Table 4. Characteristics associated with Y93H of NS5A region

	Y93		Univariate	Multivariate	
	Wild type (n=332)	H (n=78)	P-value	OR (95%CI)	P-value
Age (years)	62.4±11.8	65.9±10.6	0.02		
Male / Female	116 / 216	26 / 52	0.79		
ALT (U/L)	49.5±40.2	49.6±34.0	0.98		
Platelet ($x10^9$/L)	162±60	141±50	<0.01	2.43 (1.34-4.41)	0.003
Albumin (g/dl)	4.1±0.4	4.0±0.5	0.13		
AFP (ng/ml)	8.9±19.2	11.6±20.0	0.34		
HCV RNA (logIU/ml)	6.5±0.8	6.9±0.5	<0.01	3.42 (1.62-7.2)	0.001
Prior therapy (Naïve/experienced)	115/ 217	21/ 57	0.19		
Fibrosis stage (0-2/ 3-4)	170/ 84	44/ 24	0.73		
IL28B (rs8099917) (TT/ TG or GG)	159/ 141	59/ 14	<0.01	3.44 (1.69-7.01)	0.001
ISDR mutation (0/ ≥1)	141/ 106	43/ 21	0.14		
Core amino acid 70 (Wild type/ Mutant)	115/ 75	33/ 14	0.22		

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Hallmarks of Hepatitis C Virus in Equine Hepacivirus

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ABSTRACT

Equine hepacivirus (EHcV) has been identified as a closely related homologue of hepatitis C virus (HCV) in the United States, the United Kingdom, and Germany, but not in Asian countries. In this study, we genetically and serologically screened 31 serum samples obtained from Japanese-born domestic horses for EHcV infection and subsequently identified 11 PCR-positive and 7 seropositive serum samples. We determined the full sequence of the EHcV genome, including the 3′ untranslated region (UTR), which had previously not been completely revealed. The polyprotein of a Japanese EHcV strain showed approximately 95% homology to those of the reported strains. HCV-like *cis*-acting RNA elements, including the stem-loop structures of the 3′ UTR and kissing-loop interaction were deduced from regions around both UTRs of the EHcV genome. A comparison of the EHcV and HCV core proteins revealed that Ile^{190} and Phe^{191} of the EHcV core protein could be important for cleavage of the core protein by signal peptide peptidase (SPP) and were replaced with Ala and Leu, respectively, which inhibited intramembrane cleavage of the EHcV core protein. The loss-of-function mutant of SPP abrogated intramembrane cleavage of the EHcV core protein and bound EHcV core protein, suggesting that the EHcV core protein may be cleaved by SPP to become a mature form. The wild-type EHcV core protein, but not the SPP-resistant mutant, was localized on lipid droplets and partially on the lipid raft-like membrane in a manner similar to that of the HCV core protein. These results suggest that EHcV may conserve the genetic and biological properties of HCV.

IMPORTANCE

EHcV, which shows the highest amino acid or nucleotide homology to HCV among hepaciviruses, was previously reported to infect horses from Western, but not Asian, countries. We herein report EHcV infection in Japanese-born horses. In this study, HCV-like RNA secondary structures around both UTRs were predicted by determining the whole-genome sequence of EHcV. Our results also suggest that the EHcV core protein is cleaved by SPP to become a mature form and then is localized on lipid droplets and partially on lipid raft-like membranes in a manner similar to that of the HCV core protein. Hence, EHcV was identified as a closely related homologue of HCV based on its genetic structure as well as its biological properties. A clearer understanding of the epidemiology, genetic structure, and infection mechanism of EHcV will assist in elucidating the evolution of hepaciviruses as well as the development of surrogate models for the study of HCV.

The *Flaviviridae* family is composed of four genera: *Flavivirus*, *Pestivirus*, *Pegivirus*, and *Hepacivirus*. *Flaviviridae* family viruses are enveloped and contain a single-stranded, positive-sense RNA genome, which encodes a single large precursor polyprotein composed of approximately 2,800 to 3,000 amino acids. The genus *Hepacivirus* had included only two species, hepatitis C virus (HCV) and GB virus B (GBV-B), until 2010. GBV-B was isolated from serum samples obtained from laboratory tamarins by 11 passages of serum obtained from a human patient with idiopathic hepatitis (1). Although GBV-B experimentally infects tamarins and common marmosets, but not chimpanzees, *in vivo* (2, 3), the natural host of GBV-B has not yet been clarified. Several hepacivirus species were recently detected in dogs, horses, bats, and rodents and tentatively designated nonprimate hepaciviruses (NPHVs). Bat hepaciviruses have been isolated from some species of bats in Kenya (4), while rodent hepaciviruses have been isolated from several species of rodents in Germany, the Netherlands, South Africa, and Namibia (5, 6). GBV-B is phylogenetically more similar to rodent hepacivirus than to HCV (5). Several strains of equine hepacivirus (EHcV) have been isolated from domestic horses in the United States, the United Kingdom, and Germany (5, 7, 8). The canine hepacivirus was isolated from dogs in the United States (9) but has not yet been genetically or serologically detected in any dogs other than those from the first report (5, 7, 8). The polypeptides of canine hepacivirus show approximately 95% amino acid homology to those of the EHcV strains, suggesting that canine hepacivirus may belong to the same species as EHcV and

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that infections may be rare in dogs (5, 7, 8, 10). Recent phylogenetic analyses identified EHcV as the most closely related viral homologue of HCV among the reported NPHV strains; however, epidemiological and virological information on EHcV is limited. The open reading frames of EHcV strains show approximately 95% homology to one another, suggesting that previously reported EHcV strains may be classified into one species. Several genome sequences of rodent hepacivirus have already been completely determined (5). The 3′ untranslated region (UTR) of HCV was found to include three stem-loop (SL) structures, while variable stem-loop structures were found in that of rodent hepacivirus and GBV-B (5). However, the nucleotide sequence of the EHcV 3′ UTR has not yet been determined completely because the adenine-rich [(A)-rich] sequence downstream of the stop codon in the EHcV genome interrupts an ordinary 3′-rapid amplification of cDNA ends (RACE) reaction (8). The RNA secondary structure of the hepacivirus 3′ UTR may indicate species specificity (5).

On the basis of amino acid similarities among the polyproteins of NPHVs and HCV, the N-terminal one-fourth of the NPHV polyprotein has been predicted to be cleaved by signal peptidase into mature structural proteins and a viroporin (core, E1, E2, and p7), while the C-terminal three-fourths has been predicted to be cleaved by viral proteases into maturated nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (6). Core, E1, and E2 have been predicted to form viral particles with host lipids, although it remains unclear whether p7 is incorporated into a viral particle. Signal peptide peptidase (SPP) was shown to further cleave the C-terminal transmembrane region of HCV and GBV-B core protein after signal peptidase-dependent cleavage (11, 12). However, whether SPP cleaves the C-terminal transmembrane region of the NPHV core protein remains unknown.

The mature core proteins of HCV and GBV-B are localized mainly on lipid droplets (LDs) (13, 14). The core proteins of dengue virus are also localized on LDs but are not cleaved by SPP (15), suggesting that localization of the core protein on LDs may be one of the common characteristics of the *Flaviviridae* family. The HCV core protein is known to be partially localized in the detergent-resistant membrane (DRM), which originates from lipid raft-like membranes (16, 17). The DRM is composed of cholesterol and sphingolipids, which are included in the replication compartment known as the membranous web (18, 19). Therefore, LDs and DRM are considered to be the intracellular compartments for the replication and viral assembly of HCV, but it is currently unknown whether NPHV core proteins are localized on LDs and DRM.

Epidemiological information on EHcV is still limited. The results of the present study demonstrated that Japanese-born domestic horses were infected with EHcV, which showed high homology to the reported strains on the basis of its nucleotide and amino acid sequences. We predicted the RNA secondary structures around the 5′ and 3′ UTRs of the EHcV genome and analyzed the biological properties of the EHcV core protein in relation to the HCV core protein.

MATERIALS AND METHODS

Samples. Serum samples 1 to 13 were collected from Japanese-born domestic horses raised on one farm, farm A, located in Hokkaido, Japan, while groups of serum samples numbered 14 to 18 and 19 to 31 were from horses on farms B and C, respectively, located in Tokyo, Japan (Fig. 1). The distance between Hokkaido and Tokyo is about 1,000 km. All sample collections conformed to guidelines for the care and use of laboratory animals (Yamanashi University) and were approved by the Institutional Committee of Laboratory Animal Experimentation (Yamanashi University). All samples were divided into small aliquots and stored at −80°C until nucleic acid extraction.

RT-PCR. Total RNAs were prepared from horse sera using a Qiagen viral RNA extraction kit (Qiagen, Valencia, CA). RNAs were converted to cDNA using a PrimeScript reverse transcription-PCR (RT-PCR) kit (TaKaRa, Shiga, Japan) with random primers. The viral gene was amplified by PCR using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ) with three pairs of primers: NPHV-F1 (5′-TGTCACCT ACTATCGGGG-3′) and NPHV-R1 (5′-TCAAGCCTATACAGCAAAG G-3′), NPHV-F2 (5′-ATCATTTGTGATGAGTGCC-3′) and NPHV-R2 (5′-CATAAGGGCGTCCGTGGC-3′), and NPHV-F3 (5′-GTGGTCGCC ACGGATGCC-3′) and NPHV-R3 (5′-ACCCTATGAAGACGCTCTCC-3′). PCR was carried out as follows: one cycle at 92°C for 5 min; 35 repeats of one cycle at 94°C for 0.5 min, 58°C for 0.5 min, and 72°C for 0.5 min, in that order; and one cycle at 72°C for 1 min followed by holding at 4°C. The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and visualized using the BioDoc-It imaging system (UVP, Upland, CA).

Determination of the EHcV genomic sequence. The viral genome of EHcV was segmentally amplified by PCR using the primers listed in Table 1. The PCR products were cloned into T vectors prepared from pBluescript II SK(−) (20). The DNA sequences of the PCR products were determined using an ABI Prism BigDye Terminator version 1.1 cycle sequencing kit and an ABI Prism 310 genetic analyzer (Life Technologies, Tokyo, Japan). More than three colonies were picked up among the transformants of *Escherichia coli* with regard to the accuracy of the sequence. The nucleotide sequences of the PCR products were determined in forward and reverse directions. The junction of two adjacent PCR products was confirmed by PCR using primers that overlapped two close regions. The 5′-terminal sequence upstream of the open reading frame was determined with a 5′-RACE core set (TaKaRa) using the 5′ phospholyrated RT primer for the NPHV 5′ UTR (5′-CATCCTATCAGACCG-3′). The 3′-terminal region downstream of the (A)-rich region was determined by the 3′-RACE method (21, 22), modified as follows: Total RNAs were prepared from horse serum using TRIzol LS reagent (Invitrogen, Carlsbad, CA) with 40 μg of glycogen (Nacalai Tesque, Kyoto, Japan). The poly(U) tail was added to the 3′ end of the RNA preparation using *Escherichia coli* poly(U) polymerase (New England BioLabs, Ipswich, MA) and was incubated for 45 min at 37°C. The resulting preparation was reverse transcribed by the SuperScript First-Strand Synthesis system (Life Technologies) using an oligo(dA) adapter primer (5′-TTGCGAGCACAGAATTAATACG ACTCACAAAAAAAAAAAAAVN-3′). The sequence of each region was determined by sequencing more than 3 clones. The primers for PCR amplification and the RACE methods are listed in Table 1. The whole sequence of the EHcV strain isolated from serum sample 3 (GenBank accession number AB863589) was determined by the method described above. The EHcV strain was designated JPN3/JAPAN/2013 in this study. The partial NS5B-coding regions and 3′ UTRs were amplified from serum samples 5 and 1. The nucleotide sequences of samples 5 and 1 (GenBank accession numbers AB921150 and AB921151, respectively) were determined by the method described above. The neighbor-joining trees of the nucleotide sequences from the NPHV, HCV, and GBV-B strains were predicted by the method of Saitou et al (23). Trees were constructed by the maximum composite likelihood method calculated by using the program MEGA5 (24) (see Fig. 3). The secondary protein structures were predicted by the method of Garnier et al. (25) (see Fig. 6). Hydrophobicity plots of the EHcV and HCV core proteins were prepared by the method of Kyte and Doolittle (26) and drawn using the software Genetyx (Nihon Genetyx, Tokyo, Japan) (see Fig. 5).

Quantification of viral genomic RNAs in horse sera. Total RNA was prepared from equine serum using a Qiagen viral RNA extraction kit and was then reverse transcribed into cDNA by using a PrimeScript RT-PCR

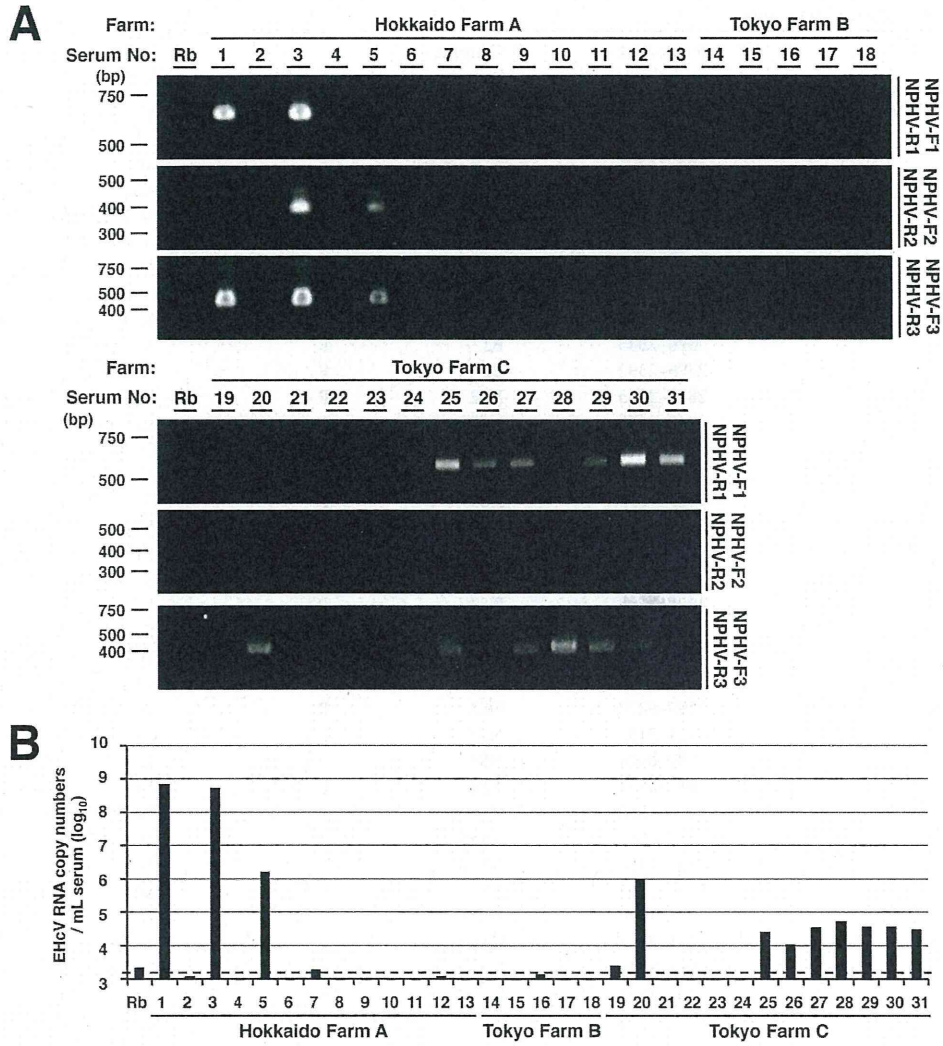

FIG 1 Detection and genetic analyses of NPHV genomic RNA in sera of Japanese domestic horses. (A) Total RNAs extracted from 31 equine sera and normal rabbit serum (Rb) as a negative control were subjected to RT-PCR analysis. Hokkaido Farm A, Tokyo Farm B, and Tokyo Farm C indicate the farms where the individual horses were reared. Three sets of primers, NPHV-F1 and NPHV-R1, NPHV-F2 and NPHV-R2, and NPHV-F3 and NPHV-R3, were used to amplify NPHV-specific gene regions. The PCR products were electrophoresed and stained with ethidium bromide. (B) Total RNAs were isolated from sera, reverse-transcribed, and estimated as a copy number per ml. Normal rabbit serum was used as a negative control. The dashed line indicates the cutoff level.

kit with random primers. The amount of targeted viral RNA was estimated using SYBR GreenER qPCR SuperMix (Life Technologies) and the ABI StepOnePlus real-time PCR system (Life Technologies). The region encoding NS3 was targeted with the primer pair NPHV-F3 (5′-GTGGTC GCCACGGATGCC-3′) and NPHV-R3 (5′-ACCCTATGAAGACGCTC TCC-3′). Total RNAs extracted from conventional rabbit serum were used as a negative control to determine the analytical threshold line. The *in vitro*-transcribed RNA of EHcV was utilized for the standard curve.

Prediction of RNA secondary structures. The 5′-UTR sequences of EHcV strains were aligned with the MUSCLE program and subjected to a manual search for covariant nucleotide substitutions. The RNA folding structure upstream of domain III in the 5′ UTR was predicted using the Mfold web server (27) with conventional phylogenetic conservation analysis due to the lack of sufficient homology to the 5′ UTR sequences of HCV strains. The NS5B-coding regions and 3′ UTRs of EHcV strains were aligned with the program MUSCLE. Conserved secondary structures were predicted as described above. The secondary structures of the 3′ UTR in EHcV were predicted by the Mfold web server without confirming phylogenetic data because of the absence of additional available sequences of the EHcV 3′ UTR and the lack of sufficient homology to the HCV X-tail sequences.

Plasmids. The PCR product encoding the EHcV core protein was amplified from serum sample 3 and was then cloned into the BamHI and XhoI sites of pcDNA3.1-Flag/HA, which encodes the FLAG and hemagglutinin (HA) epitope tags, as reported previously (28). Ala^{204} was replaced with Lys to prevent signal peptidase-dependent cleavage. The translated EHcV core protein was added to the FLAG and HA epitope tags at the N and C termini (EHcVc), respectively. A point mutation was generated using a KOD mutagenesis kit (Toyobo, Osaka, Japan). The PCR products encoding EHcVc or the mutant in which Ile^{190} and Phe^{191} were replaced with Ala and Leu (EHcVc-mt), respectively, were introduced into the AflII and EcoRV sites of pCAGGS using an In-Fusion HD cloning kit (TaKaRa). The introduced fragments of all plasmids were confirmed by sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems). The plasmid encoding the N-terminally FLAG-tagged and C-terminally HA-tagged HCV core protein (HCVc) and the mutant in which

TABLE 1 List of PCR primers used in this study

Primer	Nucleotide position	Genome location	F or R[a]	Sequence (5′→3′)
Primer for cloning of the NPHV genome	92–111	5′ UTR	F	ATGTGTCACTCCCCCTATGG
	367–386	5′ UTR	R	CTATGGTCTACGAGACCGGC
	268–285	5′ UTR	F	AGCCGAAATTTGGGCGTG
	1207–1224	E1	R	AAACAGAAGCCATAGCGG
	1116–1132	E1	F	AGTGCTTGTTGGGTGCC
	1697–1713	E2	R	GTCCTTTGCACTTCGGG
	1605–1623	E2	F	ACTGTTAAGCAGATGTGGG
	2103–2121	E2	R	CACAGAGTTGGTAAGTAGC
	2007–2023	E2	F	AAGCAGTGTGGTGCTCC
	2526–2545	E2	R	AAACAGAACCAGAGAATTGC
	2375–2392	E2	F	CCCTGCCTTCACTACTGG
	2898–2913	NS2	R	CGAGATAGCGCCAAGC
	2847–2867	NS2	F	TTTATGCTAGTAAAGTGGTGG
	3396–3415	NS2	R	GGTGATAAAAGTCTCCATCC
	3318–3334	NS2	F	ATCCTCCATGGCTTGCC
	3819–3835	NS3	R	GGGCCACCTGAACTACC
	3732–3750	NS3	F	ACCAGGACGGGTCAGGTCG
	4254–4270	NS3	R	ATAATGTCATAAGCACC
	4177–4195	NS3	F	CTAGTTGCAAGACAACGGG
	4682–4700	NS3	R	AGTGTTGCAGTCAGTGACG
	4574–4591	NS3	F	TGTCACCTACTATCGGGG
	5199–5218	NS3	R	TCAAGCCTATACAGCAAAGG
	4574–4591	NS3	F	TGTCACCTACTATCGGGG
	5199–5218	NS3	R	TCAAGCCTATACAGCAAAGG
	5134–5152	NS3	F	CTCCCAGCAAAGATGAACG
	5997–6014	NS4B	R	AGCACCCACACCAACAGC
	5919–5934	NS4B	F	AAGATCTTGAGTGGTG
	6651–6632	NS5A	R	GCCGATAACTCTGACAGC
	6547–6564	NS5A	F	ACACCTGGAAAACAGCCG
	7293–7310	NS5A	R	AGATTCCGTGGCCGAAGG
	7235–7252	NS5A	F	AGCTCTCGTTTCCGGGTG
	7573–7590	NS5B	R	TAGCTGACGCTGTTGTGG
	7511–7527	NS5B	F	ACGCCACCCTATAGGCC
	8027–8046	NS5B	R	GTTGACGGGGAGTGTATTGG
	7926–7943	NS5B	F	ATCGTTTACCCCGATTTG
	8528–8545	NS5B	R	CAAGATGTTATCTGCTCC
	8457–8474	NS5B	F	CGTGACTTCACTAATGCC
	9069–9086	NS5B	R	GTCAATCGAGTTTACGCC
Primer for 5′ RACE	235–252	5′ UTR	F	AATCGCGGCTTGAACGTC
	213–230	5′ UTR	R	TGTACTCACGGATTCACG
Primer for 3′ RACE	8979–8999	NS5B	F	CTTAAAGTACGTGGTGGTCGC
Adapter primer			R	GCGAGCACAGAATTAATACGAC

[a] F, forward; R, reverse.

Ile[176] and Phe[177] were replaced with Ala and Leu (HCVc-mt), respectively, were described previously (28). The gene encoding human signal peptide peptidase (SPP) or its mutant was introduced into pcDNA3.1-myc/His C (Invitrogen) instead of the plasmids described previously (28). The resulting plasmids encoded C-terminally myc-$His_{\times 6}$-tagged wild-type SPP (SPP-wt) or the mutant protein in which Asp[219] was replaced with Ala (SPP-D219A).

Cell culture and transfection. The human embryonic kidney cell line 293FT and the human hepatoma cell line Huh7OK1 (29) were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids (Sigma, St. Louis, MO), sodium pyruvate (Sigma), and 10% fetal bovine serum (FBS) and were then cultured at 37°C under the conditions of a humidified atmosphere and 5% CO_2. Plasmids were transfected into cell lines using XtremeGene 8 (Roche) according to the manufacturer's protocol.

Western blot analysis. 293FT cells were cultured in 6-well plates and transfected with the appropriate plasmids. The transfected cells were harvested at 18 h posttransfection, washed with cold phosphate-buffered saline (PBS), and suspended in 50 μl of the lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 135 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitor cocktail (Merck Bioscience, Calbiochem, San Diego, CA). The lysates were centrifuged at 19,000 × *g* for 5 min at 4°C. The supernatants were mixed with 16 μl of 4× SDS sample buffer and then boiled at 60°C for 20 min. The resulting mixtures were subjected to SDS-PAGE. The proteins in a gel were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with mouse anti-FLAG antibodies (Sigma), mouse anti-HA antibodies (Covance, Princeton, NJ), mouse

anti-c-*myc* antibodies (BD Pharmingen, San Diego, CA), or mouse anti-beta-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunocomplexes were visualized with SuperSignal West Femto substrate (Thermo Scientific, Rockford, IL) and detected using an LAS-4000 Mini image analyzer (GE Healthcare, Buckinghamshire, United Kingdom).

Detection of antibodies against EHcV. To detect anti-EHcV antibodies in horse sera, we subjected lysates prepared from 293FT cells expressing EHcVc, which is an N-terminally FLAG-tagged and C-terminally HA-tagged EHcV core protein (a positive reference), or cells transfected with an empty plasmid (a negative reference) to Western blotting, as described above. The resulting PVDF membranes were incubated with Blocking One solution (Nacalai Tesque) for blocking at room temperature for 30 min and then incubated with 1,000-fold-diluted horse serum in 10-fold-diluted Blocking One. Mouse anti-FLAG or rabbit anti-EHcV core antibody was used as a positive serum control. The resulting membrane was incubated with an HRP-conjugated antibody to mouse, rabbit, or horse IgG (Abcam, Cambridge, UK) at room temperature for 1 h. Protein bands with a molecular mass of 28 kDa were detected in the positive reference, but not in the negative reference, using positive serum or an antibody to the FLAG epitope tag or EHcV core protein. The rabbit polyclonal antibody against the EHcV core protein was generated by immunization using peptides of the residues from 2 to 15, GNKSKNQKPQPQRG (Scrum Inc., Tokyo, Japan).

Pulldown assay for SPP binding. Human embryonic kidney 293FT cells expressing EHcVc or HCVc with or without SPP-D219A were harvested at 18 h posttransfection, washed with cold PBS, suspended in 100 μl of the lysis buffer, and centrifuged at 14,000 ×g for 5 min at 4°C. Twenty microliters of the lysate was mixed with 20 μl of 2× SDS sample buffer. The remaining lysate was adjusted to 250 μl with the lysis buffer and incubated for 2 h at 4°C after the addition of 20 μl of His-Select nickel affinity gel (Sigma) equilibrated 50% (vol/vol) with lysis buffer. The nickel beads that included SPP-wt or SPP-D219K were washed five times with 500 μl of lysis buffer by centrifugation at 5, 000 × *g* for 1 min at 4°C and then suspended in 40 μl of 1× SDS sample buffer. After being boiled at 60°C for 20 min, the supernatant was subjected to Western blotting to detect the coprecipitated core proteins.

Immunofluorescence microscopy. Huh7OK1 cells were incubated with fresh DMEM containing Bodipy 558/568 (2 μg/ml; Molecular Probes) for 1 h at 37°C to visualize lipid droplets (LDs). The cells were washed once with prewarmed DMEM and incubated for 30 min at 37°C. The treated cells were then fixed in 4% paraformaldehyde for 30 min at room temperature. After two washes with PBS, the cells were permeabilized with permeabilization buffer containing 0.1% saponin (eBioscience, San Diego, CA) for 30 min at 37°C and blocked with PBS containing 2% FBS (blocking buffer) for 30 min at room temperature. The cells were incubated with an appropriate antibody, as indicated in the figure legends. The cells were washed three times with PBS. The mounted cells were observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI).

Flotation assay. A flotation assay was carried out according to the method described previously (17). Briefly, 293FT cells expressing EHcVc or EHcVc-mt were cultured on a 10-cm dish. The transfected cells were washed once with cold PBS at 18 h posttransfection and harvested using a cell scraper. The cells were suspended in 1.2 ml of 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail (Merck, Calbiochem) (TNE buffer) and were then homogenized by 10 passes through a 26-gauge needle. Each 0.6-ml aliquot of the homogenates was incubated for 30 min on ice with or without 1% Triton X-100 and was then mixed with 0.4 ml of OptiPrep (Axis-Shield, Oslo, Norway). An appropriate concentration of OptiPrep was adjusted with TNE buffer. This mixture was overlaid with 1.2 ml of 30% OptiPrep, 1.2 ml of 25% OptiPrep, and 0.8 ml of 5% OptiPrep, in that order, and was centrifuged at 42,000 rpm for 5 h at 4°C in an SW50.1 rotor (Beckman Coulter, Fullerton, CA). Each fraction, with a volume of 0.4 ml, was collected from the top of the centrifugation tube and was then precipitated by mixing with 4 volumes of cold acetone at −30°C. The resulting pellet was resolved in 50 μl of 1× sample buffer and then subjected to Western blot analysis using a mouse anti-FLAG antibody (Sigma), a rabbit anti-calreticulin antibody (Sigma), and a rabbit anti-caveolin-1 antibody (Sigma). The fractions containing calreticulin in the absence and presence of Triton X-100 were defined as the membrane and detergent-soluble membrane fractions, respectively. In the presence of the detergent, fractions 3 to 5, which contained caveolin-1 but only small amounts of calreticulin, were defined as the detergent-resistant membrane fractions.

Nucleotide sequence accession numbers. The whole sequence of the EHcV strain isolated from serum sample 3 was deposited in GenBank under accession number AB863589. The nucleotide sequences of the partial NS5B-coding regions and 3′ UTRs from samples 5 and 1 were registered as AB921150 and AB921151, respectively.

RESULTS

Detection of the EHcV genome and antibody to EHcV in sera of Japanese-born horses. To clarify whether NPHVs were distributed in Japan, we collected 31 horse serum samples and examined them in order to detect the EHcV genome and antibody to the core protein. We prepared total RNAs from horse sera and screened them using RT-PCR analyses with three sets of PCR primers (NPHV-F1/NPHV-R1, NPHV-F2/NPHV-R2, and NPHV-F3/NPHV-R3) that targeted the NS3-coding region that is relatively conserved among NPHVs. Total RNA prepared from conventional rabbit serum was used as a negative control. PCR products with the expected sizes were found in horse serum samples 1, 3, 25, 26, 27, and 29 to 31 using NPHV-F1/NPHV-R1, in horse serum samples 3 and 5 using NPHV-F2/R2, and in horse serum samples 1, 3, 5, 20, and 25 to 31 using NPHV-F3/R3 (Fig. 1A). The EHcV genome was detected in 11 of 31 (35%) serum samples by RT-PCR (Fig. 1A and B). Copy numbers of the EHcV genome in horse sera varied from 10^4 to 10^9 copies per ml of sera (Fig. 1B). Although a PCR product was slightly amplified from serum sample 19 by PCR using the primer pair NPHV-F1/R1, the copy number of the virus genome in serum sample 19 was estimated to be low, at a level similar to that of the negative control. Thus, we could not determine whether serum sample 19 included a viral genome. We then immunologically surveyed horse sera by Western blotting. Western blotting analyses using horse sera to detect antibodies to the EHcV core protein (Fig. 2) showed that the sera of samples 1, 2, 3, 5, 14, 20, and 25 were immunoreactive to the EHcV core protein (7 positive serum samples of a total of 31 samples; 22.6%). The sera of samples 1, 3, 5, and 20 were PCR positive and seropositive. Serum samples 2 and 14 were PCR negative and seropositive, whereas samples 26 to 31 were PCR positive and seronegative. These results suggest that EHcV has infected Japanese-born domestic horses.

Genetic analysis of EHcV. PCR products corresponding to the 5′ UTR and the open reading frame were segmentally amplified from serum sample 3 by 5′ RACE and RT-PCR, respectively. In the present study, we successfully determined the 3′-terminal sequence downstream of a stop codon using the 3′-RACE method with poly(U) polymerase. We determined the nucleotide sequence of the putative full genome, which was designated JPN3/JAPAN/2013 (GenBank accession number AB863589). The full-length genome of strain JPN3/JAPAN/2013 is composed of 9,355 nucleotides, consisting of the 5′ UTR with a nucleotide length of 389, the 3′ UTR with a nucleotide length of 134, and an open

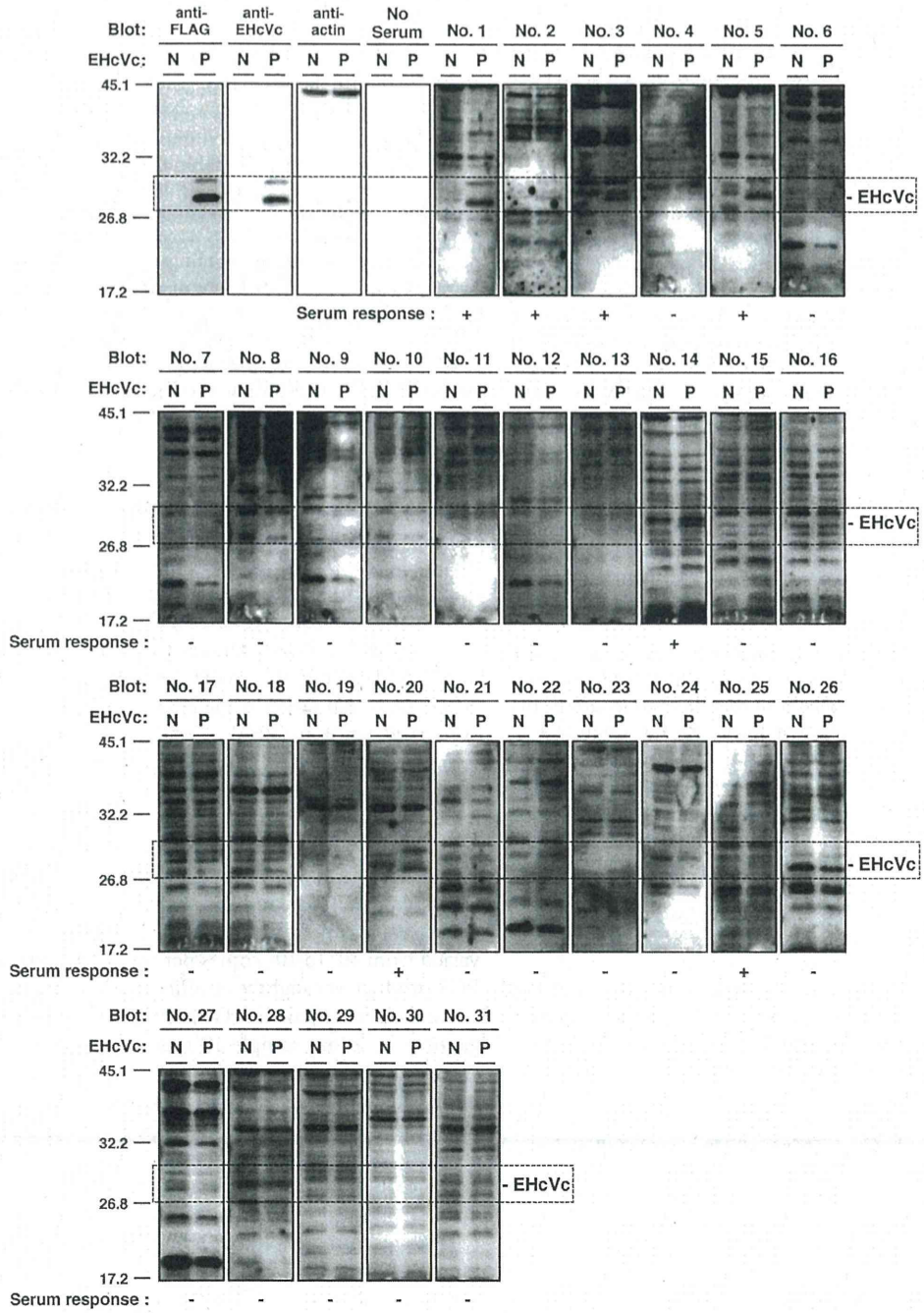

FIG 2 Serological screening of Japanese-born domestic horses. Lysates of 293FT cells transfected with an empty plasmid (a negative reference, N) or the plasmid encoding EHcVc (a positive reference, P) were subjected to Western blotting using serum from each horse. The serum response "+" indicates that the protein band with the same molecular size as the EHcV core protein was specifically detected in the "P" lane, but not in the "N" lane, while the serum response "−" indicates that the protein band with the same molecular size as the EHcV core protein was detected in neither the "P" lane nor the "N" lane. Both antibodies to the FLAG tag and to the EHcV core protein were used as serum positive controls, while protein amounts were standardized with blotting using the antibody to beta-actin. "No serum" indicates the membrane was incubated without primary antibodies but with HRP-conjugated anti-horse IgG antibodies as a background of the secondary antibody.

reading frame with a nucleotide length of 8,832. The open reading frame encodes 2,943 amino acids. Table 2 summarizes the amino acid homology of the JPN3/JAPAN/2013 polyprotein with the polyproteins of the other EHcV strains. The polyprotein of JPN3/JAPAN/2013 shared more than 94% homology with the other EHcV polyproteins and exhibited the highest homology, 97.8%, with NPHV-H10-094 (GenBank accession number JQ434007), which was isolated from a horse in the United States (8). The NS3- and NS5B-coding regions of the EHcV strains were phylogenetically analyzed by the neighbor-joining method. The phylogenetic

TABLE 2 Amino acid sequence homologies of the polyproteins

	Non-primate hepaciviruses					
	H10-094 (JQ434007)	B10-022 (JQ434004)	NZP1 (JQ434001)	AAK-2011 (JF44991)	H3-011 (JQ434008)	A6-066 (JQ434003)
JPN3/JAPAN/2013 (AB863589)	97.8[a]	96.7	95.7	95.7	95.6	95.3

	Non-primate hepaciviruses		HCV			
	G1-073 (JQ434002)	F8-068 (JQ434005)	HCV1a (NC004102)	HCV1b (AB779562)	JFH1 (AB047639)	GBV-B (NC001655)
JPN3/JAPAN/2013 (AB863589)	94.9	94.1	46.5	45.6	44.5	28.9

	Non-primate hepaciviruses		
	JPN3/JAPAN/2013 (AB863589)	AAK-2011 (JF44991)	GBV-B (NC001655)
HCV1a (NC004102)	46.1	46.0	33.3

[a], percent identity.

trees of the NS3 (Fig. 3A) and NS5B regions (Fig. 3B) showed that JPN3/JAPAN/2013 was included in the clade comprising the U.S. strains NPHV-H10-094 (GenBank accession number JQ434007) and B10-022 (GenBank accession number JQ434004).

Putative RNA secondary structures around the UTRs of EHcV. The 5′-terminal region of JPN3/JAPAN/2013 was compared with those of the EHcV genomes (Fig. 4A). The HCV internal ribosome entry site (IRES)-like structure was embedded in the 5′ UTRs of NPHVs (5, 6). The 5′-UTR region was well conserved among the EHcV strains and showed a mean diversity of approximately 4% among the EHcV strains (Fig. 4A). The 3′-terminal sequence downstream of the (A)-rich region in the EHcV genome had not been reported because the (A)-rich region downstream of the stop codon of EHcV interrupted the reaction in the ordinary 3′-RACE method (5, 6). In the present study, we determined the nucleotide sequences downstream of the (A)-rich region from serum sample 3 (JPN3/JAPAN/2013; GenBank accession number AB863589), sample 5 (JPN5/JAPAN/2015; GenBank accession number AB921150), and sample 1 (JPN1/JAPAN/2015; GenBank accession number AB921151) by the modified 3′-RACE method using poly(U) polymerase, although the region in serum sample 1 was incompletely amplified (Fig. 4B). The regions downstream of the (A)-rich region were conserved between serum samples 3 and 5, whereas the (A)-rich regions varied among the three strains (Fig. 4B).

The secondary structure of 5′ UTR in strain JPN3/JAPAN/2013 was predicted according to the method described previously (8) (Fig. 4C). The stem-loops in the 5′ UTR were designated according to the stem-loops of the HCV 5′-UTR structures (30). Stem-loops (SLs) I, II, IIIa to IIIf, and the pseudoknot interaction were predicted within the 5′ UTR of strain JPN3/JAPAN/2013. These structures were the same as that of the strain reported previously (9), although several nucleotide insertions and deletions were more predominant in the apical loop of subdomain IIIb than in the other strains reported previously (Fig. 4A and C). Two seed sites of the microRNA miR-122 (Fig. 4A and C) were found in the 5′ UTR of strain JPN3/JAPAN/2013 at nucleotide residues 81 to 89 (UCCACAUUA) and 98 to 103 (CACUCC), which also corresponded to the predicted miR-122 seed sites in the 5′ UTRs of the other EHcV strains (9).

The HCV 3′ UTR, which is generally 200 to 300 nucleotides in length, consists of a short variable region, the poly(U/UC) stretch sequence, and the 3′-X-tail region, in that order (31–33). Although the EHcV 3′ UTR, which is composed of 138 nucleotides, is shorter than the HCV 3′ UTR, the 3′ UTR of EHcV consists of the (A)-rich sequence and 3′-X-tail region, in that order. The (A)-rich sequence of EHcV may vary in length (Fig. 4B). We subsequently predicted the secondary structure of the EHcV 3′ UTR. Although the EHcV 3′ UTR, which is composed of 138 nucleotides, is shorter than the HCV 3′ UTR, the 3′ UTR includes three predicted SL structures (Fig. 4C). Based on the SL structures in the HCV 3′ X-tail, these SL structures in the EHcV 3′ UTR were designated 3′SL I, 3′SL II, and 3′SL III, in that order from the 3′ terminus (Fig. 4C). Interestingly, the (A)-rich sequence was partially incorporated into the 3′SL III, although the poly(U/UC) stretch sequence in the HCV 3′ UTR is separated from any 3′SL structures (31–33). Furthermore, the two SL structures in the 3′ side of the EHcV NS5B-coding region were predicted to correspond to 5BSL3.2 and 5BSL3.3 in the NS5B-coding region of HCV. HCV 5BSL3.2 was previously shown to interact with 3′SL II to form the kissing-loop interaction, which is required for HCV replication (33). The secondary structure prediction shown in Fig. 4C suggests that the kissing-loop interaction may be conserved between 5BSL3.2 and the 3′SL II of the EHcV genome through their complementary sequences. The long-range RNA-RNA interaction between the apical loop of subdomain IIId in HCV IRES and the bulge of 5BSL3.2 supports IRES-dependent translation and viral RNA replication (34–36). In the case of the EHcV genome, the complement sequences were detected in the apical loops of subdomain and the 5BSL3.2-like subdomain (Fig. 4C), suggesting that the long-range RNA-RNA interaction may reside in the EHcV genome. These results indicated that HCV-like RNA secondary structures may be conserved around both UTRs of the EHcV genome.

Cleavage of the EHcV core protein by SPP. The C-terminal transmembrane region of the HCV core protein was previously shown to be cleaved by SPP following the cleavage of the core-E1 junction by signal peptidase (11, 28, 37). The core protein is known to be released from the precursor polyprotein embedded in the endoplasmic reticulum (ER) membrane, and it then moves

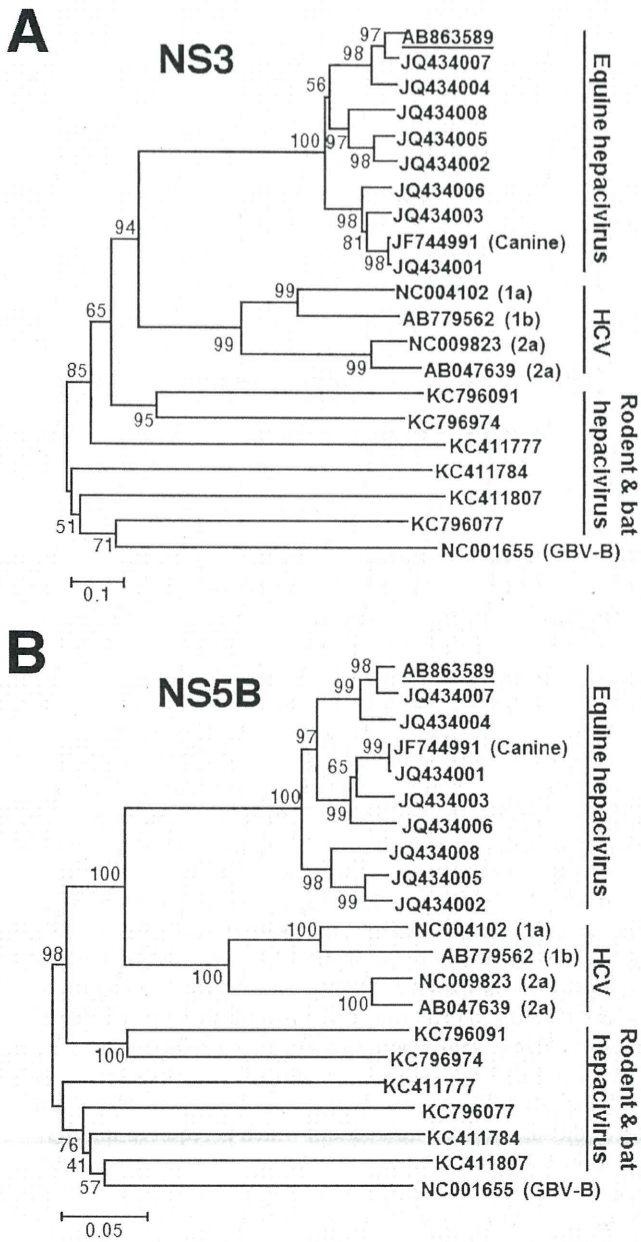

FIG 3 Phylogenetic analysis of the EHcV gene. Neighbor-joining trees of the nucleotide sequences from the NS3 (A) and NS5B (B) regions of the NPHV, HCV, and GBV-B strains are shown (23). Trees were constructed by the maximum composite likelihood method calculated using the program MEGA5 (24). The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1,000 replicates) is indicated next to the branches. Analyses were carried out using 10 strains of EhcV, JPN3/JAPAN/2013, A6-066 (GenBank accession no. JQ434003), B10-022 (GenBank accession no. JQ434004), F8-068 (GenBank accession no. JQ434005), G1-073 (GenBank accession no. JQ434002), G5-077 (GenBank accession no. JQ434006), H3-011 (GenBank accession no. JQ434008), H10-094 (GenBank accession no. JQ434007), NZP1 (GenBank accession no. JQ434001), and AAK-2011 (canine hepacivirus; GenBank accession no. JF744991); 4 strains of HCV, H77 (genotype 1a; GenBank accession no. NC004102), LyHCV (genotype 1b; GenBank accession no. AB779562), HC-J6CH (genotype 2a; GenBank accession no. NC009823), and JFH1 (genotype 2a; GenBank accession no. AB047639); 3 strains of bat hepacivirus, PDB-112 (GenBank accession no. KC796077), PDB-445 (GenBank accession no. KC796091), and PDB-829 (GenBank accession no. KC796074); 3 strains of rodent hepacivirus, RMU10-3382 (GenBank accession no. KC411777), NLR-AP-70 (GenBank accession no. KC411784), and SAR-46 (GenBank accession no. KC411807); and another primate hepacivirus, GBV-B (GenBank accession no. NC001655). The Japanese strain JPN3/JAPAN/2013 (GenBank accession no. AB863589) is underlined.

mainly to lipid droplets (LDs) (13, 14). Although SPP-dependent cleavage and LD translocation of the capsid protein are features common to HCV and GBV-B (13), it currently remains unknown whether the EHcV core protein shows these properties. The EHcV core protein shared 49.5% amino acid homology with the HCV core protein (genotype 1b) (Fig. 5A) and exhibited a hydrophobic/hydrophilic pattern similar to that of the HCV core protein (Fig. 5B). The EHcV core protein was predicted to be composed of domains 1, 2, and 3 relative to the HCV core protein. The transmembrane region of the EHcV core protein was predicted to span from Asn^{177} to Val^{199} by TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The transmembrane region of the EHcV core protein was 65% identical to that of the HCV core proteins (Fig. 5A). The C-terminal residue of the mature HCV core protein was found to be Phe^{177} in human and insect cell lines (17, 38). Our previous findings suggest that Ile^{176} and Phe^{177} of the HCV core protein may be responsible for SPP-dependent cleavage, because the replacement of Ile^{176} and Phe^{177} with Ala and Leu, respectively, abrogated intramembrane cleavage by SPP and impaired virus production (17, 28, 39). Weihofen et al. reported that SPP cleaved a peptide bond of the alpha-helix-breaking structure in a transmembrane region of the membrane protein (40). The replacement of Ile^{176} and Phe^{177} with Ala and Leu, respectively, in the HCV core protein converted the beta-sheet structure (alpha-helix-breaking structure) to an alpha-helix structure in the transmembrane region, as reported previously (28) (Fig. 6A and B). Ile^{190} and Phe^{191} of the EHcV core protein, which correspond to Ile^{176} and Phe^{177}, respectively, of the HCV core protein, reside in the alpha-helix-breaking structure of the transmembrane region (Fig. 6A and B). In contrast, the replacement of Ile^{190} and Phe^{191} with Ala and Leu, respectively, in the EHcV core protein were predicted to convert the beta-sheet to an alpha-helix structure in a manner similar to that for the HCV core protein (Fig. 6A and B). To investigate the involvement of SPP in the maturation of the EHcV core protein, we expressed EHcVc or HCVc in 293FT cells with an SPP or SPP mutant. These core proteins were expected to be resistant to signal peptidase-dependent processing because the C-terminal residue Ala of both core proteins was replaced with Arg, resulting in the detection of an immature core protein by the anti-HA antibody (Fig. 6A) (28). The core proteins with molecular masses of 23 kDa and 28 kDa were detected mainly with the anti-FLAG antibody in 293FT cells expressing HCVc and HCVc-mt, respectively (Fig. 6C, lanes 2 and 3); however, the 23-kDa band was not detected with the anti-HA antibody (Fig. 6C, lane 2). When EHcVc was expressed in 293FT cells, it was detected at a molecular mass of 27 kDa with the anti-FLAG antibody, but not with the anti-HA antibody (Fig. 6C, lane 4). In contrast, EHcV-mt, in which the 190th and 191st residues were Ala and Leu instead of Ile and Phe, respectively, was detected mainly at a molecular mass of 30 kDa with the anti-FLAG and anti-HA antibodies (Fig. 6C, lane 5). A loss-of-function SPP mutant (SPP-D219A) in which the 219th residue was Ala instead of Asp was shown to have a dominantly negative effect on SPP-dependent cleavage of the

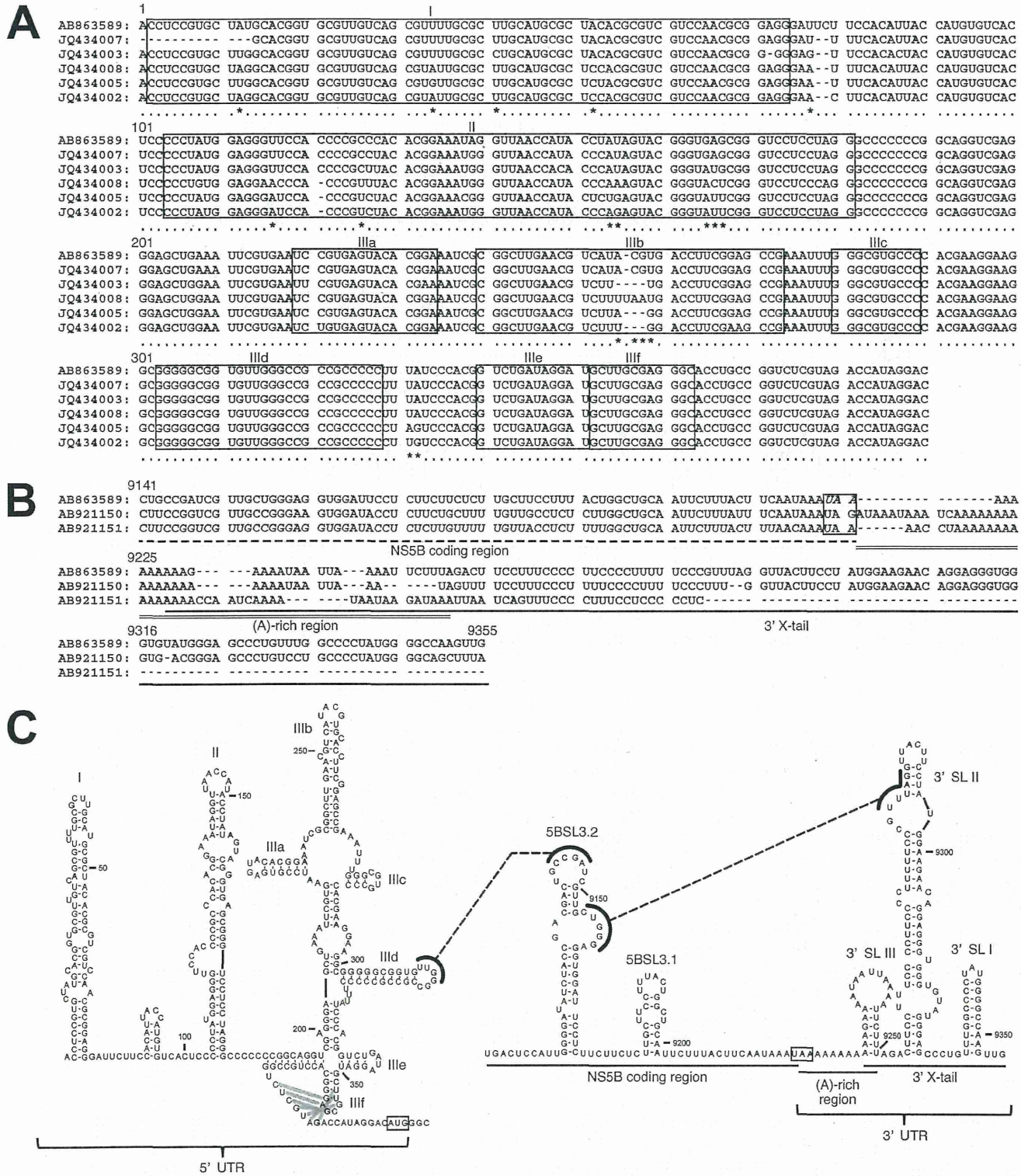

FIG 4 RNA structure analysis of the 5′ and 3′ ends of EHcV. (A) Alignment of the 5′ UTRs of the EHcV strains. Asterisks and black lines indicate variable residues and miRNA-targeting regions, respectively. SL structures are enclosed in rectangles. GenBank numbers are listed in the legend to Fig. 3. (B) Alignment of the 3′ UTRs of the EHcV strains. The 3′-terminal sequences with GenBank numbers AB921150 and AB921151 were determined using serum samples 5 and 1, respectively, and were aligned with that of JPN3/JAPAN/2013 (GenBank accession no. AB863589). (C) The secondary structures of the 5′ UTR, NS5B-coding region, and 3′ UTR were predicted on the basis of minimum free energy predictions. The stem loops in the 5′ UTR (left) were designated according to the stem loops of the HCV 5′-UTR structures (30). A gray line, double line, and single line indicate a pseudoknot structure, miR-122-target region, and complementary sequence, respectively. The stem loops in the NS5B-coding region and 3′ UTR (right) were designated to correspond to the stem loops embedded in the HCV 3′-terminal region (32, 33). The NS5B-coding region, (A)-rich region, and 3′-X-tail sequence are indicated under the schematic structure. Start and stop codons are enclosed by rectangles.