cently, Miyanari et al. reported that the association of core protein with the NS proteins and replication complexes around lipid droplets (LDs) is critical for producing infectious viruses (33).

In the present study, we demonstrated that NS5A is a prerequisite for HCV particle production via its interaction with core protein, and we identified serine residues in the C-terminal region of NS5A that play an important role in virion production. Substitution of the serine residues with alanine residues inhibited not only the interaction of NS5A with core protein but also HCV RNA-core association and led to a decrease in HCV particle production with no effect on RNA replication.

MATERIALS AND METHODS

DNA construction. Plasmids pJFH1, which contains the full-length JFH-1 cDNA downstream of the T7 RNA promoter sequence, and pSGR-JFH1/Luc, in which the neomycin resistance gene of pSGR-JFH1 has been replaced by the firefly luciferase reporter gene, have been previously described (24, 56). To generate the fluorochrome gene-tagged full-length JFH-1 plasmid, pJFH1/NS5A-GFP, the region encompassing the RsrII site of NS5A and the BsrGI site of NS5B was amplified by PCR, the amplification product was cloned into pGEM-T Easy vector (Promega, Madison, WI), and the resultant plasmid was designated pGEM-JFH1/RsrII-BsrGI. A GFP reporter gene was amplified by PCR from pGreen Lantern-1 (Invitrogen, Carlsbad, CA) with primers containing the XhoI sequence and inserted, after restriction digestion with XhoI, into the XhoI site of pGEM-JFH1/RsrII-BsrGI. The resulting plasmid was digested by RsrII and BsrGI and ligated into pJFH1 similarly digested by RsrII and BsrGI to produce pJFH1/NS5A-GFP. For generation of the fluorochrome gene-tagged subgenomic reporter plasmid, pJFH1/NS5A-GFP was digested by RsrII and SnaBI and ligated into pSGR-JFH1/Luc similarly digested by RsrII and SnaBI. The mutations in the NS5A gene were generated by oligonucleotide-directed mutagenesis (57). To construct plasmids expressing N-terminally FLAG-tagged HCV core protein or hemagglutinin (HA)-tagged NS5A, DNA fragments encoding core protein or NS5A (wild type or mutants) were generated from the full-length JFH-1 cDNA by PCR. The core protein coding sequence, together with a FLAG sequence linked to its N terminus, was cloned into the pCAGGS vector (37). The coding sequences of NS5A, together with an HA sequence linked to their N termini, were also cloned into pCAGGS vectors. All PCR products were confirmed by automated nucleotide sequencing with an ABI Prism 3130 Avant Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Cells and viruses. The human hepatoma cell line, Huh-7, and JFH1/4-1 cells, which are Huh-7 cells carrying a subgenomic replicon of JFH-1 (32), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with minimal essential medium nonessential amino acids (Invitrogen), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Huh/c-p7 cells, which are Huh-7 cells stably expressing the proteins core to p7 derived from the JFH-1 strain (18), were incubated in DMEM containing 300 μg/ml of zeocin (Invitrogen). HCV particles derived from JFH-1 were produced by transient transfection of Huh-7 cells with in vitro transcribed RNA, as described previously (56, 58). Recombinant vaccinia virus strain DIs, which expresses the bacteriophage T7 RNA polymerase under the control of the vaccinia virus early/late promoter P7.5, was generated and propagated as previously described (19).

DNA transfection, immunoprecipitation (IP), and immunoblotting. For coexpression of FLAG-tagged core protein and HA-tagged NS5A, cells were seeded onto 35-mm wells of a six-well cell culture plate and cultured overnight. Plasmid DNAs (2 μg) were transfected into cells using TransIT-LT1 transfection reagent (Mirus, Madison, WI). Cells were harvested at 48 h posttransfection, washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.25 ml lysis buffer (20 mM Tris-HCl [pH 7.4] containing 135 mM NaCl, 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS], and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). 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. After preclearing, the supernatant was immunoprecipitated with 10 μl of anti-FLAG M2-agarose beads (Sigma, St. Louis, MO). For expression of the full-length HCV polyprotein, Huh-7 cells transfected with 10 μg of in vitro transcribed RNAs by electroporation were resuspended in 20 or 30 ml of culture medium, and 10-ml aliquots were seeded into 100-mm culture dishes. At 72 h posttransfection, the cells were incubated in 0.5 ml of lysis buffer (20 mM Tris-HCl [pH 7.4] containing 135 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol) supplemented with 50 mM NaF, 5 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. After preclearing, the supernatant was immunoprecipitated with 5 μg of polyclonal anti-NS5A antibody (34a) or polyclonal anti-C/EBPβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and 20 μl of protein G-agarose beads (Invitrogen). The immunocomplex was precipitated with the beads by centrifugation at 800 × *g* for 30 s and then was washed five times with lysis buffer by centrifugation. The proteins binding to the beads were boiled in 20 μl of SDS sample buffer and then subjected to SDS–12.5% polyacrylamide gel electrophoresis (PAGE). The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) and then reacted with a primary antibody and a secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with an ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, United Kingdom) and detected using an LAS-3000 imaging analyzer (Fujifilm, Tokyo, Japan).

In vitro synthesis of HCV RNA and RNA transfection. Plasmid DNAs were digested with XbaI and treated with mung bean nuclease (New England Biolabs, Ipswich, MA) to remove the four terminal nucleotides, resulting in the correct 3′ end of the HCV cDNA. Digested DNAs were purified and used as templates for RNA synthesis. HCV RNA was synthesized in vitro using a MEGAscript T7 kit (Ambion, Austin, TX). Synthesized RNA was treated with DNase I (Ambion), followed by acid guanidinium thiocyanate-phenol-chloroform extraction to remove any remaining template DNA. Synthesized HCV RNAs were used for electroporation. Trypsinized Huh-7 cells were washed with Opti-MEM I reduced-serum medium (Invitrogen) and resuspended at 3×10^6 cells/ml with Cytomix buffer (54). RNA was mixed with 400 μl of cell suspension and transferred into an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybaid, Middlesex, United Kingdom). Cells were then pulsed at 260 V and 950 μF using a Gene Pulser II unit (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred onto six-well culture plates or 100-mm culture dishes.

Luciferase assay. Cells were harvested at different time points posttransfection of subgenomic reporter replicons and lysed in passive lysis buffer (Promega). The luciferase activity in cells was determined using a luciferase assay system (Promega).

Quantification of HCV core protein. HCV core protein in transfected cells or cell culture supernatants was quantified using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA Kit; Ortho Clinical Diagnostics, Tokyo, Japan). To determine intracellular core protein amounts, cell lysates were prepared as described previously (41). To determine the efficiency of core protein release, the ratio of extracellular core protein to total core protein (the sum of intra- and extracellular core protein amounts) was calculated.

Intra- and extracellular infectivity assay. Culture supernatants were harvested 72 h posttransfection, and virus titers were determined by a 50% tissue culture infectious dose ($TCID_{50}$) assay as described previously (28, 46). Virus titration was performed by seeding naïve Huh-7 cells in 96-well plates at a density of 1×10^4 cells/well. Samples were serially diluted fivefold in complete growth medium and used to infect the seeded cells (six wells per dilution). At 72 h after infection, the inoculated cells were fixed and immunostained with a mouse monoclonal anti-core protein antibody (2H9) (56), followed by an Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (IgG) (Invitrogen). Wells that showed at least one core protein-expressing cell was counted as positive. Cell-associated infectivity was determined essentially as described previously (12, 47). Briefly, cells were extensively washed with PBS, scraped, and centrifuged for 3 min at 120 × *g*. Cell pellets were resuspended in 1 ml of DMEM containing 10% FBS and subjected to four cycles of freezing and thawing using dry ice and a 37°C water bath. Samples were then centrifuged at 2,400 × *g* for 10 min at 4°C to remove cell debris, and cell-associated infectivity was determined by $TCID_{50}$ assay.

Expression of HCV proteins using vaccinia viruses, metabolic labeling of cells, and radioimmunoprecipitation analysis. Metabolic labeling of cells and radioimmunoprecipitation analysis were performed as described by Huang et al. (17) with some modifications. A total of 4×10^5 Huh-7 cells were seeded onto each well of six-well cell culture plates and cultured overnight. A 2-μg amount of subgenomic replicon DNAs carrying defined NS5A mutations was transfected into cells using TransIT-LT1 transfection reagent, and at 12 h posttransfection the cells were then infected at a multiplicity of infection of 10 with recombinant vaccinia viruses expressing the T7 RNA polymerase. After 40 h of transfection, cells were incubated in methionine- and cysteine-deficient DMEM (Invitrogen) or phosphate-deficient DMEM (Invitrogen) for 2 h and labeled for 6 h with [^{35}S]methionine and [^{35}S]cysteine (200 μCi/well; GE Healthcare) or

[^{32}P]orthophosphate (250 μCi/well; GE Healthcare). The cells were then washed twice with cold PBS and lysed with SDS lysis buffer (50 mM Tris-HCl [pH 7.6], 0.5% SDS, 1 mM EDTA, 20 μg/ml of PMSF). The cell lysates were passed through a 27-gauge needle several times to shear cellular DNA. After a 10-min incubation at 75°C, the lysates were clarified by centrifugation and diluted fivefold with HNAET buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.67% bovine serum albumin, 1 mM EDTA, 0.33% Triton X-100). After preclearing by incubation with 20 μl of protein G-agarose beads for 1 h at 4°C, the supernatant was incubated with 2 μg of rabbit polyclonal anti-NS5A antibody overnight at 4°C. A 20-μl aliquot of protein G agarose beads was further added and incubated for 2 h at 4°C. The cell pellets were washed three times with 0.5 ml of HNAETS buffer (HNAET containing 0.5% SDS), followed by washing once with 0.5 ml of HNE buffer (50 mM HEPES [pH 7.5], 150 mM NaCl and 1 mM EDTA). After treatment with or without λ protein phosphatase (New England Biolabs), the cell pellets were suspended in 20 μl of SDS sample buffer and boiled for 10 min. The proteins were resolved on 10% SDS-polyacrylamide gels and analyzed by autoradiography.

Subcellular fractionation analysis. All steps were carried out at 4°C in the presence of a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) as described previously (20), with some modifications. Cells were suspended in four cell volumes of homogenization buffer (50 mM NaCl, 10 mM triethylamine [pH 7.4], 1 mM EDTA), snap frozen in liquid nitrogen, stored at −80°C, and thawed in a water bath at room temperature. Supernatants (0.4 ml) were layered on linear 10-ml iodixanol gradients from 2.5 to 25% and centrifuged at 37,000 rpm for 3.5 h in an SW41 rotor (Beckman, Fullerton, CA), followed by collection of 0.8-ml fractions from the top. Each fraction was concentrated by Centricon YM30 (Millipore), separated by SDS-PAGE, and immunoblotted with a rabbit polyclonal anti-calnexin antibody (Stressgen Biotechnologies, Victoria, Canada), a mouse monoclonal anti-adipose differentiation-related protein (ADRP) antibody (Progen Biotechnik, Heidelberg, Germany), or a rabbit polyclonal anti-NS5A antibody. The core protein amount in each fraction was also determined by enzyme-linked immunosorbent assay (ELISA).

IP-RT-PCR. The process of cell lysis to RNA purification was carried out essentially as described by Johnson et al. (21) with some modifications. A total of 3×10^6 Huh-7 cells were transfected with 10 μg of in vitro transcribed HCV RNAs and resuspended in 20 or 30 ml of culture medium, after which 10-ml aliquots were seeded into 100-mm culture dishes. At 72 h posttransfection, the cells were scraped and incubated in 500 μl of hypotonic buffer (10 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF) per dish. The cells were passed through a 20-gauge needle several times, lysed with Nonidet P-40 at a final concentration of 1%, and incubated on ice for an additional 10 min. After centrifugation at 4,000 × *g* at 4°C for 15 min, glycerol was added to the supernatants at a final concentration of 5%. The cell lysates were incubated with 20 μl of protein G-agarose beads for 30 min at room temperature. After the cell lysates were removed from protein G-agarose beads, 5 μg of mouse monoclonal anti-core protein antibody or normal mouse IgG (Sigma) as a negative control was added, and samples were incubated for an additional 1 h at room temperature. A 20-μl aliquot of protein G-agarose beads per sample was added to the cell lysates and incubated for 1 h. After incubation, the beads were washed three times with wash buffer (10 mM Tris-HCl [pH 7.6], 100 mM KCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol) and eluted in 100 μl of elution buffer (50 mM Tris-HCl [pH 8.0], 1% SDS, and 10 mM EDTA) at 65°C for 10 min. After treatment with 100 μg of proteinase K at 37°C for 30 min, the RNAs in immunocomplexes were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Reverse transcriptase PCR (RT-PCR) was carried out using random hexamer and Superscript II RT (Invitrogen), followed by nested PCR with LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan) and primer sets amplifying the fragments of nucleotides (nt) 129 to 2367 and nt 7267 to 9463 of the JFH-1 genome. To amplify the fragment of nt 129 to 2367, the sense primer 5′-CTGTGAGGAACTACTGTCTT-3′ and the antisense primer 5′-TCCACGATGTTCTGGTGAAG-3′ were used for first-round PCR; the sense primer 5′-CGGGAGAGCCATAGTGG-3′ and the antisense primer 5′-CATTCCGTGGTAGAGTGCA-3′ were used for second-round PCR. To amplify the fragment of nt 7267 to 9463, the sense primer 5′-GTCCAGGGTGCCCGTTCTGGACT-3′ and the antisense primer 5′-GCGGCTCACGGACCTTTCAC-3′ were used for first-round PCR; the sense primer 5′-CACCGTTGCTGGTTGTGCT-3′ and the antisense primer 5′-GTGTACCTAGTGTGTGCCGCTCTA-3′ were used for second-round PCR.

Indirect immunofluorescence analysis. Cells incubated for 3 days after transfection with JFH-1 RNAs were seeded in an eight-well chamber slide (BD Biosciences, San Jose, CA) and cultured overnight. The adherent cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature. After a washing step with PBS, the cells were permeabilized with PBS containing 0.3% Triton X-100 and 2% FBS for 1 h at room temperature and stained with a rabbit polyclonal anti-NS5A antibody and a mouse monoclonal anti-core protein antibody. The fluorescent secondary antibodies were Alexa Fluor 488- or Alexa Fluor 555-conjugated anti-rabbit or anti-mouse IgG antibodies (Invitrogen). Analyses of JFH-1 were performed on a Zeiss confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany).

RESULTS

Mutations of serine residues at the NS5A C terminus impair basal phosphorylation but have little effect on viral RNA replication. As demonstrated in a previous study, insertion of GFP into the NS5A C terminus does not significantly affect viral RNA replication but reduces the generation of infectious HCV particles (41). The C-terminal region of NS5A contains highly conserved serine residues that are involved in basal phosphorylation (1, 23, 49). To examine the involvement of the serine clusters (cluster 3-A [CL3A] and cluster 3-B [CL3B]) in the C-terminal region of NS5A in HCV particle production, we created mutated HCV genomes as well as subgenomic replicons carrying alanine substitutions for the conserved serine residues at aa 2384, 2388, 2390, and 2391 (residues are numbered according to the positions within the original JFH-1 polyprotein) (CL3A/SA); at aa 2428, 2430, and 2433 (CL3B/SA); or an in-frame deletion spanning aa 2384 to 2433 (Δ2384–2433) (Fig. 1). A construct with an in-frame insertion of GFP (NS5A-GFP) was also generated as described previously for the Con1 isolate (34).

First, we analyzed the effects of the NS5A mutations on HCV RNA replication using a transient RNA replication assay using subgenomic luciferase reporter replicons (Fig. 2A) and found that the serine-to-alanine substitutions (CL3A/SA and CL3B/SA) did not affect viral RNA replication. NS5A-GFP and Δ2384–2433 slightly reduced RNA replication, indicating that the mutations of the NS5A C terminus tested in this study do not critically affect RNA replication, which is consistent with previous reports (1, 34, 51).

Next, the phosphorylation status of the mutated NS5A was analyzed as described in Materials and Methods (Fig. 2B). NS5A was isolated from radiolabeled cells by IP and analyzed either directly by SDS-PAGE or after treatment with λ protein phosphatase. Analysis of ^{32}P-radiolabeled proteins revealed that the CL3A/SA, CL3B/SA, and Δ2384–2433 mutations resulted in marked reduction of basal phosphorylation (Fig. 2B, compare lane 1 with lanes 3, 5, and 7 in the top panel). All ^{32}P-labeled NS5A proteins were sensitive to treatment with phosphatase (lanes 2, 4, 6, and 8). The possibility that loss of signal after dephosphorylation was due to contaminating proteases present in the phosphatase preparations can be ruled out because no degradation of the ^{35}S-labeled proteins was observed (Fig. 2B, bottom panel). These results suggest that mutations in the C-terminal serine cluster of NS5A impair basal phosphorylation but have no significant effect on viral RNA replication.

Effect of mutations introduced into the NS5A C terminus on the production of infectious HCV particles. To analyze HCV particle production from cells transfected with the in vitro transcribed viral genomic RNAs, we harvested supernatants and cells at 4, 24, 48, 72, and 96 h posttransfection and measured the amounts of core protein. As shown in Fig. 3A, comparable amounts of core proteins were detected in all transfected cells 4 h after transfection, reflecting unchanged

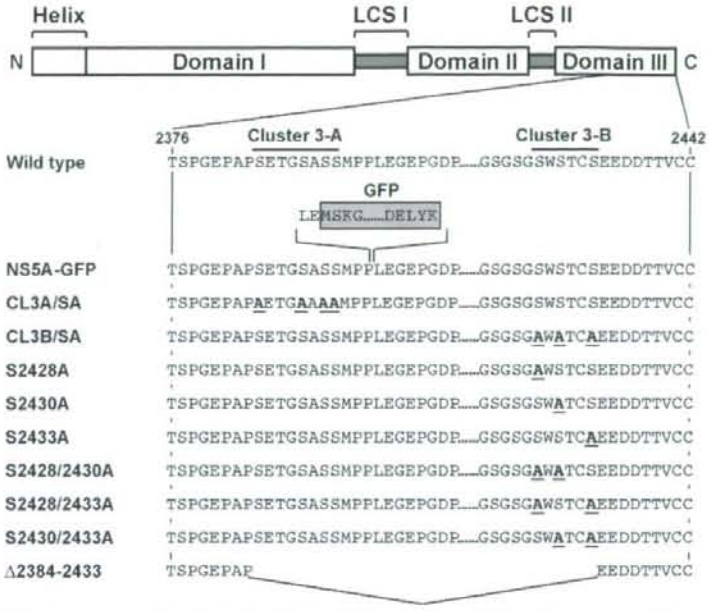

FIG. 1. Structures of HCV constructs used in this study. Schematic diagram of the NS5A structure according to Tellinghuisen et al. (52) is shown in the top panel. The three domains are indicated by white boxes and are separated by trypsin-sensitive regions with presumably low structural complexity (low-complexity sequence [LCS]). The numbers indicate amino acid residues within the original JFH-1 polyprotein. The names listed on the left represent full-length HCV constructs, subgenomic reporter replicons, or N-terminally HA-tagged NS5A constructs used in this study. NS5A-GFP carries a GFP insertion between aa 2394 and 2395 as indicated by a shaded box. CL3A/SA and CL3B/SA carry several serine-to-alanine substitutions in the NS5A C terminus constructed as described previously (1). HCV constructs from S2428A to S2430/2433A carry single or double serine-to-alanine substitutions generated by modification of the CL3B/SA construct. The Δ2384–2433 mutant possesses an in-frame deletion in the C-terminal region of NS5A. Amino acid substitutions are marked in bold and underlined. N and C represent N terminus and C terminus, respectively.

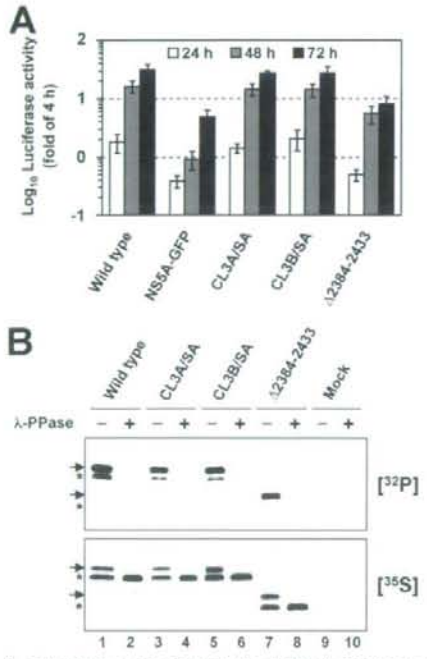

FIG. 2. Mutations at the C terminus of NS5A impair basal phosphorylation and have only a minor impact on RNA replication. (A) Replication of given mutants in transfected Huh-7 cells as determined by luciferase reporter assays performed at 24, 48, and 72 h posttransfection (white, gray, and black bars, respectively). Values given were normalized for transfection efficiency using the luciferase activity determined 4 h after transfection, which was set to 1. Mean values of quadruplicate measurements and the standard deviations are given. (B) Phosphorylation analysis of NS5A using the vaccinia virus T7 hybrid system. NS3-to-NS5B polyprotein fragments carrying the mutations specified above the lanes were transfected into Huh-7 cells, and proteins were radiolabeled with [^{32}P]orthophosphate or [^{35}S]methionine and [^{35}S]cysteine. NS5A proteins were isolated by IP and separated by SDS-PAGE (10% polyacrylamide). Mock-transfected cells served as a negative control (lanes 9 and 10). Half of the samples were treated with λ protein phosphatase (λ-PPase) (+) whereas the other half was mock treated (−) prior to SDS-PAGE. Arrows and asterisks indicate hyperphosphorylated and basally phosphorylated forms, respectively.

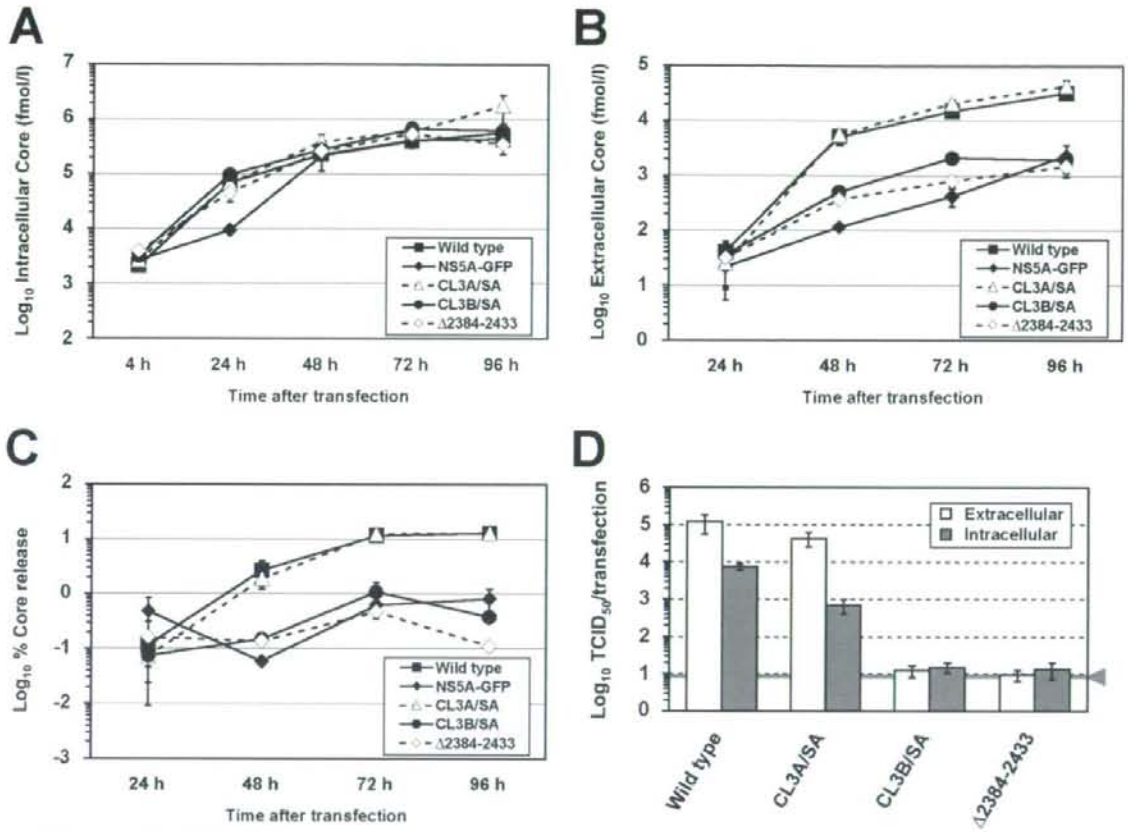

FIG. 3. Effect of mutations introduced into the NS5A C terminus on the production of infectious HCV particles. (A) Intracellular levels of core protein measured at various time points after transfection. A total of 3×10^6 Huh-7 cells were transfected with 10 μg of in vitro-transcribed HCV RNAs specified in the inset and resuspended in 10 ml of culture medium, after which 2-ml aliquots were seeded into each well of a six-well culture plate. The cells were harvested at different time points between 4 h and 96 h posttransfection, and then 500 μl of cell lysate per well was prepared. After centrifugation, supernatants were processed for a core protein-specific ELISA. (B) Release of core protein from cells transfected with the HCV genomes specified in the inset. Cell culture supernatants harvested from cells given in panel A were analyzed by a core protein ELISA. (C) Efficiency of core protein release from cells transfected with the HCV genomes specified in the inset. The percent core protein release (vertical axis) indicates the percentage of released core protein in relation to total core protein (the sum of intra- and extracellular core protein) calculated for each time point. (D) Infectivity of virus particles contained in supernatants and cells after transfection with mutants specified below the graph. Culture supernatants and cells were harvested 72 h posttransfection, and extracellular (white bars) and intracellular infectivity (gray bars) levels were determined by $TCID_{50}$ assay. The gray line and arrowhead represent the detection limit of the limiting dilution assay. Mean values and standard deviations for at least triplicates are shown in all panels.

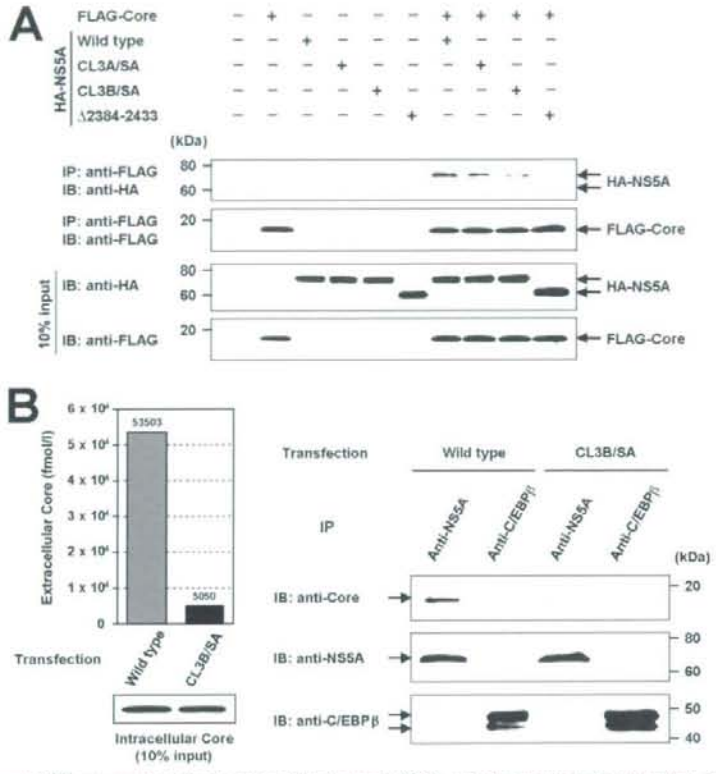

FIG. 4. aa 2428, 2430, and 2433 are essential for the interaction between NS5A and the core protein. (A) Effect of mutations at the NS5A C terminus on the interaction of NS5A with the core protein. N-terminally FLAG-tagged core protein and N-terminally HA-tagged NS5A carrying defined mutations were coexpressed in Huh-7 cells and immunoprecipitated with anti-FLAG antibody. The resulting precipitates were examined by immunoblotting using anti-HA or FLAG antibody. One-tenth of the cell lysates used in IP is shown as the 10% input. (B) Interaction between NS5A and the core protein in HCV-replicating cells. Huh-7 cells were lysed 72 h after transfection of the in vitro transcript of the HCV genome (wild type or CL3B/SA) and were immunoprecipitated with anti-NS5A antibody or anti-C/EBPβ antibody as a negative control. The resulting precipitates were examined by immunoblotting using anti-core protein, NS5A, or C/EBPβ antibody. One-tenth of cell lysates used in IP was immunoblotted with anti-core protein antibody (10% input). Cell culture supernatants harvested from transfected cells were analyzed by a core protein ELISA in parallel. IB, immunoblotting.

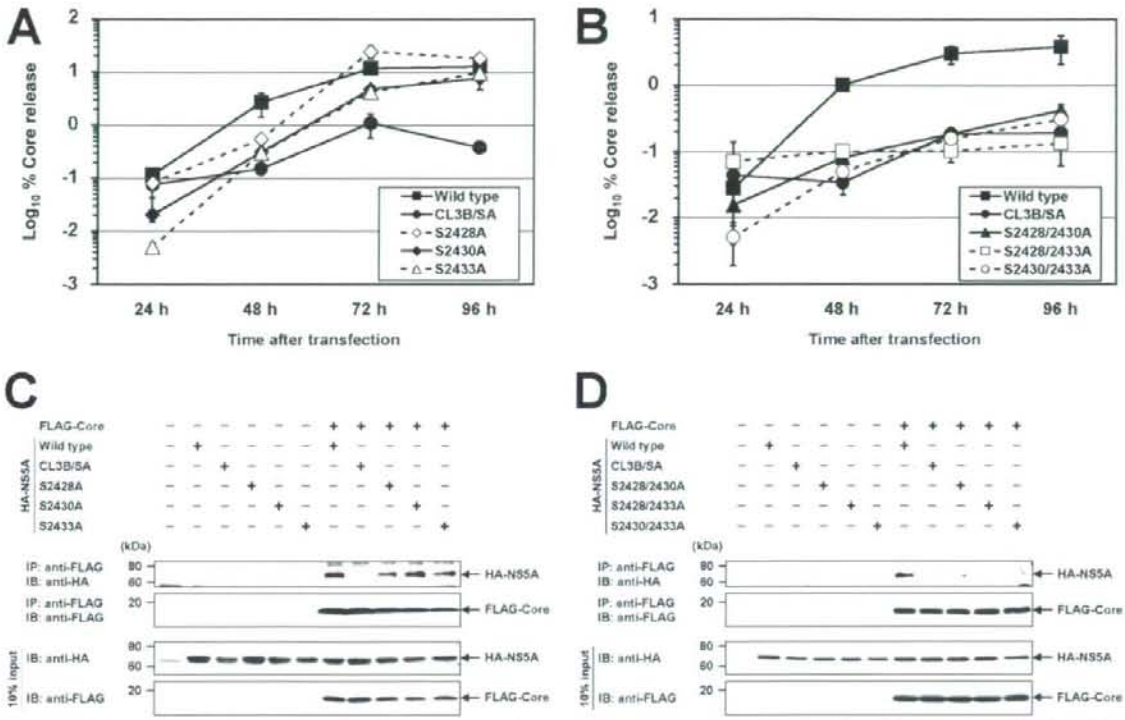

FIG. 5. Determination of critical amino acids responsible for virus production and the interaction of NS5A with the core protein. (A and B) Effect of single or double serine-to-alanine substitutions on virus production. After transfection of in vitro transcripts of the HCV genomes specified in the inset into Huh-7 cells, the cells and culture supernatants were harvested at the time points given, and the amounts of the core protein were determined by core protein-specific ELISA. Percent core protein release (vertical axis) indicates the percentage of released core protein in relation to total core protein (the sum of intra- and extracellular core protein) calculated for each time point. Mean values and standard deviations for at least triplicate experiments are shown. (C and D) Effect of single or double serine-to-alanine substitutions on the interaction between NS5A and the core protein. N-terminally FLAG-tagged core protein and N-terminally HA-tagged NS5A carrying defined mutations were coexpressed in Huh-7 cells and immunoprecipitated with anti-FLAG antibody. The resulting precipitates were examined by immunoblotting using anti-HA or FLAG antibody. One-tenth of the cell lysates used in IP is shown as the 10% input. IB, immunoblotting.

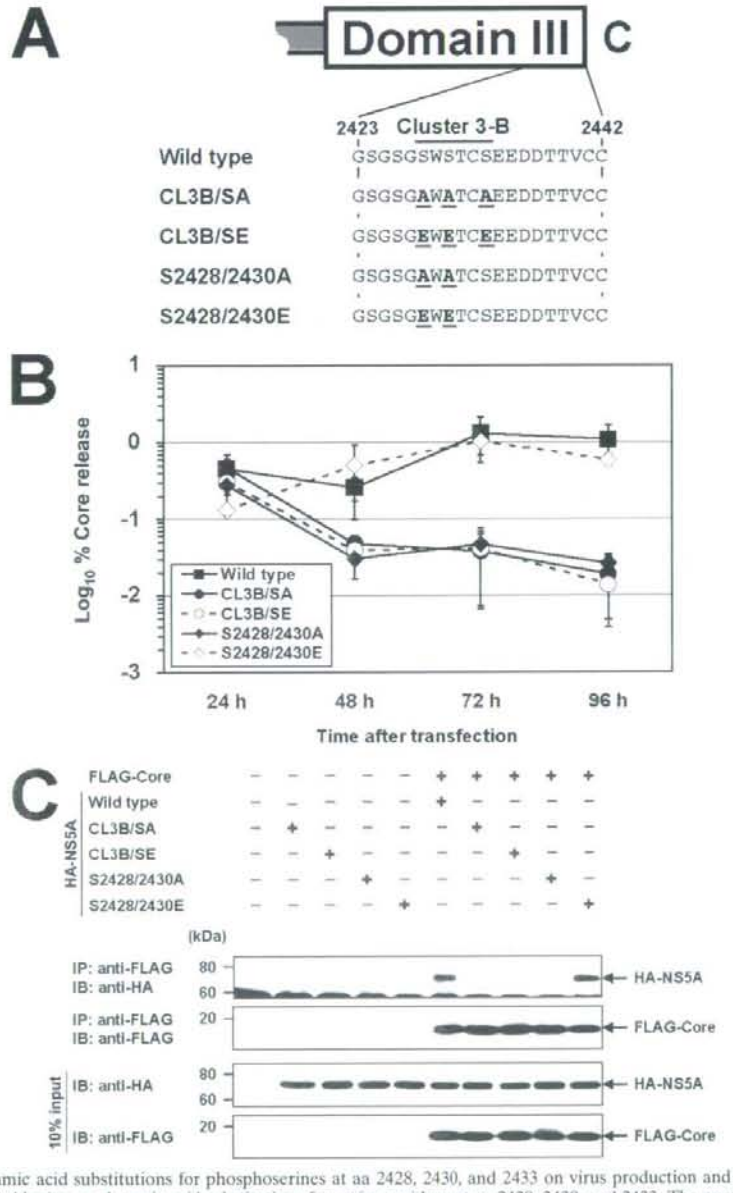

FIG. 6. Effect of glutamic acid substitutions for phosphoserines at aa 2428, 2430, and 2433 on virus production and the interaction of NS5A with the core protein. (A) Alanine or glutamic acid substitutions for serine residues at aa 2428, 2430, and 2433. The numbers indicate amino acid positions within the polyprotein of the JFH-1 isolate. The names shown on the left represent full-length HCV or N-terminally HA-tagged NS5A constructs used in this experiment. Amino acid substitutions are marked in bold and underlined. C represents the C terminus. (B) Effect of alanine or glutamic acid substitutions on virus production. After transfection of in vitro transcripts of the HCV genomes specified in the inset into Huh-7 cells, the cells and the culture supernatants were harvested at the time points given, and the amounts of core protein were determined by core protein-specific ELISA. Percent core protein release (vertical axis) indicates the percentage of released core protein in relation to total core protein (the sum of intra- and extracellular core protein) calculated for each time point. Mean values and standard deviations for at least triplicate experiments are shown. (C) Effect of alanine or glutamic acid substitutions on the interaction between NS5A and the core protein. N-terminally FLAG-tagged core protein and N-terminally HA-tagged NS5A carrying defined mutations were coexpressed in Huh-7 cells and immunoprecipitated with anti-FLAG antibody. The resulting precipitates were examined by immunoblotting (IB) using anti-HA or FLAG antibody. One-tenth of the cell lysates used in IP is as shown as the 10% input.

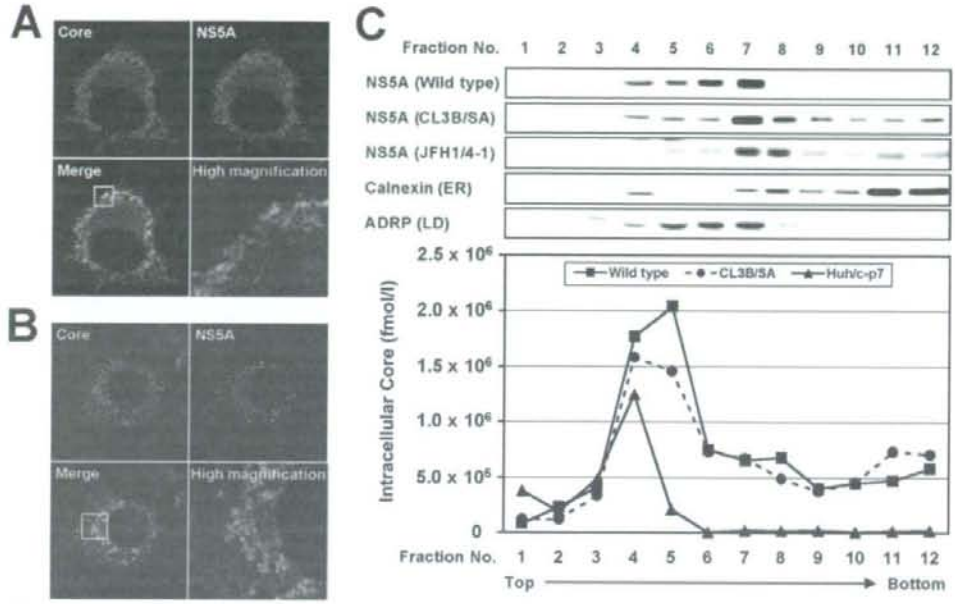

FIG. 7. Subcellular localization of NS5A and the core protein in HCV-replicating cells. Huh-7 cells were transfected with the in vitro transcript of the HCV genome, wild type (A) or CL3B/SA (B). Seventy-two hours after transfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and double stained with antibodies against the core protein (green) and NS5A (red), followed by staining with an Alexa Fluor 488- or Alexa Fluor 555-conjugated antibody. High-magnification panels are enlarged images of white squares in the merge panels. (C) HCV (wild type or CL3B/SA)-replicating cells, JFH1/4-1 cells harboring a subgenomic replicon of JFH-1, or Huh/c-p7 cells stably expressing JFH-1 structural proteins were lysed by freeze-thawing, and the cell lysates were fractionated on 5 to 25% iodixanol gradients. The distributions of NS5A, calnexin (ER marker), and ADRP (LD marker) were determined by immunoblotting, and those of the core protein were examined by core protein-specific ELISA.

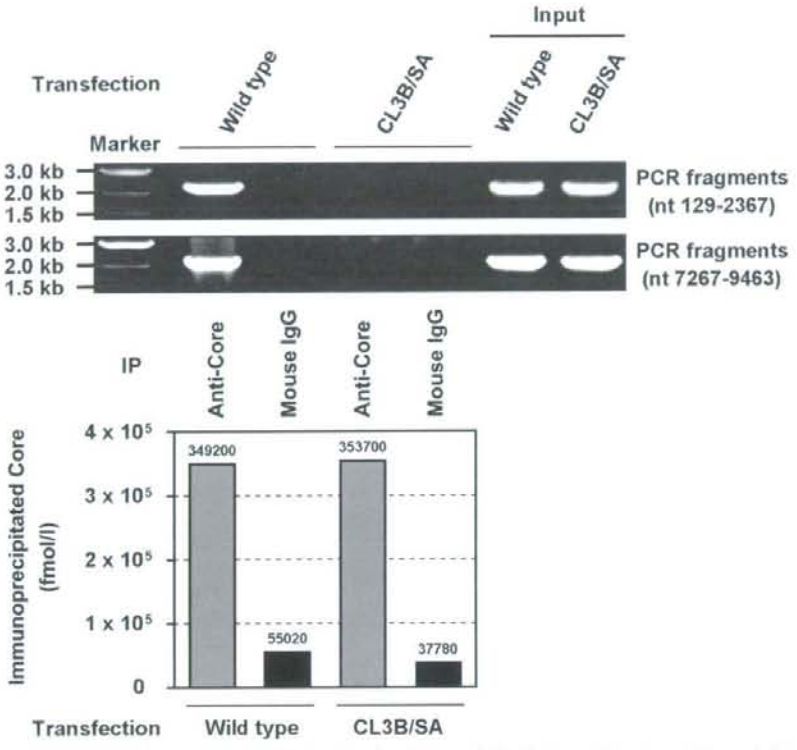

FIG. 8. IP-RT-PCR of HCV-replicating cells performed to examine the association between the core protein and the HCV genome RNA. Huh-7 cells were transfected with the in vitro transcript of the HCV genome (wild type or CL3B/SA) and lysed in 500 μl of hypotonic buffer at 72 h posttransfection. After IP with an anti-core protein antibody or mouse IgG, immunoprecipitates were eluted in 100 μl of elution buffer. RNAs in immunocomplexes were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. PCR was carried out as described in Materials and Methods with primer sets amplifying the fragments of nt 129 to 2367 and nt 7267 to 9463 of the JFH-1 genome. One-tenth (10 μl) of each eluted immunoprecipitate was used for assays of the core protein amounts to ensure IP efficiency (lower panel). RNA extracted from a small aliquot of each cell lysate used in IP-RT-PCR is shown as the input.

in the virus released from the transfected cells; however, more evident perturbation was obtained from double or triple substitutions (Fig. 5A and B). Tellinghuisen et al. determined the HCV production at 48 h after RNA transfection and found a marked inhibition by the single substitution S2433A. In our study, as indicated in Fig. 5A, the reduction caused by the S2433A mutant was approximately 90% at 48 h after transfection; however, the virus production from the mutant reached a similar level to that of the wild type at 96 h posttransfection.

Several previous studies have found that apolipoproteins B (apoB) and E (apoE), microsomal triglyceride transfer protein, and HCV p7 protein are key factors for production of the infectious HCV particles (4, 11, 16, 22, 47). Assembly and maturation of the viral particles appear to depend on the formation of very-low-density lipoprotein, a large particle containing apoB, apoE, and large amounts of neutral lipids in hepatic cells. p7 protein is primarily involved in a late step of virus particle production, and the findings support the idea that p7 acts as viroporin, which has the capacity to compromise cell membrane integrity and thus favors the release of viral progeny. How the early step in virion production regulated by the NS5A-core protein interaction links with the later step(s) involved in the very-low-density lipoprotein assembly or p7 function remains an interesting question to be addressed.

In summary, we demonstrated that the C-terminal serine cluster of NS5A (aa 2428, 2430, and 2433), which is involved in generating the basal phosphorylated form, is a determinant of NS5A interaction with the core protein and the subcellular localization of NS5A. Mutation of this cluster blocks the NS5A-core protein interaction, resulting in perturbation of association between the core protein and HCV RNA. It is thus tempting to consider that NS5A plays a key role in transporting the viral genome RNA synthesized by the replication complex to the surface of LDs or LD-associated membranes, where the core protein localizes, leading to facilitation of nucleocapsid formation. Structural analysis of the NS5A domain III-core protein complex should provide greater insight into the mode of interaction between these viral proteins. Identification of residues at the interface that are involved in important interactions will be of significant value in designing novel structure-based inhibitors to block the early step of HCV particle formation.

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Trans-encapsidation of hepatitis C virus subgenomic replicon RNA with viral structure proteins

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ABSTRACT

A trans-packaging system for hepatitis C virus (HCV) subgenomic replicon RNAs was developed. HCV subgenomic replicon was efficiently encapsidated by the HCV structural proteins that were stably expressed *in trans* under the control of a mammalian promoter. Infectious HCV-like particles (HCV-LPs), established a single-round infection, were produced and released into culture medium in titers of up to 10^3 focus forming units/ml. Expression of NS2 protein with structural proteins (core, E1, E2, and p7) was shown to be critical for the infectivity of HCV-LPs. Anti-CD81 treatment decreased the number of infected cells, suggesting that HCV-LPs infected cells in a CD81-dependent manner. The packaging cell line should be useful both for the production of single-round infectious HCV-LPs to elucidate the mechanisms of HCV assembly, particle formation and infection to host cells, and for the development of HCV replicon-based vaccines.

Hepatitis C virus (HCV) is a positive-strand RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family. The HCV genome comprises about 9600 nucleotides that encode a single polyprotein of around 3000 amino acids [1–3], which is processed by cellular and viral encoded proteases into at least 10 different structural and nonstructural proteins [4–6]. The JFH-1 strain of HCV, classified as genotype 2a strain, is the first HCV strain that can produce HCV particles in Huh7 cells [7,8]. The synthesis of HCV-like particles (HCV-LPs) using a recombinant baculovirus containing the cDNA of HCV structural proteins has been reported [9]. HCV-LP production by mammalian expression systems using vesicular stomatitis virus [10] and semliki forest virus [11] were also reported although the amount of VLP production is not as high as that of baculovirus system.

Subgenomic replicon system is a useful tool as gene expression vectors and is desirable for the development of vaccines. In the case of flaviviruses, several systems have been described for packaging flavivirus replicons, including Kunjin virus replicons [12–14], yellow fever virus replicons [15], tick-borne encephalitis virus replicons [16], and West Nile virus replicons [17,18]. In some cases, these packaging systems have utilized cell lines expressing the flavivirus structural proteins under the control of eukaryotic promoters [16,19]. These virus-like particle (VLP)-generating systems have been useful for packaging viral genomes encoding various foreign genes [14,15,20,18], the study of virus tropism and various aspects of viral assembly and entry [17].

Subgenomic replicons of JFH-1 replicate efficiently in Huh7 cells and do not require cell culture-adaptive mutations [21]. The construction of a system to package HCV replicon into HCV-LPs would not only be useful to investigate as-yet unclear steps of HCV life cycles such as genome packaging and virion assembly but also offers the possibilities of a new approach for vaccine development. In this study, we constructed subgenomic replicon cell lines constitutively expressing JFH-1 structural proteins under the control of elongation factor-1α (EF) promoter, and found stable expression of structural proteins and release of HCV-LPs from the cell line. A sucrose density gradient centrifugation of the culture medium resulted in partial purification of the HCV-LPs. Infectivity of HCV-LPs produced by this system was confirmed by colony formation assay and immunofluorescence analysis. Anti-CD81 antibody treatment decreased the infectivity of HCV-LPs, suggesting that VLPs infected to cells in CD81-dependent fashion. This is the first report that HCV structural proteins of HCV can trans-package its subgenomic replicon. The system described here should be useful to elucidate the mechanisms of HCV assembly, particle formation, and infection to host cells.

Materials and methods

Plasmid construction. Core to p7 coding region of JFH-1 was amplified using pJFH-1 [21] as a template and sense primer

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5′-GAGAATTCGTAGACCGTGCACCATG-3′ and antisense primer 5′-AAGAATTCTTAGGCATAAGCCTGCCGGGGCA-3′. Core to NS2 coding region of JFH-1 was amplified using pJFH-1 as a template and sense primer 5′-GAGAATTCGTAGACCGTGCACCATG-3′ and antisense primer 5′-AAGAATTCTTAAAGGAGCTTCCACCCCTTGG-3′. Amplified fragments were inserted into EcoRI site of pEF4 (Invitrogen) to generate pEFJFH/c-p7 and pEFJFHc-NS2, respectively.

Establishment of cell lines capable of packaging JFH-1 replicon RNA into VLPs. Huh7 cells were transfected using Lipofectamine (Invitrogen) with either pEFJFH/c-p7 or pEFJFHc-NS2 and were cultured with 0.2 mg/ml of zeocin (Invitrogen). Zeocin-resistant colonies were collected 3 weeks after transfection. The cell lines, Huh/c-p7 and Huh/c-NS2 (expressing pEFJFH/c-p7 and pEFJFHc-NS2, respectively) were then electroporated with 1 μg of JFH-1 subgenomic replicon (SGR-JFH1) RNA and were cultured with 0.375 mg/ml of G418 (Nacalai Tesque). Expression of core, E2 and NS5A was confirmed by Western blotting using anti-HCV core and anti-HCV E2 monoclonal antibodies [22] and anti-HCV NS5A polyclonal antibody [23]. The total RNA of culture media for each cell line (Huh/c-p7/SGR and Huh/p-NS2/SGR) was extracted using the QIAampViral RNA Mini spin column (Qiagen). Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems), as described previously [24,25]. The HCV core antigen in the culture media was measured by immunoassay (Ortho HCV-Core ELISA Kit; Ortho-Clinical Diagnotics), following the manufacturer's instructions. Culture medium was centrifuged at 8000g for 30 min to remove all cellular debris, after which the supernatant was concentrated to 1 ml by centrifugation using Amicon Ultracel 100k (Amicon). The concentrated medium was then layered on top of a continuous 10–60% (wt/vol) sucrose gradient in phosphate buffered saline (PBS) and then centrifuged at 40,000 rpm at 4 °C for 16 h (SW41E rotor, Beckman). Fractions (1 ml each) were collected from the top of the tube (12 fractions in total) and the density for each fraction was determined. The concentrations of replicon RNAs and core proteins of each fraction were measured as described above.

Infectivity of HCV-LPs. To determine whether these cell lines produced infectious HCV-LPs, we performed a colony formation assay using neomycin-resistant gene of SGR-JFH1 RNA. Naive Huh7 cells were infected with pooled fractions of 1.12–1.20 g/ml of both cell lines and were cultured for 3 weeks with G418 at 0.375 mg/ml. Formed colonies were stained with crystal violet and counted.

We also performed an immunofluorescence study in order to analyze the infectivity of the HCV-LPs. Following 3 days of incubation, the cells were fixed and immunostained for NS5A with anti-NS5A rabbit polyclonal antibody as described previously (Murakami et al., in press). Ffu (focus forming units) was calculated essentially based on the method as described previously [7,26]. Virus titration was performed by seeding Huh-7 cells in 96-well plates at 1×10^4 cells/well. Samples were serially diluted 5-fold in complete growth medium and used to infect the seeded cells (six wells per dilution). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI).

Results

Establishment of cell lines capable of packaging JFH-1 replicon RNA into VLPs

Stable cell lines expressing JFH-1 structural proteins were generated by transfecting with either pEFJFH/c-p7 or pEFJFHc-NS2. Zeocin-resistant colonies were collected 3 weeks after transfection and the expression of JFH-1 structural proteins was confirmed by Western blotting using anti-HCV core and anti-HCV E2 monoclonal antibodies [22] (Fig. 1A, lanes 1 and 2). The cell lines, Huh/c-p7 and Huh/c-NS2 (expressing pEFJFH/c-p7 and pEFJFHc-NS2, respectively) were then electroporated with 1 μg of SGR-JFH1 RNA. Six G418-resistant colonies were selected 3 weeks after electroporation and were termed Huh/c-p7/SGR (1-6) and Huh/c-NS2/SGR (1-6) cells. Expression of core, E2 and NS5A of Huh/c-p7/SGR-1, and Huh/c-NS2/SGR-3 was confirmed by Western blotting (Fig. 1A, lanes 3 and 4).

To investigate whether HCV-LPs were secreted from Huh/c-p7/SGR and Huh/c-NS2/SGR cells, we analyzed the culture medium of these cell lines 6 days postinfection. As shown in Fig. 1B, HCV replicon RNA and core protein were secreted from both cell lines. Fifty milliliters of culture medium from one Huh/c-NS2/SGR-1 and Huh/c-p7/SGR-3 cell line was concentrated, layered on top of a continuous 10–60% (wt/vol) sucrose gradient in PBS and then centrifuged at 40,000 rpm at 4 °C for 16 h. Fractions were collected from the top of the tube and the concentrations of replicon RNAs and core proteins of each fraction were measured. HCV RNA and core protein were predominantly detected in the 1.15–1.20 g/ml fractions, with a peak fraction of 1.16 g/ml fraction (Fig. 2A). HCV-LPs were

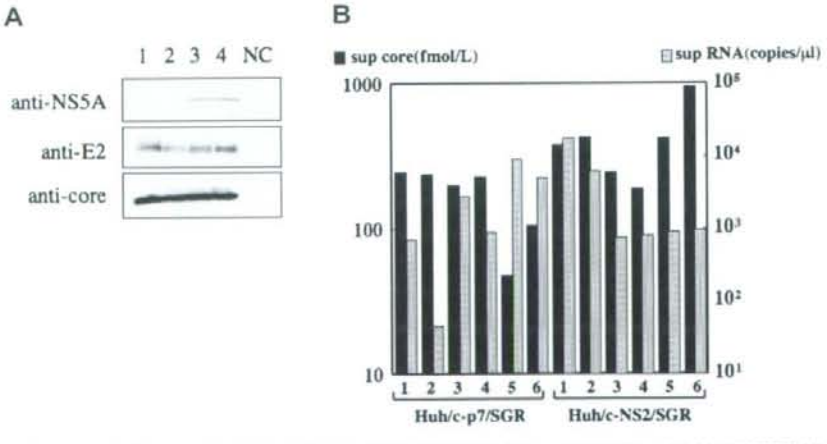

Fig. 1. (A) Western blot analysis of established cell lines. Huh/c-p7/SGR (1), Huh/c-NS2/SGR (2), Huh/c-p7/SGR-1 (3), and Huh/c-NS2/SGR-3 (4) cells were analyzed using anti-core, anti-E2, and anti-NS5A antibodies, respectively. Huh7 cells were used as a negative control. (B) Screening of G418-resistant cell lines. HCV replicon RNA and core protein of culture media of six colonies from Huh/c-p7/SGR or Huh/c-NS2/SGR cells were measured by real-time RT-PCR and ELISA, respectively. Black bars represented the concentration of core protein (fmol/l), dotted bars represented the concentration of replicon RNA (copies/μl).

observed by electron microscopy and these resembled previously reported particles (Fig. 2B)[27]. The secretion of HCV-LPs from these cell lines was maintained at almost the same level for more than 1 year (data not shown).

Infectivity of HCV-LPs

To determine whether these cell lines produced infectious HCV-LPs, we performed a colony formation assay using neomycin-resistant gene of SGR-JFH1 RNA. If HCV-LPs were infectious, SGR-JFH1 that was encapsidated in the particles would be introduced into infected cells, thus would confer neomycin resistance to the cells. To exclude the possibility that subgenomic replicon RNA in culture medium was captured by inoculated cells, Huh7 cells were also inoculated with concentrated culture medium of SGR-JFH1 cells. As shown in Fig. 3A, Huh7 cells infected with the fraction of Huh/c-NS2/SGR cells formed visible colonies 10–14 days after infection. Calculated colony forming units (cfu) of the culture medium of Huh/c-NS2/SGR cells were in the order of $5.54 \pm 2.92 \times 10^1$ cfu/ml similar to those of culture medium of JFH-1-infected cells [28]. The cells inoculated with concentrated medium of SGR-JFH1 cells formed no colonies (Fig. 3A). On the other hand, cells infected with Huh/c-p7/SGR formed no colonies, suggesting that NS2 protein was required for the infectivity of HCV-LPs. Infectivity of HCV-LPs from other cell lines of Huh/c-NS2/SGR, shown in Fig. 1, were also confirmed by colony formation assay, whereas HCV-LPs from other cell lines of Huh/c-p7/SGR showed no infectivity (data not shown).

In order to analyze the infectivity of the HCV-LPs, an immunofluorescence study was also performed. Huh7 cells infected with the Huh/c-NS2/SGR culture medium peak fraction (Fig. 2A) were positive for NS5A at 72 h postinfection (Fig. 3B), whereas the cells infected with the Huh/c-p7/SGR culture medium peak fraction were negative for NS5A (Fig. 3B), suggesting that the expression of NS2 protein in infected cells was critical for the infectivity of the HCV-LPs. The infectivity of the Huh/c-NS2/SGR culture medium was calculated to be $3.4 \pm 0.6 \times 10^2$ ffu/ml. The Cfu of this culture medium was determined to be approximately 16% of ffu, likely because only a portion of introduced replicon could render neomycin resistance to the infected cells. The cells infected with JFH-1 showed spread of infection 72 h postinfection. On the other hand, the cells infected with the Huh/c-NS2/SGR culture medium peak fraction showed very limited or no spread of infection (Fig. 3B). Moreover, no NS5A-positive cells were observed when we inoculated new Huh7 cells with the concentrated culture medium from Huh7 cells that were infected the Huh/c-NS2/SGR culture medium peak fraction (Fig. 3B, reinfection), suggesting that HCV-LPs produced by Huh/c-NS2/SGR cells supported only a single-round of infection.

We also measured the infectivity of the 12 sucrose density gradient fractions of the culture medium of Huh/c-NS2/SGR cells. The density of the peak of infectivity was lower than the peak densities of the core protein and replicon RNA (Fig. 2A), however this result agreed with a previous observation [29].

Neutralization of HCV-LPs infection by CD81-specific antibody

CD81 was shown to be involved in HCV entry. To determine whether HCV-LPs formed in Huh/c-NS2/SGR cells were infected in a CD81-dependent fashion, we incubated Huh7 cells with the peak fractions of Huh/c-NS2/SGR and Huh/c-p7/SGR cells in the presence of 10 μg/ml of CD81 specific monoclonal antibody or non-specific mouse antibody and cultured in the presence of 0.375 mg/ml of G418. After 3 weeks postinfection, colonies were fixed and the numbers of colonies were counted. CD81-specific antibody reduced the number of colonies from 132.3 ± 32.3 to 13.0 ± 11.5 ffu/

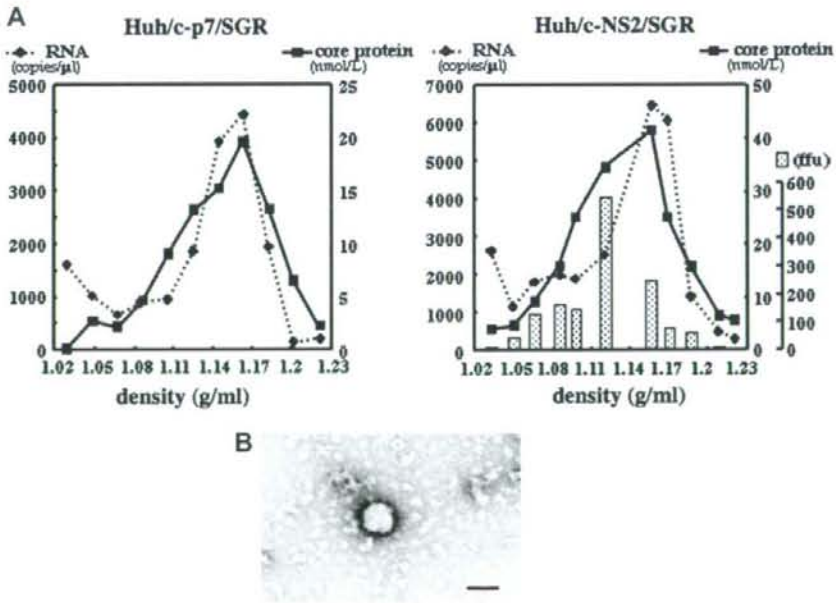

Fig. 2. (A) Sucrose density gradient analysis of culture supernatants of Huh/c-p7/SGR and Huh/c-NS2/SGR cells. Fifty milliliters of culture media collected from Huh/c-p7/SGR or Huh/c-NS2/SGR cells was concentrated to 1 ml and fractionated by ultracentrifugation at 40,000 rpm for 16 h by continuous 10–60% (wt/vol) sucrose gradient in PBS. Fractions (1 ml each) were collected from the top of the tube (12 fractions in total). HCV replicon RNA and core protein were measured by real-time RT-PCR and ELISA. The infectivity of each fraction of culture supernatant of Huh/c-NS2/SGR cells (right, lower panel) was determined by immunostaining of NS5A. (B) Electron microscopy analysis. Samples were prepared from the 1.12–1.20 g/ml fractions of culture media collected from Huh/c-NS2/SGR cells. Bar: 50 nm.

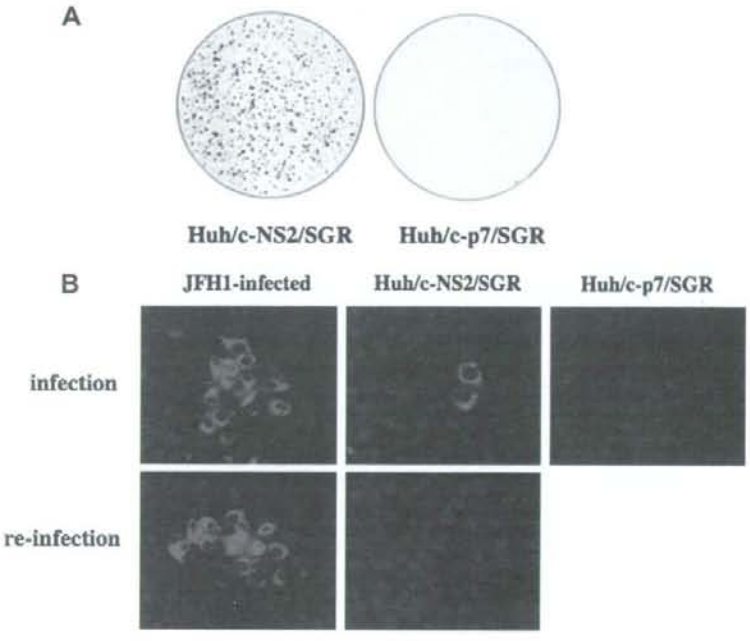

Fig. 3. (A) G418-resistant colony formation. Naive Huh7 cells were infected with 1.12–1.20 g/ml fractions of either Huh/c-p7/SGR or Huh/c-NS2/SGR cells and were cultured for 3 weeks with G418 at 0.375 mg/ml working concentration before staining with crystal violet. Experiments were performed in triplicate, and representative staining examples are shown. (B) Immunostaining experiments. Samples were serially diluted 5-fold in complete growth medium and used to infect the seeded cells (six wells per dilution). Huh7 cells in 96-well plates infected with the peak fraction of culture medium. Three days postinfection, infected cells were fixed, permeabilized with 0.3% Triton X-100 in Block Ace (Yukijirushi) and stained with anti-NS5A rabbit polyclonal antibody and Alexa488-conjugated goat anti-rabbit IgG as described previously (Murakami et al., in press). NS5A protein was shown in green. Nuclei were labeled with DAPI and were shown in blue. Re-infection shows the immunostaining of naive Huh7 cells infected with either culture media of JFH1-infected cells or that of Huh/c-NS2/SGR cells.

ml (Fig. 4), confirming that the infection of HCV-LPs to target cells is CD81-dependent and an important role of CD81 in HCV entry.

Discussion

Here we describe the development of cell lines selected to persistently harbor noncytopathic subgenomic replicons of HCV encoding neomycin resistant gene and the HCV core to NS2 cassette. The HCV-LPs secreted by this cell line are not proliferative and exhibit morphological, biophysical and antigenic properties similar to those of the putative HCV virions [27]. Jeong et al. suggested that HCV-LP is a potent immunogen for the induction of HCV-specific humoral and cellular immune responses by using baboon as a primate model [30]. Recently, replicon-based vectors of positive-stranded RNA viruses were recognized as a desirable choice of highly efficient and safe vaccines. Recent comparative analyses of vaccine potential of Kunjin virus replicons delivered as plasmid DNA, as naked RNA, and as VLPs showed a significantly better induction of immune responses to an encoded immunogen after VLP delivery than with other delivery modalities [31]. These studies suggested that HCV-LPs encapsidating its subgenomic replicon RNA are an attractive candidate for a hepatitis C vaccine. We are now constructing cell lines that secrete HCV-LPs of genotype 1a and 1b strains with this trans-packaging system and analyzing the HCV-LPs infectivity. We also showed that the expression of NS2 region is essential for infectious

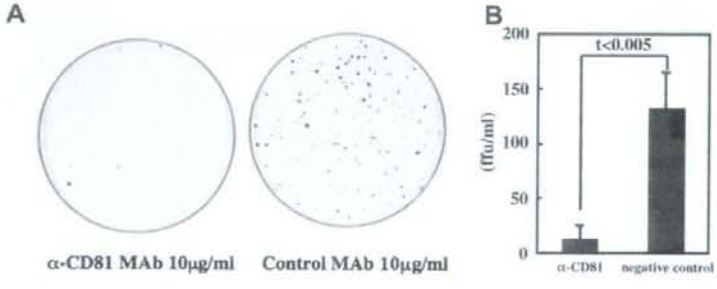

Fig. 4. Neutralization of HCV-LPs infection by CD81-specific antibody. Naive Huh7 cells were infected with peak fraction of either Huh/c-p7/SGR or Huh/c-NS2/SGR cells in the presence of 10 μg/ml of CD81 specific monoclonal antibody or nonspecific mouse antibody, then cultured 3 weeks with 0.375 mg/ml of G418. Colonies were stained with crystal violet and colony numbers were counted. (A) Colony formation. Experiments were performed in triplicate, and representative staining examples are shown. (B) Cfu of culture media per 1 ml was calculated and means ± SD was shown.

HCV-LPs production. NS2 is dispensable for RNA replication, since subgenomic replicons that lack the entire core to NS2 coding region replicate autonomously. The HCV NS2/3 protein is a highly hydrophobic protease responsible for the cleavage of the viral polypeptide between nonstructural proteins NS2 and NS3. However, many aspects of the NS2/3 protease's role in the viral life cycle and mechanism of action remain unknown. By using intergenotypic chimeras, Pietschmann et al. showed that NS2 plays an important role in the HCV morphogenesis by interacting with other NS proteins during the process of virion assembly [32]. Jones et al. reported that NS2 was required for infectious virus production and acts early in virion morphogenesis prior to the accumulation of infectious intracellular virus and indicated that the NS2 protease domain may form important interactions with other NS proteins during the process of virion assembly [33]. The results presented here also showed the importance of NS2 protein expression for the production of infectious particles, coincided with these previous observations. The mechanism NS2 plays in the process of virion morphogenesis is still unclear and remains to be determined.

In summary, we have generated a stable packaging cell line allowing production of large amounts of HCV-LPs in which the subgenomic replicon was encapsidated. The packaging cell line proved to be useful both for the production of HCV-LPs and for the encapsidation of HCV replicons for a single-round of infection.

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Research Note

Detection of Human Enteric Viruses in Japanese Clams

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ABSTRACT

A total of 57 clam packages that were collected from supermarkets and fish markets from 11 different sites in western Japan between 8 December 2005 and 6 September 2006 were examined for human enteric viruses (i.e., norovirus, Aichi virus, rotavirus, adenovirus, hepatitis A virus, and astrovirus), using PCR and reverse transcription PCR. Sixty-one percent of the packages were contaminated with one type of virus, 9% had two different types of viruses, 28% had three different types of viruses, and 9% had at least four different types of viruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses. Norovirus genogroup I and genogroup II sequences were detected in 24 and 23 packages, respectively, and these sequences belonged to nine genogroup I and eight genogroup II genotypes. Aichi viruses were found in 19 (33%) of 57 packages, and these belonged to genogroup A. Rotaviruses (group A) were detected in 14 (42%) of 33 of packages and 9 of 14 rotavirus-positive packages contained two or more rotavirus genogroup types. Adenoviruses (Ad40 and Ad41) were detected in 17 (52%) of 33 packages. One of the 57 (2%) packages was positive with hepatitis A virus (subtype IA). Astrovirus was not detected in any of the packages. This is the first study to detect such a high level of contamination in Japanese clams. These results represent an important finding because the Japanese clams were considered suitable for human consumption. Further studies are needed to determine the health risks associated with eating these highly contaminated clams.

Gastroenteritis is one of the leading causes of death by an infectious disease *(19)*, with more than 700 million cases of acute diarrheal disease occurring annually. The main viral agents that cause gastroenteritis are norovirus, rotavirus, sapovirus, astrovirus, and enteric adenoviruses. These viruses have been detected in environmental samples (e.g., lakes and sewage) as well as in foods such as oysters, clams, sandwiches, and raspberries. Other important viral agents that can accumulate in oysters and clams are hepatitis A virus (HAV) and hepatitis E virus (HEV) *(4, 17)*. The impact of viral contamination in the environment is evident in Japan, where outbreaks of norovirus oyster–associated gastroenteritis increases in winter, and this coincides with the oyster-harvesting season in winter *(21)*. The detection methods for these viruses in environmental samples and clinical specimens have greatly improved over the past 10 years and have provided a better understanding and distribution of these viruses.

The purpose of this study was to detect norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus in clam packages sold at supermarkets and fish markets, which were destined for human consumption in Japan, using PCR and reverse transcription PCR, and then describe the genetic diversity of the positive noroviruses.

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MATERALS AND METHODS

Clam samples. A total of 57 clam (*Corbicula japonica*) packages (30 to 60 clams per package) were collected from supermarkets or fish markets (nonexport) from 11 different geographically distinct sites in western Japan between 8 December 2005 and 6 September 2006. The clam packages were screened for norovirus, Aichi virus, rotavirus, adenovirus, HAV, and astrovirus using PCR and reverse transcription PCR. These 57 packages were previously screened for sapovirus *(9)*, and 46 of 57 packages were screened for HEV *(17)*.

Viral extraction. The clams were shucked, the digestive diverticulum removed by dissection on the day of harvest *(16)*, and then weighed and homogenized in nine times their weight of phosphate-buffered saline without magnesium or calcium. One gram of digestive diverticulum (10 to 15 clams per package) was homogenized with an Omni mixer (Omni International, Marietta, Ga.) in 10 ml of phosphate-buffered saline (pH 7.2). After centrifugation at 10,000 × *g* for 30 min at 4°C, the supernatant was layered onto 1 ml of 30% sucrose solution and ultracentrifuged at 154,000 × *g* for 3 h at 4°C. The pellet was resuspended in 140 μl of distilled water and stored at −80°C until use.

DNA and RNA extraction and reverse transcription. Viral DNA (for detection of adenovirus) was extracted from resuspended pellet, using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Viral RNA (for detection of norovirus, Aichi virus, rotavirus, and HAV) was extracted from the resuspended pellet, using QIAmp Viral RNA Mini Kit (Qiagen,). For reverse transcription, the RNA solution was treated with 2 U of RNase-free DNase I (Takara, Tokyo, Japan) for 30 min at 37°C, and was

TABLE 1. *Details of the clam samples*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
1	A	12/08/05	2	GI-Shijimi1 (GI/4), GII-Shijimi1 (GII/3)	Aic-1	G1/G4	−	−	−	−
2	D	12/10/05	−		−	−	+	−	−	−
3	A	12/22/05	2	GI-Shijimi2 (GI/1), GII-Shijimi2 (GII/2)	Aic-2	G9	+	−	−	−
4	D	12/17/05	−		−	G8	+	−	−	−
5	K	01/10/06	1	GII-Shijimi3 (GII/3)	Aic-3	NTa	NT	−	−	−
6	A	01/14/06	3	GI-Shijimi4 (GI/11), GII-Shijimi4a (GII/4), GII-Shijimi4b (GII/3)	Aic-4	G4	+	−	−	−
7	J	01/16/06	1	GI-Shijimi5 (GI/8)	Aic-5	NT	NT	−	−	−
8	A	01/20/06	2	GI-Shijimi6 (GI/14), GII-Shijimi6 (GII/3)	−	NT	NT	−	−	−
9	B	01/22/06	3	GI-Shijimi7 (GI/12), GII-Shijimi7a (GII/New), GII-Shijimi7b (GII/3)	Aic-6	G?	+	−	−	−
10	B	01/22/06	−		−	−	+	−	−	−
11	C	01/21/06	3	GI-Shijimi8 (GI/11), GII-Shijimi8a (GII/3), GII-Shijimi8b (GII/3), GII-Shijimi8c (GII/4), GII-Shijimi8d (GII/3)	−	−	−	−	−	−
12	D	01/24/06	3	GI-Shijimi9 (GI/8), GII-Shijimi9a (GII/3), GII-Shijimi9b (GII/4)	Aic-7	G?	+	−	−	−
13	C	01/26/06	3	GII-Shijimi10a (GII/3), GII-Shijimi10b (GII/3), GII-Shijimi10c (GII/3)	Aic-8	−	+	−	−	SaV-3
14	D	02/07/06	1	GI-Shijimi11 (GI/11)	Aic-18	NT	NT	−	+	−
15	B	02/05/06	4	GI-Shijimi12a (GI/1), GI-Shijimi12b (GI/1), GI-Shijimi12c (GI/8), GI-Shijimi12d (GI/11)	Aic-9	−	+	−	−	−
16	D	02/19/06	2	GI-Shijimi13 (GI/1), GII-Shijimi13 (GII/3)	Aic-10	G1/G8/G9	+	−	−	−
17	B	02/17/06	1	GII-Shijimi14 (GII/New)	−	G4	+	−	−	−
18	C	02/25/06	1	GII-Shijimi15 (GII/3)	Aic-11	G2/G4	−	−	−	−
19	D	03/01/06	7	GI-Shijimi16a (GI/11), GI-Shijimi16b (GI/8), GI-Shijimi16c (GI/4), GI-Shijimi16d (GI/8), GII-Shijimi16a (GII/3), GII-Shijimi16b (GII/4), GII-Shijimi16c (GII/4)	Aic-12	NT	NT	−	+	−
20	B	03/02/06	3	GI-Shijimi17 (GI/1), GII-Shijimi17a (GII/5), GII-Shijimi17b (GII/4)	Aic-19	−	+	−	−	−
21	C	03/10/06	2	GI-Shijimi18 (GI/8), GII-Shijimi18 (GII/3)	−	−	+	−	−	SaV-1
22	B	03/14/06	3	GI-Shijimi19a (GI/1), GI-Shijimi19b (GI/1), GII-Shijimi19 (GII/3)	Aic-13	−	+	−	−	−
23	A	03/14/06	−		−	NT	NT	−	−	−
24	E	03/13/06	−		−	NT	NT	−	−	−
25	E	03/14/06	−		−	NT	NT	−	−	−
26	B	03/15/06	2	GI-Shijimi20a (GI/5), GI-Shijimi20b (GI/14)	Aic-14	−	+	−	−	−
27	D	03/17/06	1	GI-Shijimi21 (GI/1)	−	G1/G2/G8	+	−	−	−
28	F	03/18/06	2	GI-Shijimi22 (GI/8), GII-Shijimi22 (GII/New)	−	G1/G2/G3/G8/G9	+	−	−	−
29	E	03/18/06	−		−	NT	NT	−	−	−
30	E	03/18/06	−		−	NT	NT	−	−	−

TABLE 1. *Continued*

Package no.	Site	Mo/day/yr	No. of norovirus genotypes	Norovirus strain name (genogroup/genotype)	Aichi virus	Rotavirus: G types	Adeno-virus	HAV	HEV (from *(17)*)	Sapovirus (from *(9)*)
31	G	03/18/06	1	GI-Shijimi23 (GI/1)	–	NT	NT	–	–	–
32	H	03/18/06	–		–	NT	NT	–	–	–
33	D	03/30/06	1	GII-Shijimi24 (GII/2)	Aic-15	G1/G8	–	–	–	–
34	B	04/07/06	3	GI-Shijimi25a (GI/2), GI-Shijimi25b (GI/8), GII-Shijimi25 (GII/4)	–	–	–	–	–	–
35	D	04/13/06	2	GI-Shijimi26 (GI/4), GII-Shijimi26 (GII/3)	Aic-16	G1/G2/G8	–	–	–	SaV-2
36	B	04/26/06	1	GII-Shijimi27 (GII/New)	–	G2/G4	–	–	–	–
37	A	05/16/06	5	GI-Shijimi28 (GI/New), GII-Shijimi28a (GII/4), GII-Shijimi28b (GII/6), GII-Shijimi28c (GII/7), GII-Shijimi28d (GII/9)	Aic-17	–	–	–	–	SaV-4
38	D	05/13/06	2	GI-Shijimi29 (GI/4), GII-Shijimi29 (GII/7)	–	NT	NT	1A	–	–
39	D	05/27/06	–		–	G8/G9	–	–	–	–
40	A	05/29/06	2	GI-Shijimi30 (GI/4), GII-Shijimi30 (GII/3)	–	–	–	–	–	–
41	I	06/14/06	–		–	–	–	–	–	–
42	D	06/16/06	–		–	G3	–	–	–	–
43	A	06/16/06	–		–	–	–	–	–	–
44	D	06/23/06	–		–	G?	–	–	–	–
45	B	07/05/06	–		–	–	–	–	–	–
46	D	07/06/06	–		–	–	–	–	–	–
47	A	07/13/06	–		–	NT	NT	–	–[b]	–
48	D	07/21/06	–		–	NT	NT	–	–[b]	–
49	B	07/21/06	–		–	NT	NT	–	–[b]	–
50	A	07/27/06	–		–	NT	NT	–	–[b]	–
51	A	08/06/06	–		–	NT	NT	–	–[b]	–
52	D	08/07/06	–		–	NT	NT	–	–[b]	–
53	B	08/10/06	–		–	NT	NT	–	–[b]	–
54	D	08/23/06	–		–	NT	NT	–	–[b]	–
55	I	09/04/06	–		–	NT	NT	–	–[b]	–
56	B	09/04/06	1	GI-Shijimi31 (GI/New)	–	NT	NT	–	–[b]	–
57	D	09/06/06	–		–	NT	NT	–	–[b]	–
Total				31/57	19/57	17/33	17/33	1/57	2/46	4/57

[a] NT, not tested.
[b] Tested in this study.

followed by the inactivation of the enzyme at 75°C for 5 min. Reverse transcription was performed with 15 μl of RNA solution and 15 μl of reverse transcription mixture that contained 1 mM dNTP mixture, 10 mM dithiothreitol, 0.75 μg of random hexamers (Takara), 33 U of RNase inhibitor (Takara), 300 U of reverse transcriptase Superscript II (Invitrogen, San Diego, Calif.), and 4.5 μl of Superscript II buffer (Invitrogen).

PCR. For the norovirus PCR, the primers were designed to amplify the 5′ end of the capsid gene *(10, 14)*. For norovirus genogroup I (GI), COG1F and G1SKR primers were used for the first PCR, and then G1SKF and G1SKR primers were used for the nested PCR. For norovirus genogroup II (GII), COG2F and G2SKR were used for the first PCR, and then G2SKF and G2SKR primers were used for the nested PCR. For the Aichi virus, C94b and 264K primers were used, and these were designed to amplify the 3C–D junction (protease-polymerase) *(26)*. For the rotavirus (group A), primers were designed to amplify the major outer capsid glycoprotein VP7, and the rotavirus type was determined by PCR size *(7)*. For the adenovirus, primers were designed to detect the E1B region of enteric adenoviruses, i.e., Ad40 and Ad41, and determined by PCR size *(1)*. For HAV, we used a set of nested in-house primers designed to amplify the capsid gene. For the first HAV PCR, we used sense HAV+2799 primer (5′-ATTCAGAT TAGACTGCCTTGGTA-3′) and antisense HAV-3273 primer (5′-CCAAGAAACCTTCATTATTTCATG-3′). For HAV nested PCR, we used sense HAV+2907 primer (5′-GCAAATTACAAT CATTCTGATGA-3′) and antisense HAV-3162 primer (5′-CTTC YTGAGCATACTTKARTCTTG-3′). The HAV PCR conditions were the same as those for the norovirus *(14)*. For the astrovirus, PreCAP1 and 12GR primers were used to amplify the first PCR product, and then Mon244 and 82b primers were used for nested PCR, which were designed to amply the 5′ end of the capsid gene *(18)*. Two types of positive controls and a virus-free negative control per five assays for norovirus PCR were used. All PCR prod-