these mutations before the mutations were able to be detected. Additionally, our data did not address the significance of the impact that mutations in regions other than NS5 may have had on viral response to therapy.

HCV populations in vivo consist of a quasispecies nature. Our previous cloning analysis detected small number of minor clones in specimens, which were determined as ISDR-wild type by direct sequencing [24]. Hence, it should be noted that our criteria for mutation could not completely distinguish between de novo mutation and selection of a minor clone.

In the present study, we found that ribavirin also expressed antiviral activity by reducing viral load, presumably because we used a highly quantitative assay for HCV-RNA measurement [17]. However, contradictory results have been reported previously [1–3]. Since the present study identified only a small reduction in viral load, further investigation is needed to confirm our result.

In conclusion, our data demonstrate that clinical administration of ribavirin induces mutations in HCV genes and suggest that, in some patients, mutagenesis may be one of the mechanisms responsible for the synergistic efficacy of ribavirin in IFN/ribavirin combination therapy.

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Journal of Gastroenterology

Editorial

Genetic changes in the interferon sensitivity-determining region of hepatitis C virus (HCV) during the natural course of infection: an implication for the gene function in the role of chronic infection

Article on page 43
Nonstructural 5A gene variability of hepatitis C virus (HCV) during a 10-year follow up
FAN W, ZHU W, WEI L, et al.

The interferon sensitivity-determining region (ISDR; aa2209–2248 of HCV-J) in the nonstructural 5A region (NS5A) of hepatitis C virus (HCV) was originally identified as the genomic functional element wherein missense mutations were closely related to the clinical efficacy of interferon treatment, as well as to serum viral loads, in genotype-1b HCV infection.[1,2] After the first reports of the ISDR, controversy arose as to its predictive value for the outcome of interferon therapy, because clinical studies in Europe and North America did not always support the relevance of ISDR,[3] although most studies in Japan, Spain, and Italy supported it.[4,5] However, recent meta-analyses have clearly supported the universal correlation between ISDR sequence and interferon resistance.[6] It is speculated that the initial discrepancy of the results might have been caused by differences in interferon regimens and patient sources.

After identification of ISDR as the key genomic element for interferon efficacy and viral replication, the molecular function of NS5A protein and its relevance to ISDR structures has been vigorously and intensively studied using NS5A protein expression in vitro or in transgenic mice. A variety of putative NS5A functions were postulated, such as binding to cellular protein kinase R (PKR),[7] TRADD,[8] Grb-2,[9] p21,[10] hVAP-33,[11] or other proteins that may influence the pathogenesis of hepatitis C by antiviral effects, apoptosis, signal transduction, cell cycle regulation, or formation of viral replication complex. Much attention has been paid to PKR, because NS5A protein was found to block the antiviral effect of PKR in an ISDR sequence-dependent manner by directly binding to PKR through the so-called PKR-binding domain, which includes the ISDR plus an additional 26 aa stretch located at the C-terminal portion (aa2209–2274). The recently developed HCV subgenomic replicon system also disclosed the importance of NS5A proteins in intracellular viral replication, because specific mutations called "cell culture-adaptive mutations" needed in its genome for efficient replication in cultured cells clustered in the central region of NS5A, particularly in the serine cluster region immediately upstream to the ISDR or the ISDR itself.[12,13] Because these mutations possibly affect phosphorylation of NS5A proteins, the role of phosphorylated NS5A protein in viral replication and interferon sensitivity has become the recent target of molecular research.

How does the HCV–ISDR structure change in a host during the natural course of disease? The answer to this question should give us important clinical information about when to start interferon therapy, whether earlier or later in HCV infection. In the current issue of the *Journal of Gastroenterology*, Fan et al.[14] report the genetic evolution of the *NS5A* gene during a 10-year follow-up of natural HCV infection in 7 patients, focusing on ISDR, PKR-binding domain, serine cluster region, and other functional domains in *NS5A* gene. To investigate changes of the genetic variability during the natural course, they performed subcloning analysis. As a result, serine residues at positions 2194, 2197, 2201, and 2204 in the serine cluster region, suggested to be important for hyperphosphorylation of NS5A protein, were highly conserved in all patients and within the quasipecies of each patient, suggesting that phosphorylation plays the crucial role in NS5A protein function. Meanwhile, subcloning analysis of the ISDR quasispecies disclosed that the wild-type ISDR (no aa substitution relative to HCV-J), or the intermediate-type ISDR (one to three substitutions) was dominant and stable throughout the observation periods in all patients. However, the ISDR quasispecies decreased over time, and the quasispecies had a tendency gradually to converge to the wild-type ISDR.

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Before the report by Fan et al., several studies already had been conducted for analyzing the natural genetic change of the ISDR.[15-19] Although these previous studies also demonstrated that the wild-type ISDR or the intermediate-type ISDR was generally stable, the observation periods were rather short, and the quasispecies complexity was not investigated in most of the studies. Importantly, Fan et al. demonstrate that the ISDR quasispecies finally tended to converge to the wild-type ISDR with a decrease of the quasispecies complexity, indicating that sequence motif of the wild-type ISDR had a crucial role functionally in establishing chronic HCV infection. Although the mutant-type ISDR (four or more aa substitutions) was not included in the study, the mutant-type ISDR was reported to be rather unstable, because nonsynonymous mutations (63%) were higher than synonymous mutations (37%), indicating that strong selective pressure of the host was exerted on the mutant-type ISDR.[16] This finding coincides with the results by Fan et al. that the HCV of wild-type ISDR ultimately survives in the course of chronic infection.

If the quasispecies complexity of the ISDR subtypes finally converges to the wild-type ISDR, it might be better to start interferon therapy early for chronic HCV infection. It is not clear why the mutant-type ISDR is unstable in a host. Part of the mechanism, however, might be explained by different interaction with cellular proteins, such as PKR induced by endogenous interferon. On the other hand, if the mutant-type ISDR was unstable and easily defeated by the wild-type ISDR in chronic infection, this weak subtype might have disappeared in the world of HCV infection. However, although the distribution is rather small, the mutant-type ISDR is still frequently found in clinical samples. Is it on the way to disappearing? Or does it have an advantage over the other types in a certain phase of infection other than the chronic phase? Recent advances in understanding of the innate immune system have disclosed that mammalian cells have two distinct innate immune pathways protecting cells from the virus: the interferon regulatory factor (IRF) system, and the interferon (IFN)—signal transduction and activator of transcription (STAT) system. In the phase of chronic infection, the IFN–STAT system might have a dominant role in viral suppression, and the wild-type ISDR is supposed to inhibit this pathway, giving a survival advantage to HCV with the wild-type ISDR. In contrast, the IRF system might be dominant in the phase of acute infection. Does the mutant-type ISDR inhibit this IRF pathway, and give a survival advantage to HCV with the mutant-type ISDR? The answer to this question requires further study, but these analyses might go far toward helping understand the mysterious function of the ISDR.

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Viral load change and sequential evolution of entire hepatitis C virus genome in Irish recipients of single source-contaminated anti-D immunoglobulin*

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SUMMARY. In hepatitis C virus (HCV) infection, serum viral load is important in the prediction of therapeutic efficacy. However, factors that affect the viral load remain poorly understood. To identify viral genomic elements responsible for the viral load, we investigated samples from a population of Irish women who were iatrogenically infected from a single HCV source by administration of HCV 1b-contaminated anti-D immune globulin between 1977 and 1978 (Kenny-Walsh, *N Engl J Med* 1999; 340: 1228). About 15 patients were divided into two groups, viral load increasing group (11 patients) and decreasing group (4 patients). Pairs of sera were collected from each patient at interval between 1.1 and 5.8 years. Full-length sequences of HCV genome were determined, and analyzed for changes in each patient. Sliding window analysis showed that the decreasing group had significantly higher mutation rates in a short segment of NS5B region that may affect the activity of RNA-dependent RNA polymerase. By comparing each coding regions, significantly higher mutation numbers were accumulated in NS5A region in the increasing group than the decreasing group (0.92 *vs* 0.16 nucleotides/site/year, $P = 0.021$). The mutation in certain positions of the HCV genome may be determinant factors of the viral load in a relatively homogeneous patient population.

Keywords: anti-D immunoglobulin, full genome sequence, hepatitis C virus.

INTRODUCTION

Hepatitis C virus (HCV) is globally distributed virus that causes chronic inflammation in liver, and may leads to liver cirrhosis and hepatocellular carcinoma over the course of 20–30 years [1–3]. Efficacy of anti-viral therapy based on pegylated interferon and ribavirin for chronic hepatitis C ranges between 40 and 80% [4–6]. Viral load is an important predictive factor for therapeutic outcome. We have previously demonstrated a close relationship between HCV genomic structures with patients' viral load [7], hepatitis activities [8,9], and ALT levels [10]. However, all of these observations were on the basis of heterogeneous hosts infected with different viral strains, making the role of the viral genetic structures ambiguous. A study using homogeneous host populations infected with a homogeneous viral strain would be ideal to clarify the role of viral gene in determination to the viral load.

There were two outbreaks of HCV infection in the world through usage of the virus-contaminated blood products from a single donor. One was in Ireland and one in Germany [11,12]. In Ireland, from May 1977 to November 1978, 704 women were iatrogenically infected with HCV through administration of a virus-contaminated anti-D immune globulin to prevent rhesus isoimmunization [12,13]. All recipients were female, had same ethnic origin, similar

*Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. There is no conflict of interests for present study.

Abbreviations: HCV, hepatitis C virus; CH, chronic hepatitis; LC, liver cirrhosis; HCC hepatocellular carcinoma; ALT, alanine aminotransferase; E, envelope; NS, nonstructural; PKR, protein kinase R; HLA, human histocompatibility leukocyte antigen; RT, reverse transcription; ISDR, interferon sensitivity determining region; PCR, polymerase chain reaction.

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duration of disease, infected with HCV genotype 1b from a single donor. In this homogeneous clinical setting, the amount of serum HCV has been shown to fluctuated [14]. HLA DR locus and DQ locus were shown to be associated with viral load in this patients group [15]. The HCV genomic determinants of the viral load set point are currently unknown.

The purpose of this study is to clarify the relationships between viral genetic structures and the serum viral load in this Irish patient population. We analyzed the full viral genomic sequences in each patient within the study population, and compared those patients whose had increased viral load with those who had decreased viral load within the time frame under retrospective investigation of approximately 20 years.

PATIENTS AND METHODS

Patients

We determined the HCV sequence of donor's preserved serum (deposited with the DDBJ/Genbank/ENBL data libraries under accession number AF313916). In total, 15 Irish female patients infected with HCV-contaminated anti-D immunogobulin were used in this study, all of them have never been treated before enrolled into this study. About 25 patients were randomly chosen from the anti-D patients, DNA sequence analysis was attempted on all specimens, and 15 pairs of sera yielded the full length viral genomic sequence information. Each patient in the study population had two sera samples retrospectively analyzed from a biobank of specimens prospectively collected as a part of the routine clinical management and viral load quantification in this patient population at Cork University Hospital. The median temporal separation between samples was 39.2 months (13–72 months). All patients were infected by HCV genotype 1b from virus-contaminated anti-D immunoglobulin injections during the period from May 1977 to November 1978. All patients attended the hepatitis C clinic at Cork University Hospital, Cork, Ireland. Serum HCV-RNA levels were determined by a polymerase chain reaction assay (PCR assay; Roche HCV Monitor kit, F. Hoffmann-La Roche Ltd., Basel, Switzerland). The study design is shown in Fig. 1. Standard deviations have previously been reported [16], the 95% confidence interval of the viral load was ±0.032 viral copies/mL. A viral load increase or decrease over the range 0.062log10 viral copies/mL, was used as the criteria for change in viral load. The patients were classified into two groups. The characteristics of group one were as follows: the viral load was increased over time, $n = 11$, hence, increasing group (group-I). The second group of patients had a decreased or remained stable in viral load over the time investigated, decreasing group (group-D, $n = 4$). All patients were seronegative for HBV markers, had no autoimmune liver disease or competing

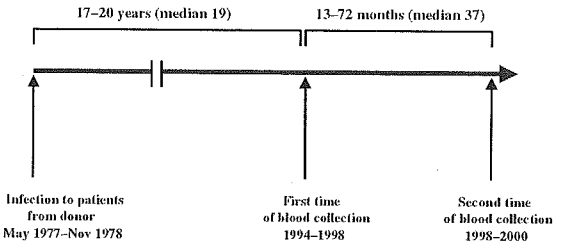

Fig. 1 Study design. A total of 30 sera were acquired from 15 patients. Each patient's sera were collected as part of the routine clinical management of chronic hepatitis C at Cork University Hospital, Ireland. Initial viral load assessment was approximately 19 years post-infection with HCV 1b contaminated anti-D immunoglobulin. The second samples selected for analysis in this study were dated to a further 6 years post-infection. Sequences were analysed by direct sequence from PCR generated amplicons.

risk factors such as excessive alcohol intake or hepatotoxic drugs. All patients had liver biopsy performed as part of their routine clinical assessment. Informed consent was obtained from each patient for liver biopsy, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Sera were stored at −80 °C storage facility.

RNA extraction and RT-PCR analysis

RNA extraction, cDNA synthesis and PCR analysis were performed as reported by us previously [17]. Briefly, serum RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan). The extracted RNA was reverse-transcribed by moloney murine leukemia virus reverse transcriptase (MMLV-RT, GIBCO BRL) using random hexamers (Takara, Kyoto, Japan). The full-length HCV genome was amplified by the nested PCR with 21 partially overlapping sets of primers using Advantage cDNA Polymerase Mix (Clontech, California, USA) according to the manufacturer's instructions. 3′UTR and X tail were not uniformly amplified probably because of the condition of storage. Therefore, these regions were not analyzed in this study.

Sequence determination

Each PCR product was purified and residual primers were removed with the column (Suprec 02, Takara, Kyoto, Japan). Thereafter, both strands of the PCR products were cycle sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Tokyo, Japan) using forward and reverse sequencing primers, respectively. The products were purified by the column (Quickspin column, Boehringer Mannheim, Indiana, USA) and sequenced using an automated DNA sequencer (model 373S; Applied Biosystems, Tokyo, Japan). Each sequence

Table 1 Patients' basic characteristics

No.	Duration from infection (year)		Interval time (month)	Viral load (log_{10}kcopy/mL)		Rate change of viral load (log_{10}kcopy/mL/year)
	1st	2nd		1st	2nd	
1	18	22	29	5.62	7.41	0.741
2	19	22	33	5.48	7.28	0.655
3	17	22	31	4.64	6.27	0.633
4	20	23	37	6.18	7.59	0.459
5	17	20	45	4.38	5.94	0.416
6	17	23	56	5.23	6.88	0.354
7	20	22	69	5.46	7.15	0.293
8	17	23	37	4.71	5.15	0.203
9	20	22	39	4.82	5.38	0.173
10	17	19	72	5.38	5.91	0.089
11	19	22	26	4.96	5.04	0.052
12	19	23	18	5.36	5.36	0.000
13	21	22	24	5.00	4.89	−0.054
14	19	22	13	5.86	5.75	−0.099
15	19	22	59	5.46	4.55	−0.184
Group-I*	18.7 ± 0.3	22.2 ± 0.2	41.0 ± 4.8	5.19 ± 0.15	6.28 ± 0.27	0.37 ± 0.07
Group-D*	19.0 ± 1.1	21.0 ± 1.0	32.0 ± 13.9	5.44 ± 0.25	5.07 ± 0.36	−0.08 ± 0.04

*no. 1–11: group-I, no. 12–15: group-D.

Table 2 Patients' basic characteristics (continued)

No.	ALT value (microkat/L)		HLA DQB1 locus	HLA DRB1 locus	DR 51–53
	1st	2nd			
1	0.50	0.44	0201, −02/0501	01/03	53
2	0.98	0.91	0201, −02/0602, −11	03/15	51/52
3	0.65	0.70	Not typed	Not typed	Not typed
4	1.43	1.56	Not typed	Not typed	Not typed
5	0.72	0.51	0201, −02/0602, −11	0701/15	51
6	0.63	0.59	05031/05031	13/14	52
7	1.26	2.29	0201, −02/0602, −11	0701/15	51/53
8	0.72	0.75	0201, −02/03032, −06	03/0701	53
9	0.56	0.57	0201, −02/03032, −06	0701/0701	52
10	1.70	0.98	0201, −02/03011	03/04	Not typed
11	0.54	0.98	0301/0501	01/13	52
12	0.71	0.59	Not typed	Not typed	Not typed
13	0.32	0.43	Not typed	Not typed	Not typed
14	0.79	0.67	0301/0301	0701/11	52/53
15	0.69	0.64	0402/0604	08/13	52
Group-I†	0.91 ± 0.12	0.97 ± 0.16			
Group-D†	0.63 ± 0.11	0.58 ± 0.06			

Normal range of ALT value was 0.12–0.60 microkat/L.
†no. 1–11: group-I, no. 12–15: group-D.

was confirmed twice with direct sequencing method for sense and anti-sense strands'. Subcloning study was not done, therefore HVR were not mentioned in the present study. HLA profile was available for 11 individuals study population and was performed as outlined previously reported [16].

Statistical analyses and phylogenetic analysis

For phylogenetic analysis, the nucleotide and deduced amino acid sequences of the patients were compared with a sequence of HCV-J strain [18] as a reference. The nucleotide and amino acid sequences were compared longitudinally in each patient. Amino acid changes were picked up between the paired samples from each patient. Serum viral load changes were defined as increasing status when they were above zero, and defined as decreasing status when equal to or under than zero. Calculation of amino acid mutation rates and the phylogenetic analyses were performed by the Mega software version 2.1 with neighbour-joining method (NJ) [19], and PHYLIP version 3.62 with maximum likelihood method (ML) [20]. NJ tree and NG distances were evaluated using 1000 bootstrap samples, *P* values for the branches of the ML tree were also calculated. Nucleotide mutation rates were calculated by the Mega ver.2.1. Statistical analyses of the two groups were done by Mann–Whitney's *U* test using the program Stat View 5.0 (SAS institute inc.). All tests of significance were two-tailed, with *P* values of <0.05 considered to indicate statistical significance.

RESULTS

Characteristics of patients

The clinical characteristics were compared between the two groups, 11 patients with increasing viral load status (group-I) and 4 patients with decreasing status (group-D), as shown

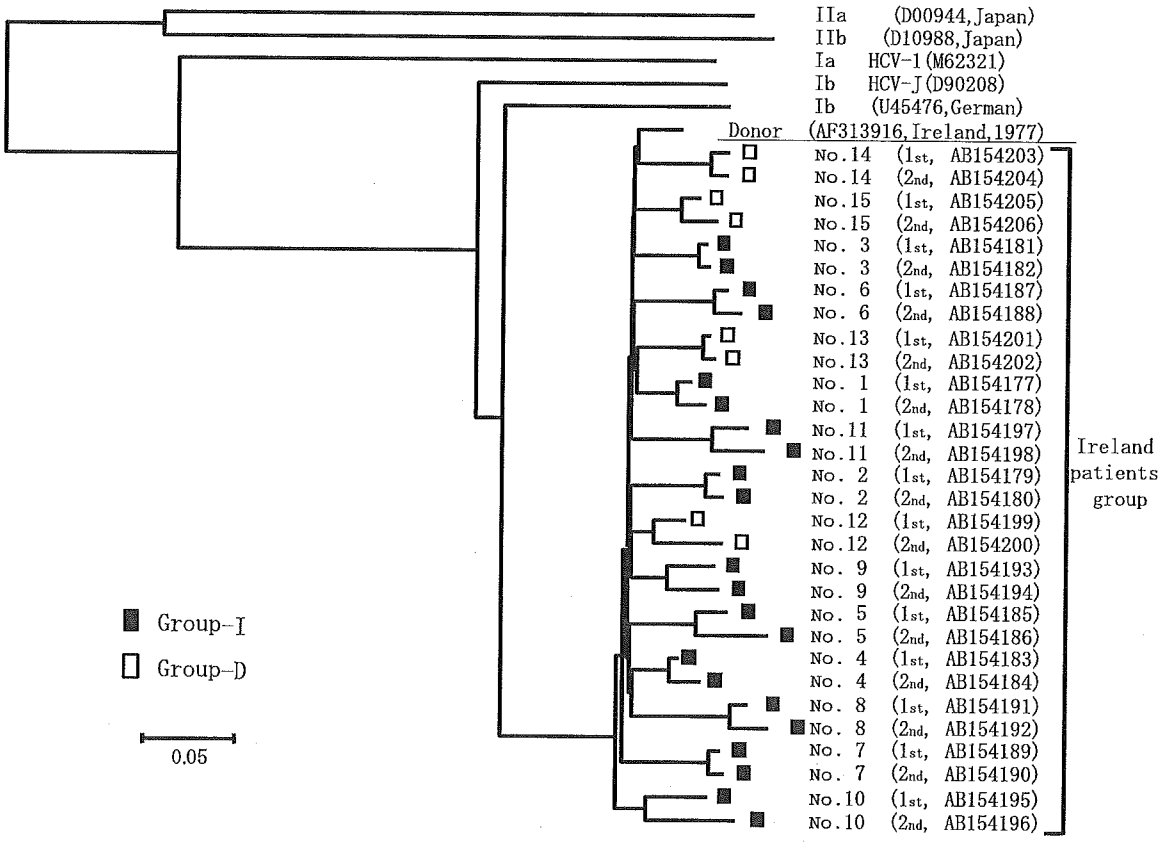

Fig. 2 Phylogenetic analysis with entire amino acid sequences of anti-D recipients, the donor, and representative sequences of genotype 1b, 2a and 2b. A phylogenetic tree was constructed by the neighbour-joining tree method with 10 000 bootstraps supports. The donor-sequence is shown in the 6th from the top. Closed squares show the recipients sequences belonging to the viral load increasing group (group-I), and open squares show them belonging to the decreasing group (group-D), and the bar at the left below is the reference for the distance which length is 0.05. Patients' numbers are matched with Table 1, and ordinal numbers after patients' number indicates the order of collection of serum from each patient. The mutations cluster in one group involving donor's sequence, but are individual to each patient. The distances from donor's sequence to patients' sequences did not segregate with change in viral load. The average distance from donor's sequence are as follows: to 1st acquired serum, 0.058 ± 0.0028 in group-I and 0.051 ± 0.0034 in group-D, and to 2nd, 0.065 ± 0.0030 in group-I and 0.060 ± 0.0021 in group-D, and within each pairs, 0.028 ± 0.0060 in group-I and 0.024 ± 0.0080 in group-D.

in Table 1. No significant difference could be found in age, time intervals between the two-blood sampling of each patient, and the intervals between the time on infection and the first blood sampling. The amounts of serum HCV-RNA were not significantly different between the two groups at first time of blood collection (5.17 ± 0.16 $\log_{10}$kcopy/mL in group-I, 5.42 ± 0.18 $\log_{10}$kcopy/mL in group-D, respectively). The viral load was significantly higher in the group-I at the second serum samples (6.37 ± 0.29 $\log_{10}$kcopy/mL in group-I, 5.14 ± 0.26 $\log_{10}$kcopy/mL in group-D, $P = 0.027$). The mean rate of change was significantly different between two groups (0.37 ± 0.07 Log_{10}kcopy/mL/year to group-I, −0.08 ± 0.04 $\log_{10}$kcopy/mL/year to group-D, $P = 0.004$). Average ALT values were not different between both the groups (Table 2).

Phylogenetic analysis

To clarify the tendency of genomic changes of HCV in each patient (deposited with the DDBJ/Genbank/ENBL data libraries under accession number AF313916 for donor sequence, AB154177 to AB154206 for recipients), phylogenetic analyses were done based on the entire amino acid sequence by the neighbour-joining tree method (Fig. 2). The phylogenetic tree analysis showed that all sequences studied in the present study belonged to genotype 1b cluster (100% bootstrap support, $P < 0.01$ in the maximum likelihood tree), and they were more closely related to the donor's sequence than any other known 1b sequences (100% bootstrap support, $P < 0.01$). The two sets of sequence data derived from the each patient segregated together on the phylogenetic tree (100% bootstrap support, $P < 0.01$). The genetic distance between each specimen of each pair was, as anticipated, less than the distance from donor's sequence (100% bootstrap support, $P < 0.01$). There was no relationship between the genetic distance calculated with amino acid sequence and time intervals of recipient's serum samplings or viral load fluctuations. This suggests that the genetic evolution speed was different among patients independent of viral loads.

Mutations from donor to recipients

We initially analyzed the average rate of HCV amino acid sequence mutation between the donor and the pair of samples from each recipient (Fig. 3). Comparison of the number of mutations between group-I and group-D in the open reading frame revealed no significant differences. Further analyses restricted to small functional regions including PKR eukaryotic initiation factor-2α phosphorylation homology domain, ALT response related element, PKR-binding domain, interferon sensitivity determining region (ISDR), nuclear localization signal and variable region 3 region showed no difference in mutations number in both groups (raw sample data not shown).

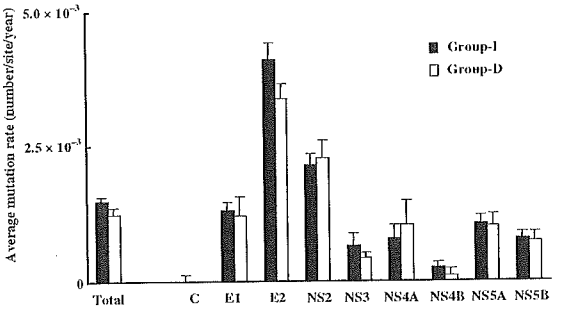

Fig. 3 Average rates of amino acid mutations from donor's sequence to recipients'. The mutation rates are those calculated from the first acquired sera from each patient in the cohort. The mutation rate was calculated for each coding region. The analysis of the number of mutations in both groups indicated that there are differences between regions, but the analysis did not achieve significance.

Genomic analysis of paired sera

The average rates of mutations in amino acids for the entire HCV genome in group-I were similar to that in group-D ($3.4 \times 10^{-3} \pm 0.6 \times 10^{-3}$ numbers/site/year in group-I and $3.3 \times 10^{-3} \pm 1.2 \times 10^{-3}$ numbers/site/year in group-D). A comparison of each coding region revealed that the average number of mutations was significantly higher in group-I than group-D in NS5A ($2.07 \times 10^{-3} \pm 0.41 \times 10^{-3}$ numbers/site/year *vs* $0.36 \times 10^{-3} \pm 0.41 \times 10^{-3}$ numbers/site/year in average, $P = 0.02$) (Fig. 4). Individual points of mutations in each patient were aligned as outlined in Fig. 5. The majority of the mutations clustered at E2, NS5A and NS5B in each patient. However, no specific position was found to be unique to group-I or group-D.

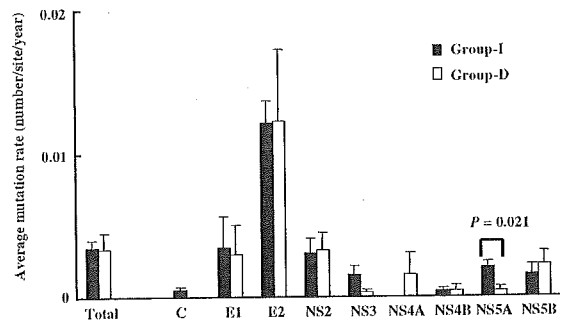

Fig. 4 Average rates of viral sequential mutations within each recipients belonging to two groups. The mutation rates are those calculated from the first to second acquired sera from each patient in the cohort. The mutation rate was calculated for each coding region. The analysis of the number of mutations indicated that there are defined differences in the observed mutation frequency between the group-I and group-D for NS5A. This difference was significantly different ($P = 0.021$).

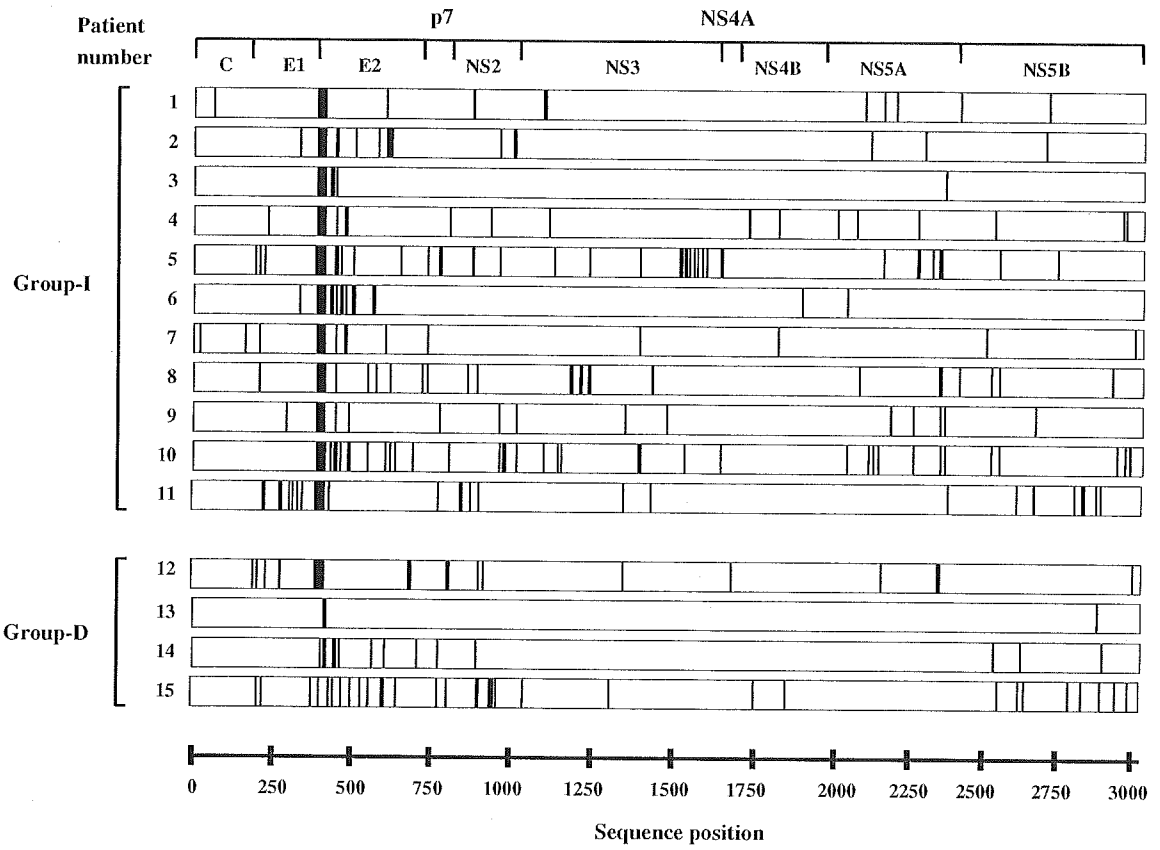

Fig. 5 Plots of mutations in full sequence in each recipients. Every mutation of amino acids has plotted on the bar for each patient. No specific point mutation was detected that was associated with change in viral load.

Sliding-window analysis

We analyzed the sequence data using a method that we previously reported [21], to find any accumulation of mutations related to viral load change across the entire sequences. We summated the total number of mutation recognized in each window constructed by 10 amino acids among all the sequence-pairs after corrected by the observation period (Fig. 6a), and compared by Mann–Whitney's *U* test (Fig. 6b). A correlation between viral load change and NS5B revealed, by collapsing the window to 2 amino acids, at position 2508–2509. These results are in agreement with those published by Qin *et al.* [22]. Qin suggested that mutations within this region have been associated with reduced NS5B activity [22].

Synonymous and nonsynonymous mutations and the molecular clock analysis

To analyze the profile of the nucleotide mutations underling the amino acid changes, we examined the synonymous and the nonsynonymous mutations of each sequence generated from this population with Nei–Gojobori model and Jukus–Cantor method. Ratios of the genetic distance of the nonsynonymous changes (d*N*) per distance of the synonymous changes (d*S*) were calculated for the respective regions as well as the entire HCV genome (Fig. 7a). High *d*N/*d*S ratio reflects the immune selective pressure, and the low *d*N/*d*S reflects the speed of genomic evolution which is dependent on the fidelity of viral genomic replication [23]. The comparison of the *d*N/*d*S ratio showed that there was no overall difference between group-I and group-D.

In addition, we investigated the viral mutational rate in each patient. Averaged mutation rates of each genome were calculated by dividing distances of synonymous changes (d*S*) by the time interval between paired specimens. The viral mutational rates of both groups were almost same across the HCV genome. The rate of mutation between the donor sequence and (1) the first sera of each pair were $2.81 \times 10^{-3} \pm 0.51 \times 10^{-3}$ nucleotides/site/year in the group-I, $2.75 \times 10^{-3} \pm 0.34 \times 10^{-3}$ nucleotides/site/year in the group-D, and (2) the second sera of each pair were $2.77 \times 10^{-3} \pm 0.49 \times 10^{-3}$ in the group-I, $2.72 \times 10^{-3} \pm 0.36 \times 10^{-3}$ nucleotides/site/year in the group-D. The average mutational rate for the entire HCV genome during

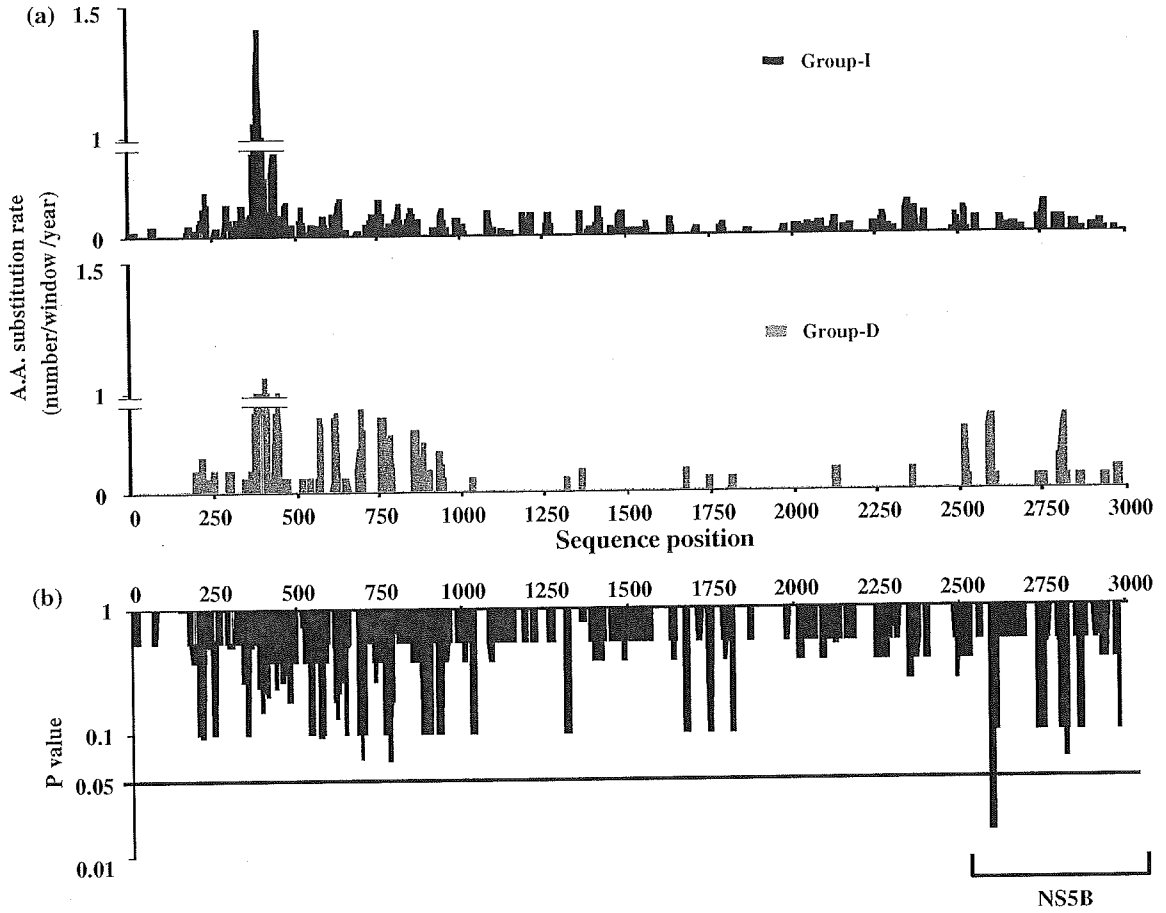

Fig. 6 Sliding-window analysis in full sequences. (a) Average mutation rates in every window are plotted according to the groups. Each window constructed with 10 amino acids. In NS5B region, calculated annual mutation numbers were high in group-D than group-I. (b) The results of statistical analysis are plotted. Longitudinal axis is appeared in logarithmic scale, areas under the bar of 5×10^{-2} indicate statistical significance. Mutation rates of two groups in E2 and NS5B region, showed difference in (a), have significance when tested by Mann–Whitney's U test ($P = 0.015$).

the two sampling points was as follows: $8.20 \times 10^{-3} \pm 2.0 \times 10^{-3}$ nucleotides/site/year for group-I and $10.0 \times 10^{-3} \pm 3.4 \times 10^{-3}$ nucleotides/site/year for group-D, respectively (Fig. 7b). The mutational rate of the viral genome did not differ between the two patient groups examined.

DISCUSSION

In the present study, we aimed to clarify the relationship between amino acid substitutions of HCV and serum viral load in Irish women, who were infected by an HCV-contaminated serum from a single donor during a period between 1977 and 1978. We analyzed full sequences of HCV derived from the patients whose viral load set point have increased (group-I), and those with patients whose viral load set point has decreased (group-D). Serum ALT values and nucleotide mutation rates were not significantly different between the two groups. These results suggested that the mutation pressure is almost equal in across the two groups. Specific points of substitution directly related to the viral load were not found. However, significantly more substitution changes of amino acid were observed in NS5A in group-I when analyzed at the level of the polyprotein. Analysis using a sliding windows method revealed that the numbers of mutations in a short segment in the NS5B region was significantly higher in group-D than in group-I. These regions of NS5B are closely related to the position that is reported to be important to RNA-dependent RNA polymerase (RdRP) activity [22]. These results suggest that the determinants of hepatitis C viral load include, at least in part, virological factors.

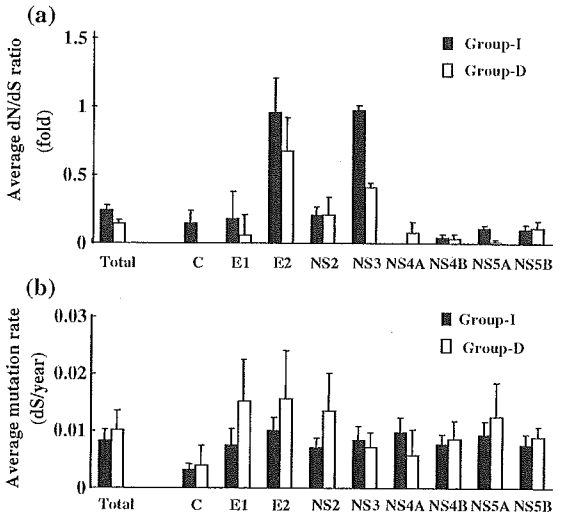

Fig. 7 The analysis of selection bias and mutation rates in RNA level. (a) Distance of nonsynonymous changes (d*N*) per distance of synonymous changes (d*S*) ratio of each coding region in comparison with two groups. Only in NS5A is the ratio significantly different between the two groups. (b) Mutation rate calculated by d*S*/interval time. There were no significant rate differences between two groups.

Clusters of mutations within the NS5B region of the genome were evident in this study group. The RdRP coded by NS5B contains conserved amino acid sequence motifs which essential for RdRP activity [24–26]. The mutation profiles correlated to viral load in the present study were not within these conserved motifs. However, Qin *et al.* and Labonte *et al.* reported that point mutations outside of this latter NS5B motif caused a change in the RdRP activity [22,27]. Qin *et al.* showed that the mutation of amino acid no. 191 of NS5B, co-incident with the region identified in the study reported here, lowered the RdRP activity from 100 to 3%. Furthermore, all mutations identified in our present study altered the polarity of amino acids, and the mutations in this site were only found in group-D. The mutations in the region may affect the replication capacity of the virus resulting in changes in the serum viral load. Further study will be needed which assess the relationship between the RdRP activity of the wild type HCV and those HCV with mutations observed in this study.

We found a significantly different pattern of the mutations in NS5A regions between group-I and group-D. NS5A possess transcriptional activator properties [28,29], and also considered to affect to virus–host interaction [30]. NS5A protein has been reported to bind to cellular RNA-dependent protein kinase R (PKR), a protein activated by double stranded viral RNA. The NS5A inhibition PKR prevents the down regulation of protein translation [31,32], mutations in ISDR, which within PKR-binding domain, have been shown to effect the efficacy of interferon based anti-viral therapy [17,33]. Several researchers have previously identified a correlation between ISDR mutation and viral load [7,17,34–36]. However sequence of ISDR were highly conserved in the present study. In addition, no such relationship was found to exist in a longitudinal study of patients which were followed 2 years [37,38], or in patients with normal ALT levels [39]. These three studies suggest that ISDR is not mutation-prone region. The role of transcriptional activator properties or affection to interaction may explain the different mutation trends found between two groups in this study.

A unique feature of the anti-D patients analyzed in this study is that they are infected with a single strain genotype, and that they are young and may have an immune system that possess different potency to influence the interaction between host and virus than other less homogeneous cohorts. Seven hundred and four individuals were identified as having being iatrogenically infected through HCV 1b contaminated anti-D immunoglobulin. Three hundred and ninety of these women were viremic [12]. Three hundred and seventy six patients had been evaluated for the clinical outcome in 1997; 55% of them had evidence of elevated alanine aminotransferase. The disease progression in this group is slower than the dogma relating to the natural progression of chronic hepatitis C. Only 7 patients had evidence of cirrhosis on the liver 17 years post-infection. Fanning *et al.* have previously reported an association between HCV RNA titers and the degree of inflammation, and between the degree of inflammation and serum ALT levels [40], but Creedon *et al.* reported no association between viral load and the progression of disease [41]. HLA class II data previously generated on this group was analyzed in an attempt to identify any association between host HLA and modulations in viral load [16,42]. HLA class II, has previously been reported to be associated with changes in viral load. The number of individuals examined does not give this study the power to fully address this question, however all of the patients with the HLA DRB1 15/DQB1 0602 phenotype belonged to the increasing group.

Two previously published studies which investigated this cohort of Irish women infected by contaminated anti-D immune globulin, estimated the mutational rate to be 2×10^{-3} synonymous substitutions per site per year by using Core E1/E2 and NS5 region sequences [43,44]. Other researcher reported that the molecular clock of HCV in Japan and USA are ranged in $1–2 \times 10^{-3}$ synonymous substitutions per site per year [45]. In the previous Japanese investigations of the entire HCV genome in all but one individual, the mutational rates were calculated about 2×10^{-3} base substitutions per site per year in human and chimpanzee over a 10 year period [46,47]. The mutation rates calculated with the first and the second samples in the present study are likely to be over estimated in comparison with those described in the previous investigations. The mutational rates between the donor sequence and the patients' samples used in this study, approximate that of these other studies [43–47]. In fact,

Allain *et al.* reported the evolutionary rate of HCV genome between blood donor and recipient, the range were 3.4×10^{-4} to 4.51×10^{-3} nucleotide substitutions per site per year, which were close to the data presented here [48]. The temporal difference between the first and second samples is too small to determine the mutation rate of the viral genome accurately.

In conclusion, we have shown that the hepatitis C viral genomic mutation patterns are associated with changes in viral load in this patient group infected from a single source. Interestingly, specific small regions within NS5B were identified as associated with changes in serum viral load. Mutations in NS5A regions were correlated with the viral load, when analyzed from the viewpoint of each polyprotein as a unit. These results suggest that the viral genome composition is a determinant of the set point of viral load for the hepatitis C virus.

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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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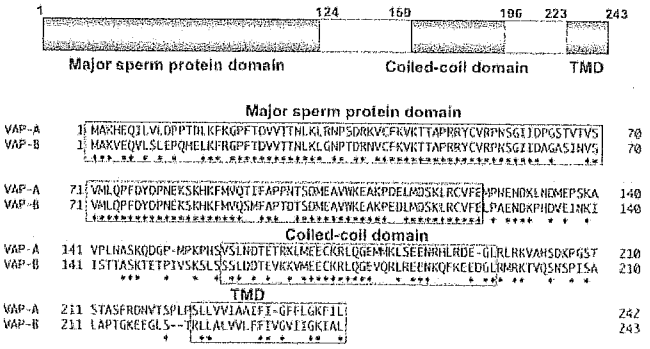

FIG. 1. Schematic representation of VAP-B and alignment of amino acid sequences of VAP-A and VAP-B. The major sperm protein domain, coiled-coil domain, and TMD are indicated. The asterisks indicate identical amino acid residues between VAP-A and VAP-B.

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).

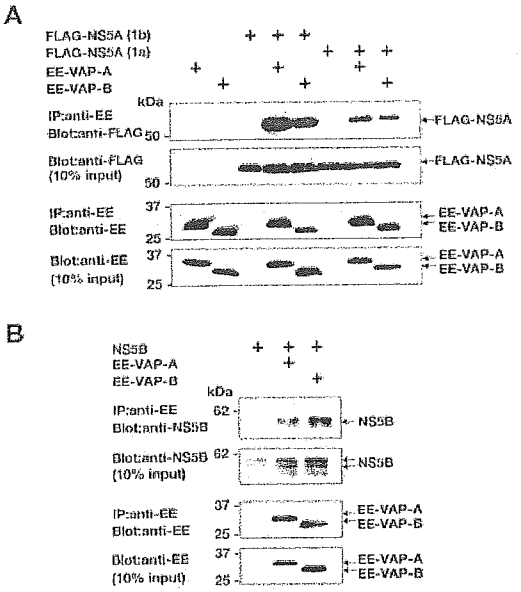

FIG. 2. VAP-A and VAP-B bind to both NS5A and NS5B in mammalian cells. N-terminally FLAG-tagged NS5A of genotype 1b, FLAG-NS5A (1b) of genotype 1a, FLAG-NS5A (1a), and N-terminally EE-tagged VAP (EE–VAP-A or EE–VAP-B) were coexpressed in HEK293T cells and immunoprecipitated with anti-EE antibody. The 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.

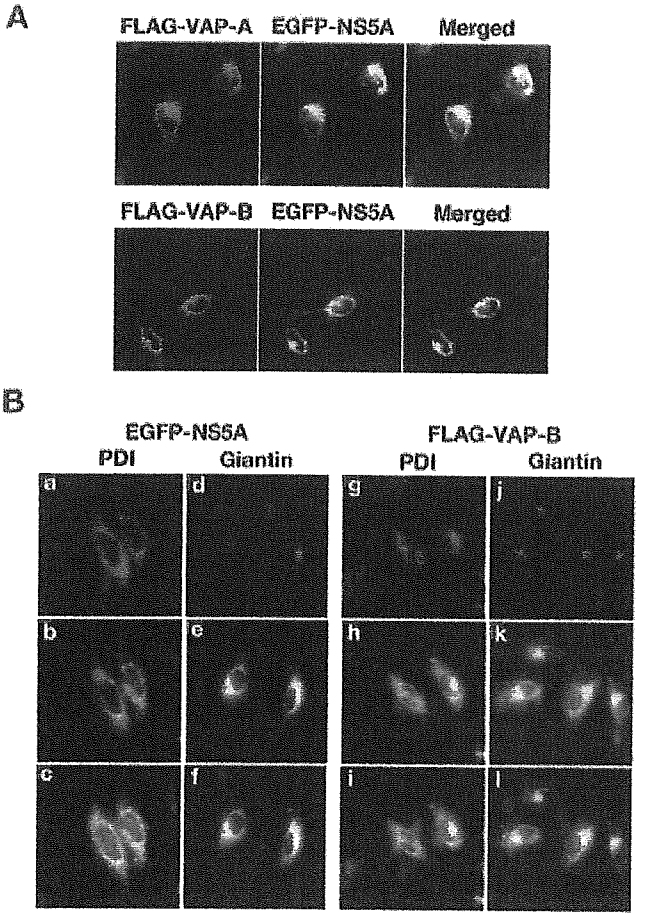

FIG. 3. Intracellular localization of VAPs and NS5A in mammalian cells. (A) N-terminally FLAG-tagged VAP (FLAG–VAP-A or FLAG–VAP-B) was coexpressed with N-terminally EGFP-fused NS5A of genotype 1b (EGFP-NS5A) in HeLa cells, fixed with 4% paraformaldehyde-PBS, permeabilized with 0.5% Triton X-100, and 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

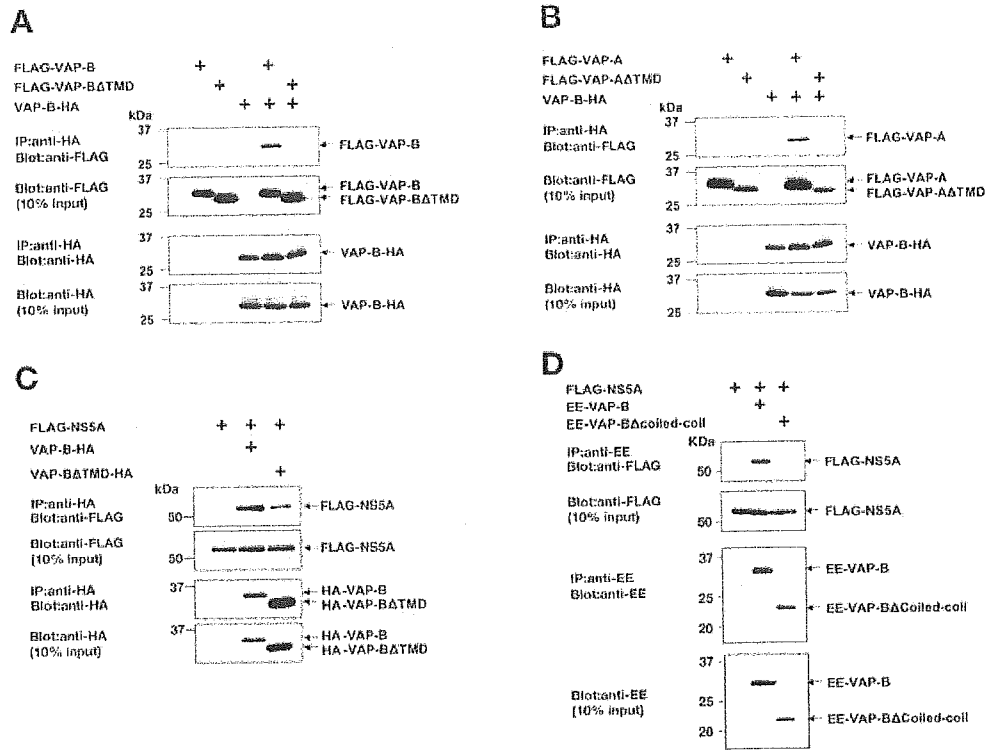

FIG. 4. VAP-B dimerizes with VAP-B and VAP-A through the TMD and interacts with NS5A via the coiled-coil domain. C-terminally HA-tagged VAP-B (VAP-B–HA) was coexpressed with FLAG–VAP-B or FLAG–VAP-B with TMD deleted (FLAG–VAP-BΔTMD). VAP-B–HA was immunoprecipitated with anti-HA antibody, and the immunoprecipitates 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 [α-^{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

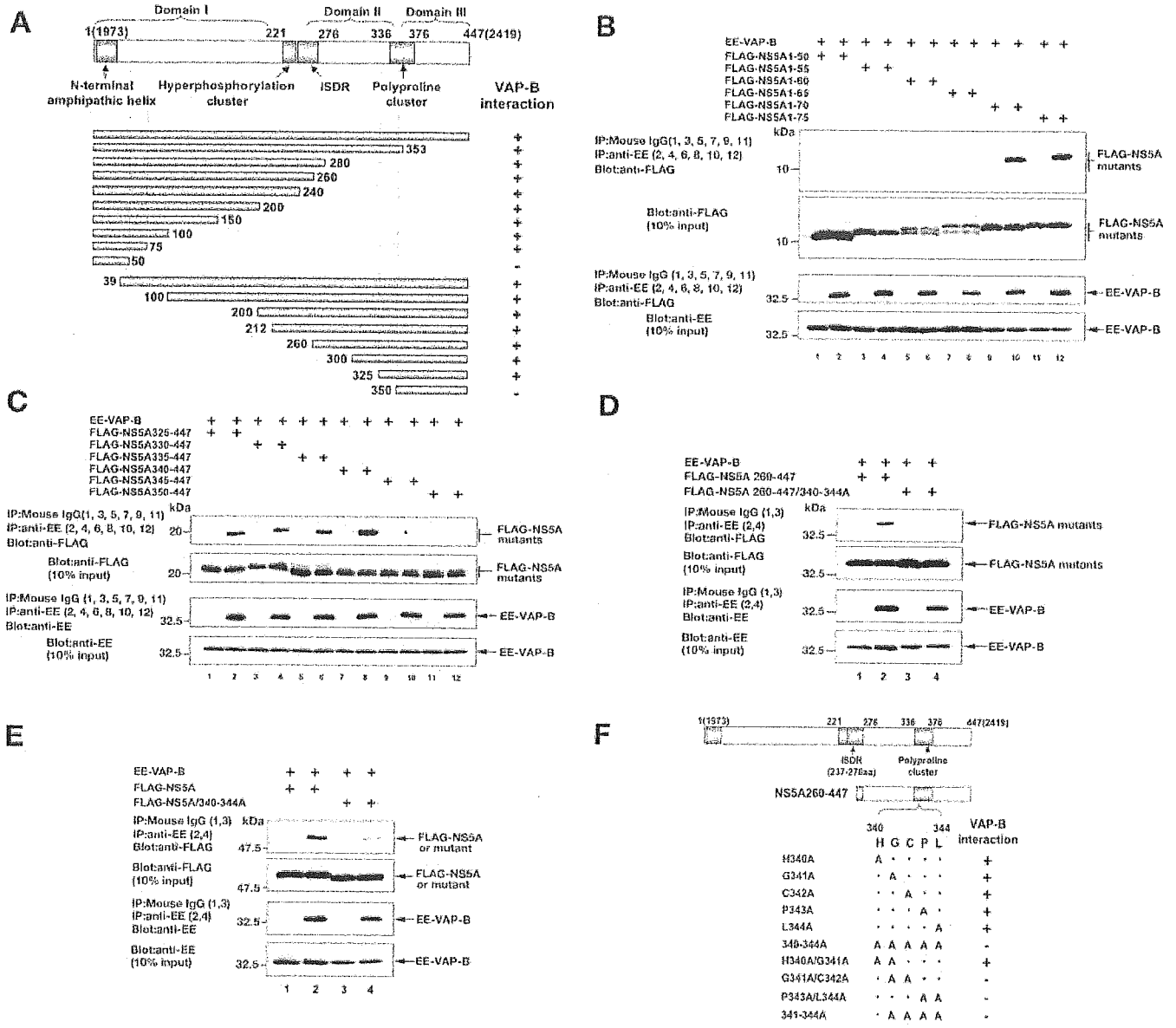

FIG. 5. Two regions of NS5A are required for VAP-B binding. N-terminal or C-terminal deletion mutants of NS5A were introduced into pEF-FLAG pGBK puro vector and coexpressed with EE–VAP-B. EE–VAP-B was immunoprecipitated with anti-EE antibody, and immunoprecipitates were immunoblotted by anti-FLAG antibody. 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