-A was amplified by PCR from a human fetal-brain library (Clontech) and was introduced into pEF-FLAG pGBK puro, pEF-EE hygro (34), pCHA3 (34), and pcDNA3.1-N-HA, in which an HA tag is inserted in the N terminus of the cloning site of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The cDNAs of human VAP-A and -B were amplified by PCR and cloned into pEF-FLAG pGBK puro, pEF-EE hygro, pcDNA3.1-N-HA, and pEGFP-C3. The genes encoding VAP lacking the transmembrane domain were amplified and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding the human VAP-B protein lacking a coiled-coil region was introduced into pEF-EE hygro. All PCR products were confirmed by sequencing them with an ABI PRSM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

**Yeast two-hybrid assay and library screening.** The NS5A-binding protein was identified by a yeast two-hybrid assay according to the user manual of MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). The DNA fragment encoding amino acids 1973 to 2419 was amplified from HCV strain J1 by PCR and then was cloned into pGBKT7 (Clontech). The resulting plasmid was designated pGBK T7 HCV NS5A. A human brain library based on pACT2 was purchased from Clontech. The yeast *Saccharomyces cerevisiae* strain AH109, which secretes alpha-galactosidase under the control of MEL1 upstream activation sequence, was grown in yeast extract-peptone-dextrose medium and transformed with the bait and library plasmids. The transformed yeast cells were grown on 2.0% agar plates of dropout medium lacking tryptophan, leucine, histidine, and adenine. The resulting colonies were inoculated on the new dropout plate containing 20 μg/ml X-alpha-Gal (5-bromo-4-chloro-3-indolyl-alpha-*O*-galactopyranoside) and lacking leucine and tryptophan. The total DNA was prepared from all positive clones and then introduced into *Escherichia coli* strain JM109. The prey plasmids of isolated yeast cells were recovered from the clones grown on LB agar plates containing 10 μg/ml ampicillin and then purified. The insert DNA fragments of isolated clones were determined by sequencing. Finally, 48 alpha-galactosidase-positive clones were identified from 2 million clones screened in the fetal-brain library. One of the positive clones contained the complete cDNA of human VAP-B in frame.

**Transfection, immunoblotting, and immunoprecipitation.** Cells were seeded onto a six-well tissue culture plate 24 h before transfection. The plasmids were transfected into cells by liposome-mediated transfection using Lipofectamine 2000 (Invitrogen). Cells were harvested 36 h posttransfection, washed five times

with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.2 ml lysis buffer (20 mM Tris-HCl, pH 7.4, containing 135 mM NaCl and 1% Triton X-100) supplemented with 1 μg/ml leupeptin, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 5 mM $NaVO_4$. Cell lysates were sonicated at 4°C for 5 min, incubated for 30 min at 4°C, and centrifuged at 14,000 × *g* for 5 min at 4°C. The supernatant was immunoprecipitated with 1 μg of antibodies and 10 μl of Protein G-Sepharose 4B Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The immunocomplex was precipitated with the beads by centrifugation at 14,000 × *g* for 30 s and then was washed five times with lysis buffer by centrifugation. The proteins binding to the beads were boiled in 30 μl of loading buffer and then subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then reacted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

**Immunofluorescence microscopy.** Cells were seeded on an eight-well chamber slide at 2 × $10^4$ per well 24 h before transfection. Transfected cells were washed twice with PBS, fixed with PBS containing 4% paraformaldehyde, and permeabilized with PBS containing 0.5% Triton X-100. The ER and Golgi apparatus of cells were stained with the mouse monoclonal antibody against luminal ER redox enzyme PDI and the rabbit polyclonal antibody against giantin, respectively, in PBS containing 5% bovine serum albumin. Bound primary antibody was revealed with Alexa Fluor 594-conjugated anti-mouse or anti-rabbit antibody. After additional washes with PBS, a coverslip was attached over PBS containing 50% glycerol and observed under an LSM 510 microscope (Carl Zeiss, Tokyo, Japan).

**Gene silencing by siRNA.** The siRNA target sequence against human VAP-B, 5′-GGUUAUGGAAGAAUGUAAGTT-3′, was synthesized and purified by Ambion (Austin, TX). Negative control siRNA, siCONTROL Non-Targeting siRNA-2, was purchased from Dharmacon (Lafayette, CO). The Huh-7 cells harboring a subgenomic HCV replicon on six-well plates were transfected with 80 nM or 160 nM of siRNA by using siFECTOR (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol. Cells were incubated in DMEM supplemented with 10% FCS and harvested at 96 h posttransfection.

**RNA replication assay.** In vitro RNA replication was determined as previously described with some modification (3). Briefly, the Huh-7 cells harboring a subgenomic HCV replicon grown in a 100-mm dish were treated with lysolecithin (Wako, Osaka, Japan) (250 μg/ml in wash buffer; 150 mM sucrose, 30 mM HEPES [pH 7.4], 33 mM $NH_4Cl$, 7 mM KCl, 4.5 mM magnesium acetate), collected by scraping in 120 μl of incomplete replication buffer (100 mM HEPES [pH 7.4], 50 mM $NH_4Cl$, 7 mM KCl, and 1 mM spermidine), and centrifuged at 1,600 rpm for 5 min at 4°C. A total of 40 μl of cytoplasmic fraction (supernatant) was treated with 1% Nonidet P-40 (Boehringer Mannheim, Quebec, Canada) at 4°C for 1 h and incubated with antibody for 4 h at 4°C with rotation. Then, samples were incubated with 1 mM of ATP, GTP, and UTP; 10 μM CTP; [α-$^{32}$P]CTP (1 MBq; 15 TBq/mmol); 10 μg/ml actinomycin D; and 800 U/ml RNase inhibitor (Promega, Madison, WI) for 4 h at 30°C. RNA was extracted from the total mixture by TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was precipitated, eluted in 10 μl of RNase-free water, and analyzed by 1% formaldehyde agarose gel electrophoresis.

**Real-time PCR.** Total RNA was prepared from cell lines by using TRIzol LS (Invitrogen), and first-strand cDNA was synthesized by using a first-strand cDNA synthesis kit (Amersham) with random primers. Each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI PRISM 7000 (Applied Biosystems). The HCV NS5A gene was amplified using the primer pairs 5′-AGTCAGTTGTCTGCGCTTTC-3′ and 5′-CGGGGAATTTCCTGGTCTTC-3′. The human beta-actin gene was amplified with the primer pairs 5′-TGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTC-3′ and 5′-CGGACTCGTCATACTCCTGCTTGCTGATCCACATC-3′, which are located at different exons to prevent false-positive amplification from contaminated genomic DNA. The value of the HCV genome was normalized with that of actin mRNA. Each PCR product was found as a single band of the correct size on agarose gel electrophoresis (data not shown).

## RESULTS

**Isolation of VAP-B as a novel binding partner for HCV NS5A.** To examine the protein(s) that interacts with NS5A in more detail, we screened a cDNA library of human fetal brain by a yeast two-hybrid system using a full-length NS5A of genotype 1b as bait. Among the 2 million transformants we screened, we obtained 48 positive clones containing cDNAs that encode proteins interactive with NS5A. A BLAST search against the GenBank database revealed each of two clones that have the cDNA encoding VAP-A and VAP-B in frame. Figure 1 shows the amino acid alignments of VAP-A and VAP-B and their predicted functional domains. VAP-A and VAP-B are composed of 242 and 243 amino acids, respectively. VAP-B shows 63% amino acid identity to VAP-A. VAP has three structural domains. The first 124 amino acids share high sequence similarity with the nematode major sperm protein and are conserved among all VAP family members (50). The central region on the protein contains an amphipathic helical structure and is predicted to form a coiled-coil protein-protein interaction motif (159 to 196 aa) and a hydrophobic carboxy-terminal transmembrane domain (TMD) (223 to 243 aa). The homology between their N-terminal regions is higher than that between their C-terminal regions (32, 48).

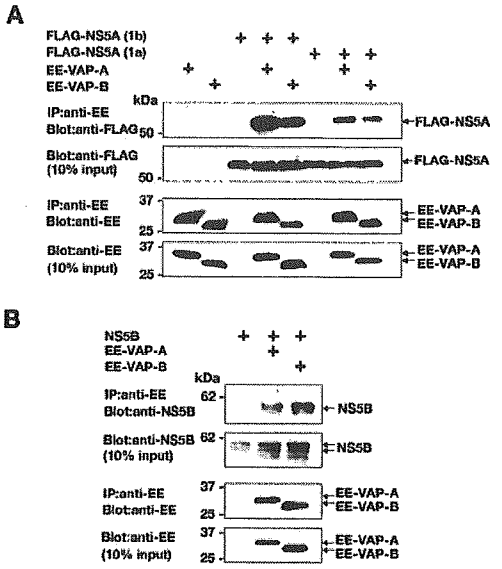


FIG. 2. VAP-A and VAP-B bind to both NS5A and NS5B in mammalian cells. N-terminally FLAG-tagged NS5A of genotype 1b, FLAG-NS5A (1b) of genotype 1a, FLAG-NS5A (1a), and N-terminally EE-tagged VAP (EE–VAP-A or EE–VAP-B) were coexpressed in HEK293T cells and immunoprecipitated with anti-EE antibody. The resulting precipitates were examined by immunoblotting using anti-FLAG antibody (A). NS5B was coexpressed with EE-tagged VAP-A or VAP-B and immunoprecipitated with anti-EE antibody, and NS5B in the precipitates was detected by anti-NS5B antibody (B). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

**VAP-B interacts with NS5A and NS5B in mammalian cells.** To confirm the specific interaction, FLAG-tagged NS5A was coexpressed with EE-tagged VAP-A or VAP-B in 293T cells, and cell lysates were immunoprecipitated by specific antibodies. NS5A was coprecipitated with VAP-A and VAP-B to similar extents (Fig. 2A). We also obtained the same results in the reverse experiments (data not shown). Recently, it was shown that hyperphosphorylation of NS5A disrupts interaction with VAP-A and negatively regulates HCV RNA replication, sug-

gesting that adaptive mutations detected in the HCV replicon prevent phosphorylation-dependent dissociation of the RNA replication complex (9). Amino acid residues at Tyr2185 and Lys2187 of NS5A genotype 1b were defined as key determinants for VAP-A binding, and the replacement of these residues with those of genotype 1a (Ala and Gly, respectively) reduced binding to VAP-A in yeast and enhanced hyperphosphorylation of NS5A (9). However, as shown in Fig. 2A, the NS5As of both the 1a and 1b genotypes were coimmunoprecipitated with VAP-A and -B in mammalian cells. Since a previous report indicated that VAP-A interacts with not only NS5A but also NS5B (12), we next examined the interaction of VAP-B with NS5B. EE-tagged VAP-A or VAP-B was coexpressed with NS5B in 293T cells and immunoprecipitated with anti-EE-tag antibody. NS5B was coprecipitated with VAP-B, as well as VAP-A (Fig. 2B). These results indicate that VAP-B participates in the complex of HCV NS proteins in a manner similar to that of VAP-A.

**NS5A colocalizes with VAP-B in ER and Golgi compartments.** To determine the subcellular localization of NS5A and VAP-B in mammalian cells, HeLa cells were cotransfected with plasmids encoding enhanced green fluorescent protein (EGFP)-tagged NS5A and FLAG-tagged VAP-B or FLAG-tagged VAP-A and examined by immunofluorescence analysis. EGFP-NS5A was colocalized exclusively with FLAG-VAP-B in the cytoplasm, as seen in FLAG-VAP-A (Fig. 3A). To further determine the precise subcellular localization of NS5A and VAP-B, the ER and Golgi apparatus were stained with specific antibodies against PDI and giantin, respectively. NS5A and VAP-B were colocalized with PDI and giantin in HeLa cells transfected with the plasmids (Fig. 3B), indicating that NS5A and VAP-B are colocalized in the membranes of the ER or ER-derived compartment. VAP-B was localized in a diffuse ER-like network, in small vesicles clustered around the nucleus, and predominantly in a perinuclear/Golgi region. Similar to the case with VAP-A, the colocalization of NS5A with VAP-B in the ER and Golgi apparatus suggests that NS5A specifically interacts with VAP-B under intracellular conditions.

**Dimerization of VAP-A and VAP-B and interaction with NS5A.** Immunoprecipitation analyses revealed that NS5A and NS5B interact with VAP-A and VAP-B. Therefore, it might be reasonable to speculate that VAP-A and VAP-B interact with each other and are involved in RNA replication through the formation of a replication complex. It has been demonstrated that VAP-A interacts with VAP-A or VAP-B through their TMDs and forms a homodimer and a heterodimer in vitro (32). We constructed expression plasmids encoding mutant VAP-A and VAP-B lacking their TMDs and examined their dimer formation with authentic VAPs in vivo. Although coprecipitation of authentic VAP (FLAG–VAP-B or FLAG–VAP-A) with VAP-B–HA was clearly detected, no interaction between TMD deletion mutants (FLAG–VAP-AΔTMD or FLAG–VAP-BΔTMD) and VAP-B–HA was observed (Fig. 4A and B). Furthermore, a TMD deletion mutant, HA–VAP-BΔTMD, which lost the ability to form a dimer with VAP-B and VAP-A, retained the ability to bind to FLAG-NS5A (Fig. 4C), although the efficiency of interaction with NS5A was reduced. These results indicate that TMDs of VAP-A and VAP-B are required for hetero- and homodimerization, but not for binding to NS5A. A region other than the TMD should be involved in the specific interaction between VAP-B and HCV NS5A. The coiled-coil domain of VAP-A was reported to be critical for binding to NS5A (48). Therefore, we examined whether the coiled-coil domain of VAP-B is also involved in interaction with NS5A. FLAG-NS5A was coimmunoprecipitated with EE–VAP-B but not with EE–VAP-BΔcoiled-coil, which lost the coiled-coil domain but retained the TMD (Fig. 4D), suggesting that the coiled-coil domain is also essential for interaction between NS5A and VAP-B.

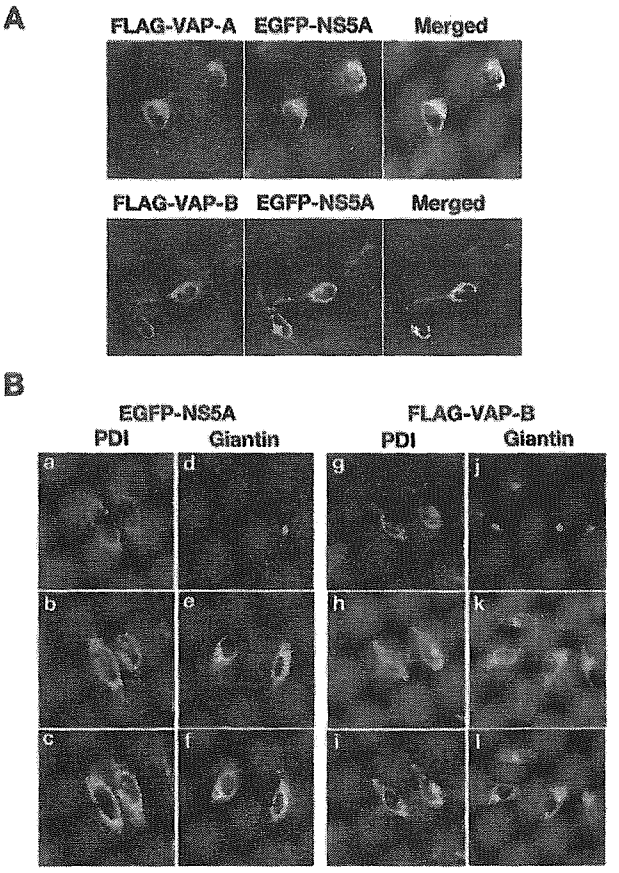


FIG. 3. Intracellular localization of VAPs and NS5A in mammalian cells. (A) N-terminally FLAG-tagged VAP (FLAG–VAP-A or FLAG–VAP-B) was coexpressed with N-terminally EGFP-fused NS5A of genotype 1b (EGFP-NS5A) in HeLa cells, fixed with 4% paraformaldehyde-PBS, permeabilized with 0.5% Triton X-100, and stained with anti-FLAG antibody and AlexaFluor 594-conjugated anti-mouse IgG antibody. (B) EGFP-NS5A of genotype 1b (b and e) or FLAG–VAP-B (h and k) was expressed and then stained with anti-PDI (a and g) or anti-giantin (d and j) antibodies and AlexaFluor 594-conjugated anti-mouse IgG antibody. FLAG–VAP-B was stained with biotinylated anti-FLAG antibody and fluorescein isothiocyanate-conjugated streptavidin. Overlapped images are shown in panels c, f, i, and l.

**Two separate domains in NS5A are critical for binding to VAP-B.** Since NS5A specifically interacts with VAP-B, we tried to determine the region of NS5A responsible for interaction with VAP-B. Various deletion mutants of FLAG-tagged NS5A were prepared as shown in Fig. 5A. The mutants covering regions from amino acids 1 to 75, but not 1 to 50, and those

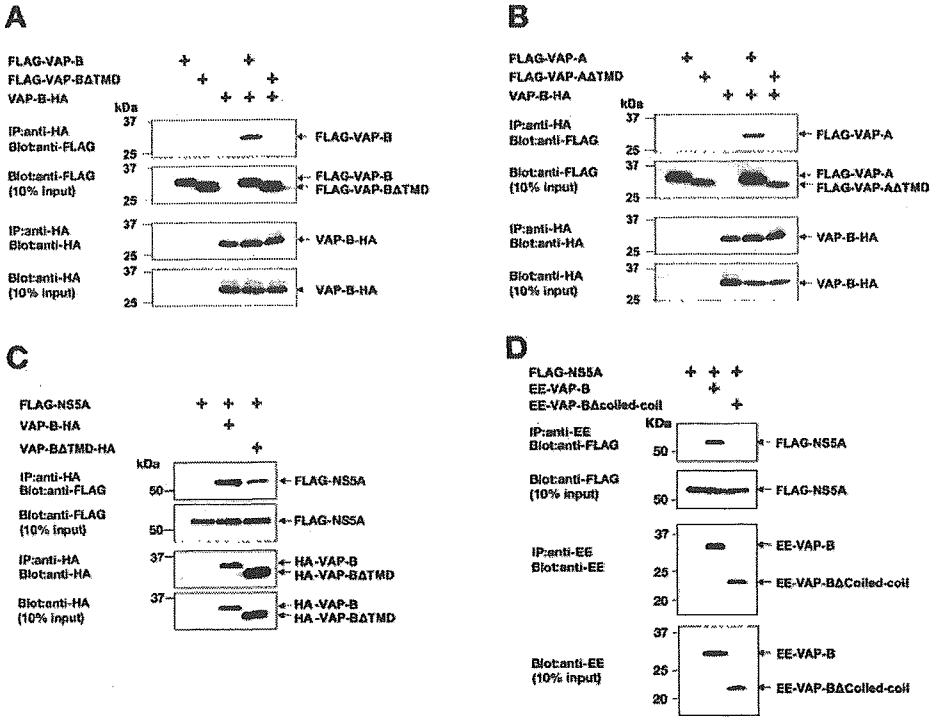


FIG. 4. VAP-B dimerizes with VAP-B and VAP-A through the TMD and interacts with NS5A via the coiled-coil domain. C-terminally HA-tagged VAP-B (VAP-B–HA) was coexpressed with FLAG–VAP-B or FLAG–VAP-B with TMD deleted (FLAG–VAP-BΔTMD). VAP-B–HA was immunoprecipitated with anti-HA antibody, and the immunoprecipitates were immunoblotted with anti-FLAG antibody (A). Interaction of VAP-B–HA with FLAG–VAP-A or FLAG–VAP-A with TMD deleted (FLAG–VAP-AΔTMD) was examined in a similar way (B). FLAG-NS5A was coexpressed with HA–VAP-B or HA–VAP-BΔTMD, and immunoprecipitates with anti-HA antibody and immunoprecipitates were immunoblotted with anti-FLAG antibody (C). FLAG-NS5A was coexpressed with EE–VAP-B or with EE–VAP-B in which the coiled-coil domain was deleted (EE–VAP-BΔcoiled-coil). EE-tagged VAP-B proteins were immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted with anti-FLAG antibody (D). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

from amino acids 325 to 447, but not 350 to 447, exhibited binding to VAP-B, suggesting that two separate regions of NS5A (amino acids 51 to 75 and 325 to 349) are involved in physical association with VAP-B. Further mutational analyses of NS5A revealed that regions from amino acids 1 to 70, but not 1 to 65, and those from amino acids 340 to 447, but not 345 to 447, interact with VAP-B (Fig. 5B and C), suggesting that amino acids 66 to 70 and 340 to 344 are required for interaction with VAP-B. According to Tellinghuisen et al., NS5A consists of three domains, domain I (amino acids 1 to 213), domain II (amino acids 250 to 342), and domain III (amino acids 356 to 477) (46, 47). In our results, the region from amino acids 340 to 344, which is essential for the physical interaction with VAP-B, belongs to the connecting segment between domains II and III of NS5A. Ala substitution analyses revealed that an NS5A construct covering amino acids 260 to 447 that replaced the five amino acid residues between 340 and 344 with Ala abrogated interaction with VAP-B (Fig. 5D), whereas that covering 75 N-terminal amino acids carrying an Ala substitution of between 66 and 70 residues retained binding activity to VAP-B (data not shown). Therefore, we focused on the region between 340 and 344 to determine the amino acid residues in NS5A responsible for specific binding to VAP-B. A FLAG-tagged full-length NS5A carrying an Ala substitution between amino acid residues 340 and 344 (FLAG-NS5A/340-344A) exhibited a clear reduction of binding to EE–VAP-B compared with the authentic NS5A (Fig. 5E). To further determine the critical amino acids of NS5A responsible for specific binding to VAP-B, each amino acid between 340 and 344 of the NS5A construct covering amino acids from 260 to 447 was replaced with Ala, and the effect of each substitution on the interaction with VAP-B was examined by immunoprecipitation. As summarized in Fig. 5F, the four amino acid residues 341 to 344 in the polyproline cluster region of NS5A, which are highly conserved among HCV genotypes, are suggested to be involved in the interaction with VAP-B.

**VAP-B plays an important role in HCV RNA synthesis.** To determine whether VAP-B is involved in HCV replication, cell lysates isolated from Huh-7 cells harboring a subgenomic HCV replicon were used for an in vitro RNA synthesis assay. Chicken anti-human VAP-B antibody raised against synthesized peptides specifically detected endogenous and overexpressed VAP-B (Fig. 6A). Cytoplasmic fraction from the HCV replicon was added to an assay mixture containing [$\alpha$-$^{32}$P]CTP and incubated at 30°C for 4 h in the presence or absence of antibodies. Labeled RNA was analyzed by 1% formaldehyde agarose gel electrophoresis as described previously (2). Replication of the subgenomic HCV RNA was inhibited by the

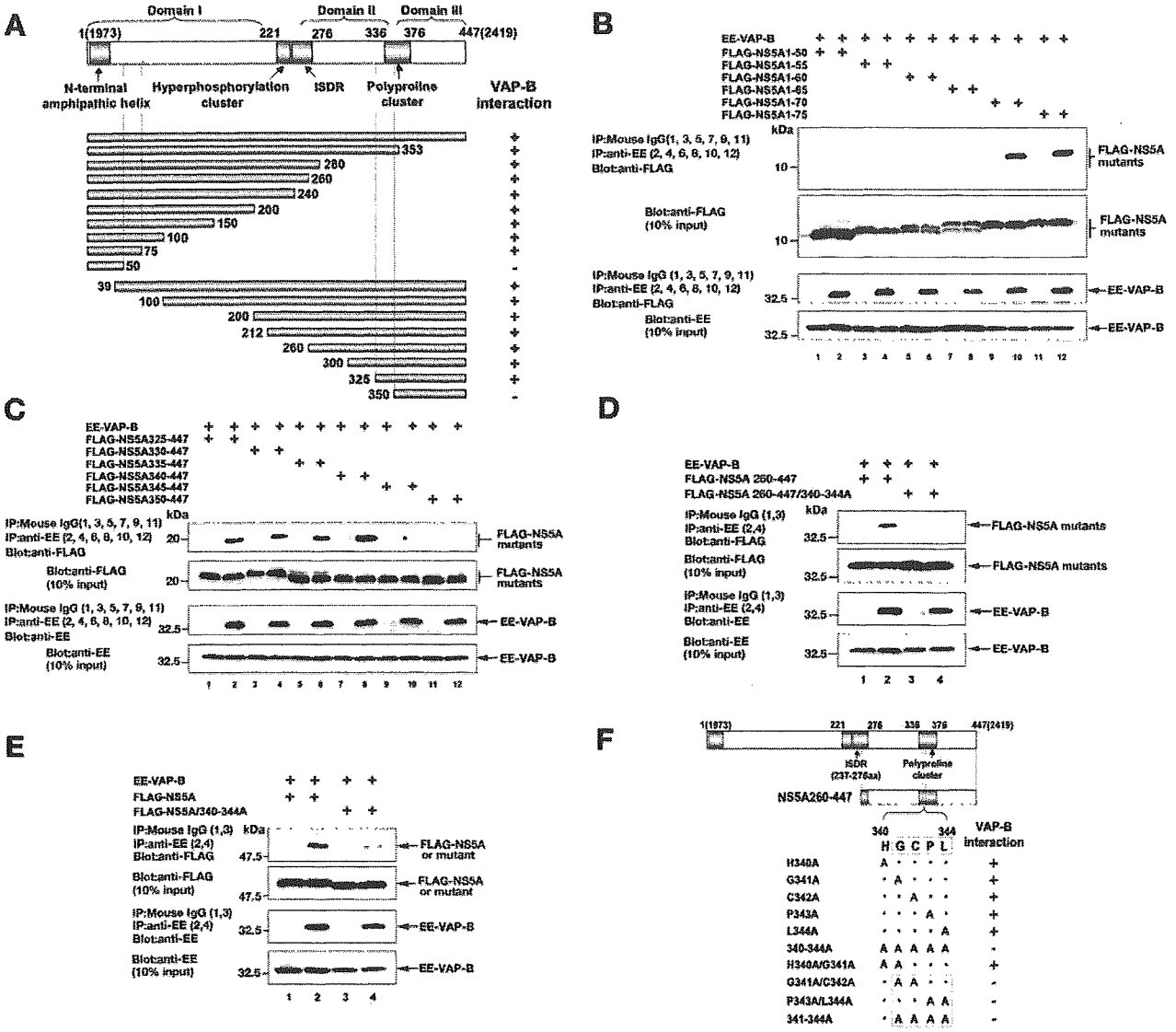


FIG. 5. Two regions of NS5A are required for VAP-B binding. N-terminal or C-terminal deletion mutants of NS5A were introduced into pEF-FLAG pGBK puro vector and coexpressed with EE–VAP-B. EE–VAP-B was immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted by anti-FLAG antibody. The reverse combination of immunoprecipitation was also examined. The results are summarized in panel A. Four functional domains in the NS5A protein and three domains based on the locations of the blocks of low-complexity sequence (46) are indicated. The numbers in parentheses indicate amino acid residues in the HCV polyprotein. To further determine the critical amino acids of NS5A for specific binding to VAP-B, deletion mutants of the N-terminal region from residues 1 to 75 (B) or those of the C-terminal region from residues 325 to 447 (C) were immunoprecipitated with EE–VAP-B. Replacement of the five residues 340 to 344 with Ala was introduced into a truncated NS5A possessing residues 260 to 447, FLAG-NS5A 260-447/340-344A (D), or full-length NS5A, FLAG-NS5A/340-(E), to examine the interaction with VAP-B. Further precise mutations were introduced into NS5A possessing residues 260 to 447. The resulting mutants were coexpressed with EE–VAP-B and immunoprecipitated as described above. The results are summarized in panel F. Four amino acids (Gly, Cys, Pro, and Leu) responsible for interaction with VAP-B are indicated by dotted squares. Plus and minus indicate binding and nonbinding, respectively (A and F). One-tenth of the lysates used in immunoprecipitation are shown as the 10% input. The data in each panel are representative of three independent experiments.

antibody to VAP-B but not by a control chicken immunoglobulin G (IgG) (Fig. 6B), suggesting that VAP-B plays a critical role in HCV replication. Aizaki et al. suggested that VAP-A sequesters NS5A at an appropriate site, such as the raft-like domain on the intracellular compartment, and that the TMD of VAP-A plays an important role in subcellular localization and dimerization (2). We demonstrated that the TMD of VAP is required for hetero- and homodimerization of VAP-A and VAP-B but not for interaction with NS5A (Fig. 4). Gao et al. indicated that a truncated VAP-A mutant lacking the TMD inhibited the association of HCV NS proteins with insoluble membrane fractions and reduced both the expression level of

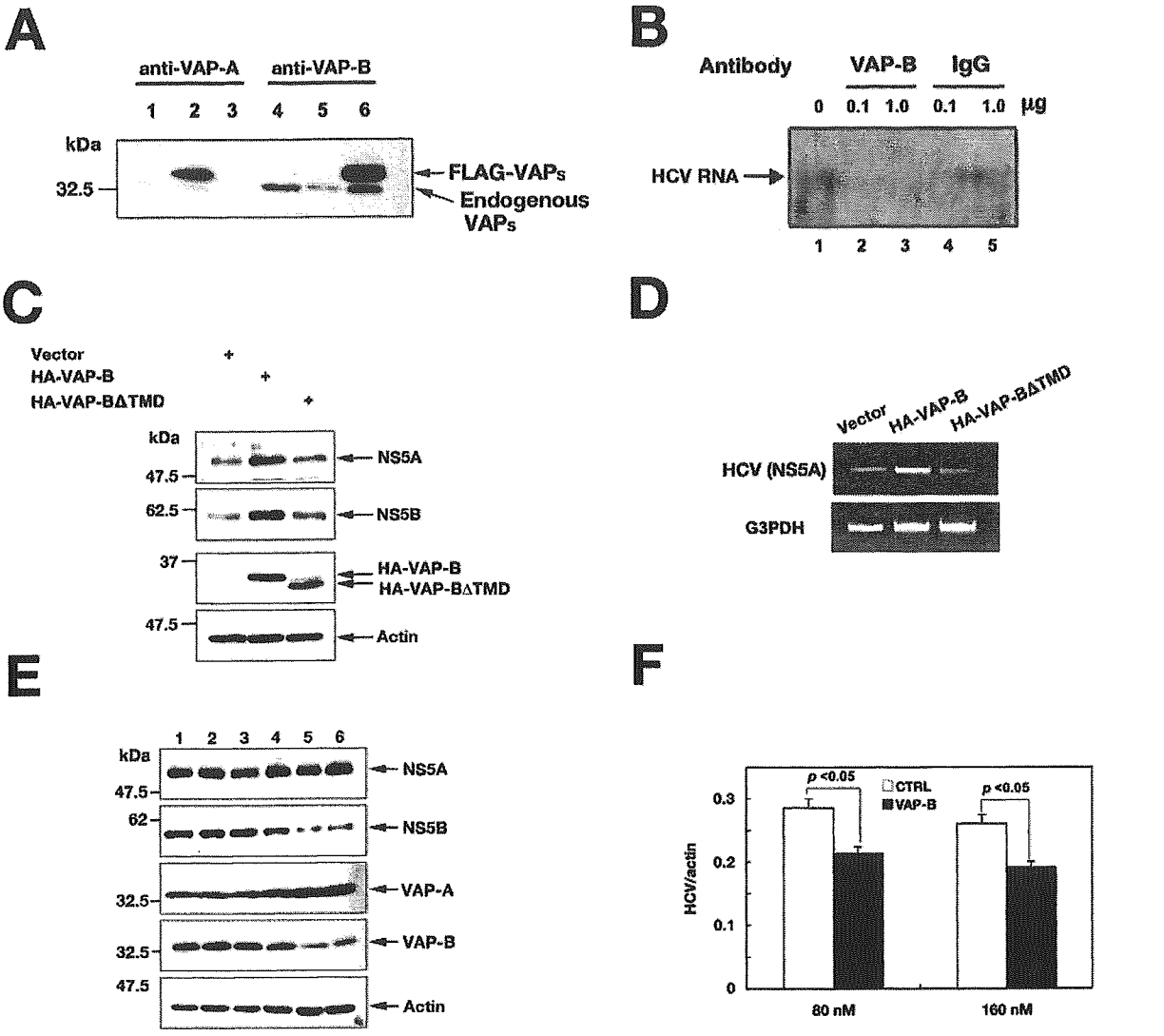


FIG. 6. VAP-B is involved in HCV replication. (A) FLAG–VAP-A (lanes 2 and 5) or FLAG–VAP-B (lanes 3 and 6) was expressed in HEK293T cells and examined by immunoblotting using anti-human VAP-A mouse monoclonal and anti-human VAP-B chicken polyclonal antibodies. (B) In vitro RNA synthesis was carried out in the presence of various concentrations of anti-human VAP-B chicken polyclonal antibody or control chicken IgG. RNA extracted from each fraction was analyzed by agarose gel electrophoresis and autoradiographed. (C) Empty plasmid, expression plasmid of N-terminally HA-tagged VAP-B (HA–VAP-B), or N-terminally HA-tagged VAP-BΔTMD (HA–VAP-BΔTMD) was transfected into HCV replicon cells. Expression of NS5A and NS5B was examined by immunoblotting. (D) HCV RNA was detected by reverse transcription-PCR using primer pairs against NS5A, and expression of G3PDH was used as a control. (E) siRNA against VAP-B or control was transfected into the HCV replicon cells. Lane 1, untreated; lane 2, treated with siFECTOR; lanes 3 and 4, control siRNA was transfected; lanes 5 and 6, VAP-B siRNA was transfected. Expression of NS5A, NS5B, VAP-A, VAP-B, and beta-actin was determined by immunoblotting at 96 h posttransfection. (F) siRNA against VAP-B or control was transfected into the HCV replicon cells. The results are expressed as standard deviations. The significance of the difference in means was determined by the Student *t* test. The data in each panel are representative of three independent experiments.

NS5A and HCV RNA replication in replicon cells (12). To determine the possible implication of VAP-B in HCV replication, VAP-B or VAP-BΔTMD was expressed in Huh-7 RNA replicon cells. In contrast with the previous data, overexpression of VAP-B increased NS5A and NS5B expression and enhanced the replication of HCV replicon cells, but no effect was observed in cells expressing VAP-BΔTMD (Fig. 6C and D). To confirm the role of VAP-B in HCV replication, we examined the effect of the knockdown of endogenous VAP-B from the HCV replicon cells by siRNA. At 96 h posttransfection, the expression of VAP-B in cells transfected with the siRNA targeted to VAP-B was reduced to half the levels of cells transfected with a control siRNA, whereas the expression of VAP-A was slightly increased. Although NS5B expression was reduced by the VAP-B knockdown, NS5A was not affected (Fig. 6E). HCV RNA