TABLE 3. Detection of NLVs by real-time quantitative RT-PCR against NLV EM-negative stool specimens

Outbreak no.	Detection by TaqMan (no. positive/no. tested)[a]	Genogroup
8	0/2	
9	2/3	GII
14	3/3	GII
16	1/1	GI
19	7/8	GII
27	2/2	GII
28	1/3	GII
33	2/3	GII
34	1/2	GII
37[b]	1/1[c]	GI + GII
Total	20/28	

[a] All stool specimens were NLV negative by EM.
[b] From an oyster-related outbreak (February 1999) not included in Table 1.
[c] Stool code SzU.

KU19, and KU36) from five EM-positive outbreaks (outbreaks 9, 26, 30, 33, and 35) contained a mixture of both NLV GI and GII (Table 1).

The detection limits of NLV GI and GII RNA had Ct values of 36.6 and 35.3, respectively, corresponding to 10 copies per reaction tube. These Ct values corresponded to more than 2.0×10^4 copies of NLV RNA per g of stool specimen. For each gram of stool specimen in EM-positive specimens, Ct values of 17.7 to 30 and 14.1 to 23.8 corresponded to 1.4×10^{10} to 3.2×10^6 and 6.3×10^{10} to 9.2×10^7 copies/g of NLV GI and GII RNAs, respectively.

We also used our method to test EM-negative stool specimens from nine EM-positive outbreaks (outbreaks no. 8, 9, 14, 16, 19, 27, 28, 33 and 34) and one oyster-related but EM-negative outbreak (outbreak 37). Of 28 samples tested, 20 were NLV RNA positive by our method (Table 3). Ct values were 31.9 (9.6×10^5 copies/g) for NLV GI (SzU) and 21.0 to 33.3 (6.1×10^8 to 1.5×10^5 copies/g) for NLV GII.

Since the real-time quantitative RT-PCR method was designed to distinguish NLV GI and GII, we confirmed the genogroup by sequencing the capsid N/S region of the PCR products from the 70 stool specimens. Using these sequence data, we constructed the phylogenetic dendrogram and found that the genogroups resulting from the phylogenetic analysis matched completely with the results from the real-time RT-PCR method (the dendrograms for the majority of the strains sequenced were shown in our previous studies [19, 21]).

In this real-time quantitative RT-PCR, we observed no cross-reactions in stool specimens containing other enteric viruses, such as rotavirus, enterovirus, adenovirus, and poliovirus, or enteropathogenic bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Vibrio parahaemolyticus* (data not shown).

DISCUSSION

We have established a sensitive and broadly reactive NLV RNA detection system that uses real-time quantitative RT-PCR. The primers were based on the ORF1-ORF2 junction region, perhaps the most conserved region of the virus genome. Similarity plots (Fig. 1) and multiple alignment analysis of sequences in this region (Fig. 2) indicated a high degree of conservation in all isolates sequenced. Thus, it seemed an ideal site for designing primers for an RT-PCR-based amplification assay. The system does not require any other detection procedures, such as electrophoresis, Southern hybridization, nested PCR, or sequence analysis. Moreover, to prevent false-positives from carryover contamination, we treated all pre-PCR samples with UNG to destroy any dUTP-containing PCR products (24, 25, 29).

Because our method uses the TaqMan system, we could quantitatively detect NLV cDNA. Linearity was obtained by use of serially diluted standard DNA plasmids (10^7 copies to 10^1 copies) containing the ORF1-ORF2 junction sequence of each genogroup (Fig. 3). With serially diluted standard DNA plasmids, we found a detection limit of 10 copies of cDNA per reaction tube with a cutoff value of 35 to 37 cycles, indicating that our method is highly sensitive. For example, the slopes of the curves of threshold cycle (Ct) values versus serial 10-fold dilutions (range of 10^{10} to 10^1) for KU4 (GI), KU115 (GI), KU98 (GII), and U201 (GII) suspended stool specimen were −3.381, −3.411, −3.422, and −3.412 cycles/$\log_{10}$ unit, respectively. These slopes correlated well with the slopes of the curves of Ct versus serially diluted NLV GI (−3.4075 cycles/$\log_{10}$ unit) and GII (−3.4166 cycles/$\log_{10}$ unit) standard DNA plasmid ($P < 0.01$). We estimated the number of copies of NLV RNA in the original sample. Ct values of 17.0 (GI) and 15.3 (GII) are equivalent to 1×10^7 copies of standard plasmid in the reaction tube and correspond to about 2.0×10^{10} copies of NLV RNA per g of stool specimen. Ct values for stool codes U2, KU44, KU109, and KU111 of 20.1, 14.1, 16.7, and 23.8 corresponded to about 1.1×10^9, 6.3×10^{10}, 1.1×10^{10}, and 9.2×10^7copies of NLV RNA per g of stool specimen, respectively.

The numbers of copies of NLV RNA in positive stool specimens determined by the real-time quantitative RT-PCR were considerably higher than previous estimates of 10^5 to 10^6 copies by EM (17). The higher titers are probably due to the ability of RT-PCR to detect NLV RNAs from additional sources (e.g., from complete and incomplete virus particles and from amplifiable RNA inside the infected intestinal epithelial cells). Our findings are also consistent with those of others who found higher numbers of NLV RNA by PCR methods (2, 13) than by EM.

Given the greater sensitivity of our assay, we wanted to examine more carefully EM-negative samples from gastroenteritis subjects within the same NLV-positive outbreaks. Of 28 EM-negative specimens, 20 were positive for NLV RNA (Table 3). In our assay, EM-negative, RT-PCR-positive stool specimens contained 3 to 30% as much NLV RNA as the EM-positive stool specimens.

The one outlier in this analysis was stool code U32 (outbreak 10), which was positive by EM (i.e., showed an image resembling that of an NLV particle) but negative by all RT-PCR methods. We speculate that the reason might be the presence of an unidentified small round virus that is morphologically similar to NLVs by EM. Conventional RT-PCR methods did not detect other RNA viruses, such as hepatitis A virus, hepatitis E virus, poliovirus, Sapporo-like viruses, and Astro virus (data not shown). Further investigation is needed to explain the results for this sample.

Interestingly, 10 stool specimens from five EM-positive outbreaks (outbreaks 9, 26, 30, 33, and 35) (Table 1), and one stool specimen from outbreak 37 that was negative by EM but positive in our assay (Table 3), showed NLV GI and GII strains. All of these outbreaks occurred in restaurants, and we suspect that they have resulted from ingesting contaminated foods. Four outbreaks (outbreaks 26, 33, 35 and 37) were shellfish related. Similar results of coinfection have been reported previously (36). Oysters, cockles, and mussels have been implicated in food-borne outbreaks. Shellfish are filter-feeding organisms and can concentrate NLV particles from a contaminated habitat. Therefore, coinfection by multiple strains is possible in shellfish-related NLV outbreaks.

The high sensitivity of our method allowed us to detect NLVs that were undetectable by EM or other PCR methods. Our method may have applications in the detection of NLV pathogens in foods, such as oysters, as well as in the monitoring of the levels and seasonal differences of NLV contamination in sewage, river water, and seawater. Furthermore, our primer and probe sequences may be used in other detection methods such as the Light Cycler PCR technique. We believe that this method will be useful for routine diagnosis as well as for clarifying the epidemiology of NLV infections and thus for the public health control of this disease in the future.

ACKNOWLEDGMENTS

We thank Sakae Inouye (Otsuma Women's University, Tokyo, Japan) for critical review of the manuscript.

This work was supported by Research on Health Sciences focusing on Drug Innovation grant KH51048 from the Japan Health Sciences Foundation.

REFERENCES

1. **Ando, T., S. S. Monroe, J. R. Gentsch, Q. Jin, D. C. Lewis, and R. I. Glass.** 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. J. Clin. Microbiol. **33**:64–71.
2. **Atmar, R. L., F. H. Neill, J. L. Romalde, F. Le Guyader, C. M. Woodley, T. G. Metcalf, and M. K. Estes.** 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. Appl. Environ. Microbiol. **61**:3014–3018.
3. **Beller, M., A. Ellis, S. H. Lee, M. A. Drebot, S. A. Jenkerson, E. Funk, M. D. Sobsey, O. D. Simmons, S. S. Monroe, T. Ando, J. Noel, M. Petric, J. P. Middaugh, and J. S. Spika.** 1997. Outbreak of viral gastroenteritis due to a contaminated well. International consequences. JAMA **278**:563–568.
4. **Berg, D. E., M. A. Kohn, T. A. Farley, and L. M. McFarland.** 2000. Multistate outbreaks of acute gastroenteritis traced to fecal-contaminated oysters harvested in Louisiana. J. Infect. Dis. **181**(Suppl. 2):S381–S386.
5. **Caul, E. O., and H. Appleton.** 1982. The electron microscopical and physical characteristics of small round human fecal viruses: an interim scheme for classification. J. Med. Virol. **9**:257–265.
6. **De Leon, R., S. M. Matsui, R. S. Baric, J. E. Herrmann, N. R. Blacklow, H. B. Greenberg, and M. D. Sobsey.** 1992. Detection of Norwalk virus in stool specimens by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. J. Clin. Microbiol. **30**:3151–3157.
7. **Dingle, K. E., P. R. Lambden, E. O. Caul, and I. N. Clarke.** 1995. Human enteric Caliciviridae: the complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. J. Gen. Virol. **76**:2349–2355.
8. **Dowell, S. F., C. Groves, K. B. Kirkland, H. G. Cicirello, T. Ando, Q. Jin, J. R. Gentsch, S. S. Monroe, C. D. Humphrey, C. Slemp, et al.** 1995. A multistate outbreak of oyster-associated gastroenteritis: implications for interstate tracing of contaminated shellfish. J. Infect. Dis. **171**:1497–1503.
9. **Green, J., C. I. Gallimore, J. P. Norcott, D. Lewis, and D. W. Brown.** 1995. Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. J. Med. Virol. **47**:392–398.
10. **Green, S. M., K. E. Dingle, P. R. Lambden, E. O. Caul, C. R. Ashley, and I. N. Clarke.** 1994. Human enteric Caliciviridae: a new prevalent small round-structured virus group defined by RNA-dependent RNA polymerase and capsid diversity. J. Gen. Virol. **75**:1883–1888.
11. **Green, S. M., P. R. Lambden, E. O. Caul, and I. N. Clarke.** 1997. Capsid sequence diversity in small round structured viruses from recent UK outbreaks of gastroenteritis. J. Med. Virol. **52**:14–19.
12. **Gunn, R. A., W. A. Terranova, H. B. Greenberg, J. Yashuk, G. W. Gary, J. G. Wells, P. R. Taylor, and R. A. Feldman.** 1980. Norwalk virus gastroenteritis aboard a cruise ship: an outbreak on five consecutive cruises. Am. J. Epidemiol. **112**:820–827.
13. **Hafliger, D., M. Gilgen, J. Luthy, and P. Hubner.** 1997. Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood. Int. J. Food Microbiol. **37**:27–36.
14. **Inouye, S., K. Yamashita, S. Yamadera, M. Yoshikawa, N. Kato, and N. Okabe.** 2000. Surveillance of viral gastroenteritis in Japan: pediatric cases and outbreak incidents. J. Infect. Dis. **181**(Suppl. 2):S270–S274.
15. **Jiang, X., P. W. Huang, W. M. Zhong, T. Farkas, D. W. Cubitt, and D. O. Matson.** 1999. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J. Virol. Methods **83**:145–154.
16. **Jiang, X., M. Wang, K. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. Virology **195**:51–61.
17. **Kapikian, A. Z., and R. M. Chanock.** 1990. Norwalk group viruses, p. 671–693. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed., vol. 1. Raven Press, New York, N.Y.
18. **Kaplan, J. E., L. B. Schonberger, G. Varano, N. Jackman, J. Bied, and G. W. Gary.** 1982. An outbreak of acute nonbacterial gastroenteritis in a nursing home. Demonstration of person-to-person transmission by temporal clustering of cases. Am. J. Epidemiol. **116**:940–948.
19. **Katayama, K., H. Shirato-Horikoshi, S. Kojima, T. Kageyama, T. Oka, F. B. Hoshino, S. Fukushi, M. Shinohara, K. Uchida, Y. Suzuk, T. Gojobori, and N. Takeda.** 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology **299**:225–239.
20. **Kobayashi, S., T. Morishita, T. Yamashita, K. Sakae, O. Nishio, T. Miyake, Y. Ishihara, and S. Isomura.** 1991. A large outbreak of gastroenteritis associated with a small round structured virus among schoolchildren and teachers in Japan. Epidemiol. Infect. **107**:81–86.
21. **Kojima, S., T. Kageyama, S. Fukushi, F. B. Hoshino, M. Shinohara, K. Uchida, K. Natori, N. Takeda, and K. Katayama.** 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. J. Virol. Methods **100**:107–114.
22. **Lambden, P. R., E. O. Caul, C. R. Ashley, and I. N. Clarke.** 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science **259**:516–519.
23. **Lew, J. F., M. Petric, A. Z. Kapikian, X. Jiang, M. K. Estes, and K. Y. Green.** 1994. Identification of minireovirus as a Norwalk-like virus in pediatric patients with gastroenteritis. J. Virol **68**:3391–3396.
24. **Longo, M. C., M. S. Berninger, and J. L. Hartley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene **93**:125–128.
25. **McGoldrick, A., E. Bensaude, G. Ibata, G. Sharp, and D. J. Paton.** 1999. Closed one-tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. J. Virol. Methods **79**:85–95.
26. **Moe, C. L., J. Gentsch, T. Ando, G. Grohmann, S. S. Monroe, X. Jiang, J. Wang, M. K. Estes, Y. Seto, C. Humphrey, et al.** 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J. Clin. Microbiol. **32**:642–648.
27. **Nakayama, M., Y. Ueda, H. Kawamoto, Y. Han-jun, K. Saito, O. Nishio, and H. Ushijima.** 1996. Detection and sequencing of Norwalk-like viruses from stool samples in Japan using reverse transcription-polymerase chain reaction amplification. Microbiol. Immunol. **40**:317–320.
28. **Noel, J. S., T. Ando, J. P. Leite, K. Y. Green, K. E. Dingle, M. K. Estes, Y. Seto, S. S. Monroe, and R. I. Glass.** 1997. Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995. J. Med. Virol. **53**:372–383.
29. **Pellett, P. E., T. J. Spira, O. Bagasra, C. Boshoff, L. Corey, L. de Lellis, M. L. Huang, J. C. Lin, S. Matthews, P. Monini, P. Rimessi, C. Sosa, C. Wood, and J. A. Stewart.** 1999. Multicenter comparison of PCR assays for detection of human herpesvirus 8 DNA in semen. J. Clin. Microbiol. **37**:1298–1301.
30. **Pether, J. V., and E. O. Caul.** 1983. An outbreak of food-borne gastroenteritis in two hospitals associated with a Norwalk-like virus. J. Hyg. **91**:343–350.
31. **Saito, H., S. Saito, K. Kamada, S. Harata, H. Sato, M. Morita, and Y. Miyajima.** 1998. Application of RT-PCR designed from the sequence of the local SRSV strain to the screening in viral gastroenteritis outbreaks. Microbiol. Immunol. **42**:439–446.
32. **Schreier, E., F. Doring, and U. Kunkel.** 2000. Molecular epidemiology of outbreaks of gastroenteritis associated with small round structured viruses in Germany in 1997/98. Arch. Virol. **145**:443–453.
33. **Schvoerer, E., F. Bonnet, V. Dubois, A. M. Rogues, J. P. Gachie, M. E. Lafon, and H. J. Fleury.** 1999. A hospital outbreak of gastroenteritis possibly related to the contamination of tap water by a small round structured virus. J. Hosp. Infect. **43**:149–154.

34. **Seah, E. L., J. A. Marshall, and P. J. Wright.** 1999. Open reading frame 1 of the Norwalk-like virus Camberwell: completion of sequence and expression in mammalian cells. J. Virol **73**:10531–10535.
35. **Stevenson, P., R. McCann, R. Duthie, E. Glew, and L. Ganguli.** 1994. A hospital outbreak due to Norwalk virus. J. Hosp. Infect. **26**:261–272.
36. **Sugieda, M., K. Nakajima, and S. Nakajima.** 1996. Outbreaks of Norwalk-like virus-associated gastroenteritis traced to shellfish: coexistence of two genotypes in one specimen. Epidemiol. Infect. **116**:339–346.
37. **Vinje, J., and M. P. Koopmans.** 1996. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. J. Infect. Dis. **174**:610–615.
38. **Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, et al.** 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. **68**:5982–5990.
39. **Yamazaki, K., M. Oseto, Y. Seto, E. Utagawa, T. Kimoto, Y. Minekawa, S. Inouye, S. Yamazaki, Y. Okuno, and I. Oishi.** 1996. Reverse transcription-polymerase chain reaction detection and sequence analysis of small round-structured viruses in Japan. Arch. Virol. Suppl. **12**:271–276.

Microbiol. Immunol., 48(3), 201–204, 2004

Immunomagnetic Capture RT-PCR for Detection of Norovirus from Foods Implicated in a Foodborne Outbreak

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Received October 20, 2003; in revised form, December 5, 2003. Accepted December 15, 2003

***Abstract*: In February 2001, an outbreak of acute gastroenteritis due to Norovirus (NV) occurred among employees of 11 companies in Aichi Prefecture. The illness was strongly associated with eating a delivered box-lunch. The use of magnetic beads coated with the antibody to the baculovirus-expressed recombinant capsid proteins of the Chiba virus (rCV) facilitated capture of NV from the food items implicated in the outbreak. Following immunomagnetic capture, NV bound to the beads was detected by reverse transcription-polymerase chain reaction (RT-PCR). Of the nine food items tested, two were positive for a genogroup 1 NV. Sequence analysis of RT-PCR products indicated that the nucleotide sequences of NV strains from foods were almost identical to those of NV strains detected in stool samples of ill patients. As the immunocapture RT-PCR method is simple and easy to perform, this technique should be useful for the detection of NV from outbreak-implicated foods.**

***Key words*: Norovirus, Immunomagnetic beads, RT-PCR**

Norovirus (NV), previously called as "Norwalk-like virus," is a genetically diverse group of viruses that belong to the family *Caliciviridae* (4, 11). NV has been recognized as a major cause of outbreaks of acute non-bacterial gastroenteritis (2, 12, 14, 15). NV-associated gastroenteritis is transmitted by fecally contaminated water, food and person-to-person contact (8). In Japan, NV outbreaks resulting from consumption of raw oysters are common, but large-scale foodborne outbreaks are frequently caused by school lunches and catered meals served at schools, banquet halls and hospitals (6). As there is no tissue culture system for NVs, reverse transcription-polymerase chain reaction (RT-PCR) has been developed for specific and sensitive detection of NV (1, 7). RT-PCR has been successfully used for the detection of NV in clinical samples, but the detection of NV in contaminated foods other than oysters has been difficult due to the low levels of viral contamination (3, 5, 13). Several steps for the purification and the concentration of the virus from food samples are usually required to obtain a suitable volume for RT-PCR. However, the possibilities of viral loss and cross-contamination among samples are increased with multiple processing steps.

In this study, immunomagnetic beads was used to capture and concentrate the virus from the contaminated foods prior to RT-PCR for the detection of NV. This method was applied to detect NV from foods implicated in a non-oyster associated foodborne outbreak. In February 2001, an outbreak of acute gastroenteritis occurred among employees of 11 companies in Aichi Prefecture. Overall, 52 (7.1%) of 736 company employees developed gastrointestinal illness 20 to 58 hr (median=42 hr) after eating a delivered box-lunch on February 7. The main symptoms reported by the 52 patients were abdominal pain (71.2%), diarrhea (51.9%), fever (40.4%), nausea (30.8%), and vomiting (26.9%). Stool samples were obtained from nine ill employees, then tested for NV by RT-PCR and antigen ELISA.

Viral RNA was extracted from the samples with TRIzol reagent (Invitrogen) and the RNA was converted to complementary DNA using a random primer, oligo (dT) primer and reverse transcriptase, as described previously (10). PCR was performed using 2 primer sets,

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; NV, Norovirus; PBS-BSA, phosphate buffered saline containing 0.1% bovine serum albumin; rCV, recombinant capsid proteins of Chiba virus; RT-PCR, reverse transcription-polymerase chain reaction.

G1F1/G1R1 and G2F1/G2R1, which preferentially amplify partial capsid genes of genogroup 1 and genogroup 2 NV strains, respectively (10). PCR was run for 35 cycles of 94 C (30 sec), 62 C (30 sec) and 72 C (30 sec), with a final elongation at 72 C for 7 min.

The stool samples were assayed for the presence of Chiba virus antigen using ELISA according to the previous method (9). Rabbit antibody to the baculovirus-expressed recombinant capsid proteins of Chiba virus (rCV) was used as the capture, and guinea pig antiserum to rCV was used as the detector. Two wells were used for each sample, one coated with rabbit pre-immune serum (*N*) and the other with rabbit post-immune serum (*P*). The reaction was visualized using horseradish peroxidase-conjugated rabbit anti-guinea pig antibody, *o*-phenylenediamine and H_2O_2. $P/N>1.89$ (Absorbance with rabbit post-immune serum/Absorbance with rabbit pre-immune serum) and $P-N>0.10$ was considered to be positive.

Epidemiological investigation showed that a box-lunch catered by a delivery company was likely the common source of the outbreak. Nine leftover food items implicated in the outbreak were collected from the delivery company. Food samples were processed for recovery of NV by the immunomagnetic capture method. Rabbit antibody to rCV was prepared as described previously (9). Magnetic beads M-280 (Dynal, Oslo, Norway) were coated with rabbit antibody to rCV according to the manufacturer's instructions. Five mg of anti-Chiba virus antibody was used to coat 10^7 beads for 2 hr at room temperature. Coated beads were washed four times with phosphate buffered saline (PBS) pH 7.2 containing 0.1% bovine serum albumin (PBS-BSA), and the beads were suspended at a concentration of 1 mg/ml in PBS-BSA. Eight ml of PBS was added to about 15 g of each individual food item and mixed well. After centrifugation at 5,000 rpm for 10 min, the supernatant was retained. The obtained solution was treated with 4 ml of Freon and centrifuged at 5,000 rpm for 10 min. The aqueous phase was collected. Fifty μl of immunomagnetic beads were added to the food extract solution and incubated on a roller at 37 C for 1 hr. After magnetizing the beads, the supernatant was removed. The beads were washed three times with PBS-BSA and the washed beads were suspended in 100 μl of distilled water. RNA extraction from the beads and the subsequent RT-PCR were performed in the same way as from the stool samples (see above). Second-round amplification was performed using 0.5 μl of the first-round amplicon and the 2 primer sets, G1F2/G1R1 and G2F2/G2R1, for genogroup 1 and genogroup 2 viruses, respectively (10). PCR was run for 30 cycles of 94 C (30 sec), 55 C (30 sec) and 72 C (1 min), with a final elongation at 72 C for 7 min. The nucleotide sequences of RT-PCR amplified products were analyzed according to the previous method (9).

Table 1. Detection of *Norovirus* in stool samples from patients by RT-PCR and ELISA

Patient No.	PCR		ELISA
	G1F1/R1	G2F1/R1	
1	−	−	−
2	+	−	+
3	+	−	+
4	−	−	−
5	+	−	+
6	+	−	+
7	+	−	+
8	−	−	−
9	+	−	+

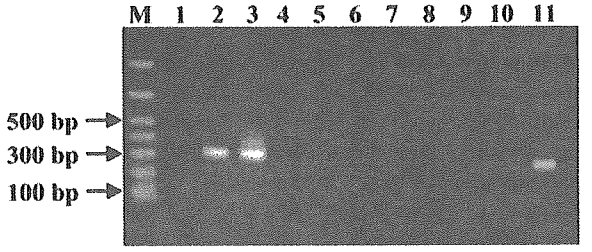

Fig. 1. Detection of *Norovirus* from foods implicated in a food-borne outbreak by immunomagnetic capture RT-PCR. M, 100 bp ladder marker; 1, fried tofu; 2, rolled cabbage; 3, macaroni; 4, devil's-tongue jelly; 5, baked tofu; 6, cabbage; 7, cooked bamboo shoot; 8, cucumber; 9, cooked radish; 10, negative control; 11, positive control.

The stool samples collected from gastroenteritic patients were assayed for NV by RT-PCR and antigen ELISA (Table 1). Six of the nine stool samples were positive for NV by RT-PCR, and all the detected NV belonged to genogroup 1. These six PCR-positive samples were also positive according to serotype-specific ELISA for Chiba virus. All stool samples were negative for pathogenic bacteria such as *Salmonella, Shigella, Campylobacter, Vibrio, Listeria, Yersinia, Escherichia coli, Bacillus cereus*, and *Staphylococcus aureus*.

Since NV strains from patients reacted with the antibody to rCV, immunobeads coated with the antibody to rCV were prepared to recover NV from the leftover food items implicated in the outbreak. After washing the food samples with PBS, the immunobeads were added to the extracted PBS solution of foods to capture NV. NV bound to the beads was detected by nested RT-PCR. Of the nine food items examined, two samples were positive for NV by RT-PCR (Fig. 1).

In our preliminary experiments, the two immunocapture RT-PCR-positive food samples were also positive

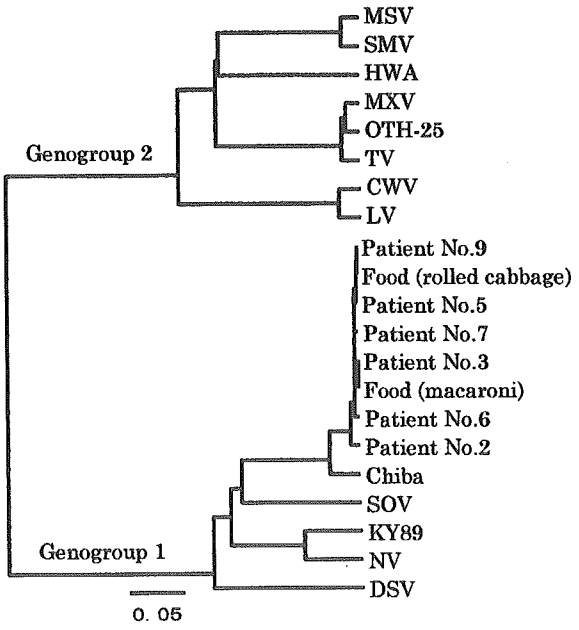

Fig. 2. Phylogenetic relationship of the outbreak strains and reference strains based on the partial capsid gene. Nucleotide sequences were aligned using Genetyx, and a phylogenetic tree was constructed by unweighted pair-group with arithmetic mean method. GenBank accession numbers of the reference strains were as follows: DSV, Desert Shield virus, U04538; NV, Norwalk virus, M87661; KY89, L23827; SOV, Southampton virus, L070418; Chiba, AB022679; LV, Lordsdale virus, X86557; CWV, Camberwell virus, U46500; TV, Toronto virus, U02030; OTH-25, L23830; MXV, Mexico virus, U22498; HWA, Hawaii virus, U07611; SMV, Snow Mountain virus, U70059; and MSV, Melksham virus, X81879. The bar indicates the evolutionary distance.

according to the conventional NV detection method including ultracentrifuge concentration and subsequent RT-PCR, but the conventional method produced many extra bends (data not shown). The immunomagnetic capture method has several advantages over the conventional methods: Manipulations are simple, special equipment is not required, the processing time is shorter, RT-PCR interfering substances are reduced, and testing of many food samples is possible.

The amplified products were cloned and sequenced. The sequences of the NV strains from foods were compared with those obtained from NV strains in stool samples. The nucleotide sequences of NV strains from the two food items and the nine ill patients were closely related to each other (96 to 99% nucleotide sequence identities). The phylogenetic analysis classified the NV strains from food items and patients into the Chiba virus cluster of genogroup I (Fig. 2).

We could not test for NV infection of food handlers in the delivery company. It is unclear whether food items were contaminated through an NV-infected food handler during the preparation of box-lunch.

In this study, we were able to identify the cause and source of illness using immunomagnetic capture RT-PCR and sequencing of NV strains in food items and in stool samples. The immunomagnetic capture RT-PCR is thus shown to be a rapid and simple method for recovery of NV from foods and identification of NV. This method will give useful information about the mode of food contamination and NV transmission in foodborne outbreaks.

References

1) DeLeon, R., Matsui, S.M., Baric, R.S., Herrmann, J.E., Blacklow, N.R., Greenberg, H.B., and Sobsey, M.D. 1992. Detection of Norwalk virus in a stool specimen by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. J. Clin. Microbiol. **30**: 3151–3157.
2) Fankhauser, R.L., Noel, J.S., Monroe, S.S., Ando, T., and Glass, R.I. 1998. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. J. Infect. Dis. **178**: 1571–1578.
3) Green, J., Wood, K.H., Gallimore, C.I., Brown, D.W.G., and Lees, D.N. 1998. A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish. Appl. Environ. Microbiol. **64**: 858–863.
4) Green, K.Y., Ando, T., Balayan, M.B., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neil, J.D., Studdert, M.J., and Thiel, H.-J. 2000. Taxonomy of the caliciviruses. J. Infect. Dis. **181** (Suppl 2): S322–330.
5) Guyader, F.L., Neill, F.H., Estes, M.K., Monroe, S.S., Ando, T., and Atmar, R.L. 1996. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute Gastroenteritis. Appl. Environ. Microbiol. **62**: 4268–4272.
6) Inoue, S., Yamashita, K., Yoshikawa, M., Kato, N., and Okabe, N. 2000. Surveillance of viral Gastroenteritis in Japan: Pediatric cases and outbreak incidents. J. Infect. Dis. **181** (Suppl 2): S270–274.
7) Jiang, X., Wang, J., Graham, D.Y., and Estes, M.K. 1992. Detection of Norwalk virus in stool by polymerase chain reaction. J. Clin. Microbiol. **30**: 2529–2534.
8) Kapikian, A., Estes, M., and Chanock, M. 1996. Norwalk group viruses, p. 783–810. *In* Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Monath, T.P., Roizman, B., and Straus, S.E. (eds), Fields virology, 3rd ed, Lippincott Williams & Wilkins, Philadelphia.
9) Kobayashi, S., Sakae, K., Natori, K., Takeda, N., Miyamura, T., and Suzuki, Y. 2000. Serotype-specific antigen ELISA for detection of chiba virus in stools. J. Med. Virol. **62**: 233–238.
10) Kobayashi, S., Sakae, K., Suzuki, Y., Ishiko, H., Kamata, K., Suzuki, K., Natori, K., Miyamura, T., and Takeda, N. 2000. Expression of recombinant capsid proteins of Chitta virus, a genogroup II Norwalk virus, and development of an ELISA to detect the viral antigen. Microbiol. Immunol. **44**: 687–693.
11) Mayo, M.A. 2002. A summary of taxonomic changes recent-

ly approved by ICTV. Arch. Virol. **147**: 1655–1656.
12) Norcott, J.P., Green, J., Lewis, D., Estes, M.K., Barlow, K.L., and Brown, D.W.G. 1994. Genomic diversity of small round structured virus in the United Kingdom. J. Med. Virol. **44**: 280–286.
13) Sugieda, M., Nakajima, K., and Nakajima, S. 1996. Outbreaks of Norwalk-like virus-associated gastroenteritis traced to shellfish: coexistence of two genotypes in one specimen. Epidemiol. Infect. **116**: 339–346.
14) Vinje, J., and Koopmans, M.P.G. 1996. Molecular detection and epidemiology of small round structured viruses in outbreaks of gastroenteritis in the Netherlands. J. Infect. Dis. **174**: 610–615.
15) Wright, P.J., Gunesekere, I.C., Doultree, J.C., and Marshall, J.A. 1998. Small round-structured (Norwalk-like) viruses and classical human calicivirus in southeastern Australia. J. Med. Virol. **55**: 312–320.

JOURNAL OF VIROLOGY, Mar. 2003, p. 3542–3548 Vol. 77, No. 6
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.6.3542–3548.2003

The 5′-End Sequence of the Genome of Aichi Virus, a Picornavirus, Contains an Element Critical for Viral RNA Encapsidation

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Received 22 April 2002/Accepted 6 December 2002

Picornavirus positive-strand RNAs are selectively encapsidated despite the coexistence of viral negative-strand RNAs and cellular RNAs in infected cells. However, the precise mechanism of the RNA encapsidation process in picornaviruses remains unclear. Here we report the first identification of an RNA element critical for encapsidation in picornaviruses. The 5′ end of the genome of Aichi virus, a member of the family *Picornaviridae*, folds into three stem-loop structures (SL-A, SL-B, and SL-C, from the most 5′ end). In the previous study, we constructed a mutant, termed *mut*6, by exchanging the seven-nucleotide stretches of the middle part of the stem in SL-A with each other to maintain the base pairings of the stem. *mut*6 exhibited efficient RNA replication and translation but formed no plaques. The present study showed that in cells transfected with *mut*6 RNA, empty capsids were accumulated, but few virions containing RNA were formed. This means that *mut*6 has a severe defect in RNA encapsidation. Site-directed mutational analysis indicated that as the mutated region was narrowed, the encapsidation was improved. As a result, the mutation of the 7 bp of the middle part of the stem in SL-A was required for abolishing the plaque-forming ability. Thus, the 5′-end sequence of the Aichi virus genome was shown to play an important role in encapsidation.

Picornaviruses, including human pathogens such as poliovirus, rhinoviruses, and hepatitis A virus (HAV), are small nonenveloped, icosahedral viruses. Their genomes are single-stranded, positive-sense RNAs of 7,200 to 8,500 nucleotides, and each encodes a single long polyprotein (22). After virus cell entry, the genomic RNA released into the cytoplasm serves as a mRNA for the synthesis of polyproteins that are processed to functional proteins by virus-encoded proteases. The genomic RNA also acts as a template for negative-strand RNAs which, in turn, are transcribed into positive strands. The synthesized positive-strand RNAs are encapsidated to form virions.

Picornavirus positive-strand RNAs are selectively encapsidated despite the coexistence of viral negative-strand RNAs and cellular RNAs in infected cells. The mechanism of the RNA encapsidation process is not well understood. The RNA sequence essential for encapsidation or for interaction with capsid proteins during encapsidation has not been identified in picornaviruses. However, studies on poliovirus have provided some clues for the identification of determinants of specific encapsidation. Poliovirus RNA in which the capsid-coding region is replaced with a foreign gene can be encapsidated by capsid proteins that are provided in *trans* by a helper virus (4, 5, 20). In addition, chimeric polioviruses harboring a different picornaviral 5′ untranslated region (5′-UTR) or 2A protein-coding region are viable (16, 26). These observations suggest that neither the poliovirus 5′-UTR, the capsid-coding region nor the 2A-coding region contains a signal essential for specific encapsidation. Based on the finding that only newly synthesized positive-strand RNAs are encapsidated, it is proposed that viral RNA replication and encapsidation are functionally coupled to each other and that the specific interaction between the RNA replication complex and the assembling capsids determines the specificity of poliovirus RNA encapsidation (17).

Aichi virus, which is associated with acute gastroenteritis in humans (27), is a new member of the family *Picornaviridae* (28). Computer-based secondary-structure prediction suggested the presence of three stem-loop structures (SL-A, SL-B, and SL-C) within the 5′-end 120 nucleotides of the Aichi virus genome (Fig. 1A). In the previous study, we investigated the function of the most 5′-end stem-loop structure (SL-A) by using various site-directed mutants derived from an infectious cDNA clone, and we showed that SL-A is an element required for viral RNA replication (24). One (*mut*6) of the mutants showed an interesting property. In *mut*6, the 7-nucleotide stretches of the middle part of the stem were exchanged with each other to maintain the base pairings of the stem (Fig. 1B). *mut*6 exhibited efficient RNA replication ability in transfected cells but formed no plaques (24). This finding suggested two possibilities; one is that *mut*6 RNA is not encapsidated, and the other is that *mut*6 RNA can be encapsidated, but the mutant has a defect in a certain early step of the infection cycle.

In the present study, we showed that *mut*6 has a severe defect in encapsidation, and we precisely mapped the region in SL-A relevant to encapsidation by characterizing several more mutants bearing changes in the middle part of SL-A. As a result, it was indicated that the 7 bp of the middle part of the stem of SL-A at the 5′ end of the Aichi virus genome are critical for viral RNA encapsidation. This is the first report of the identification of an RNA element critical for encapsidation in picornaviruses.

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MATERIALS AND METHODS

Cell culture and viruses. Vero cells were grown in Eagle minimal essential medium (MEM) containing 5% fetal calf serum (growth medium) at 37°C. As

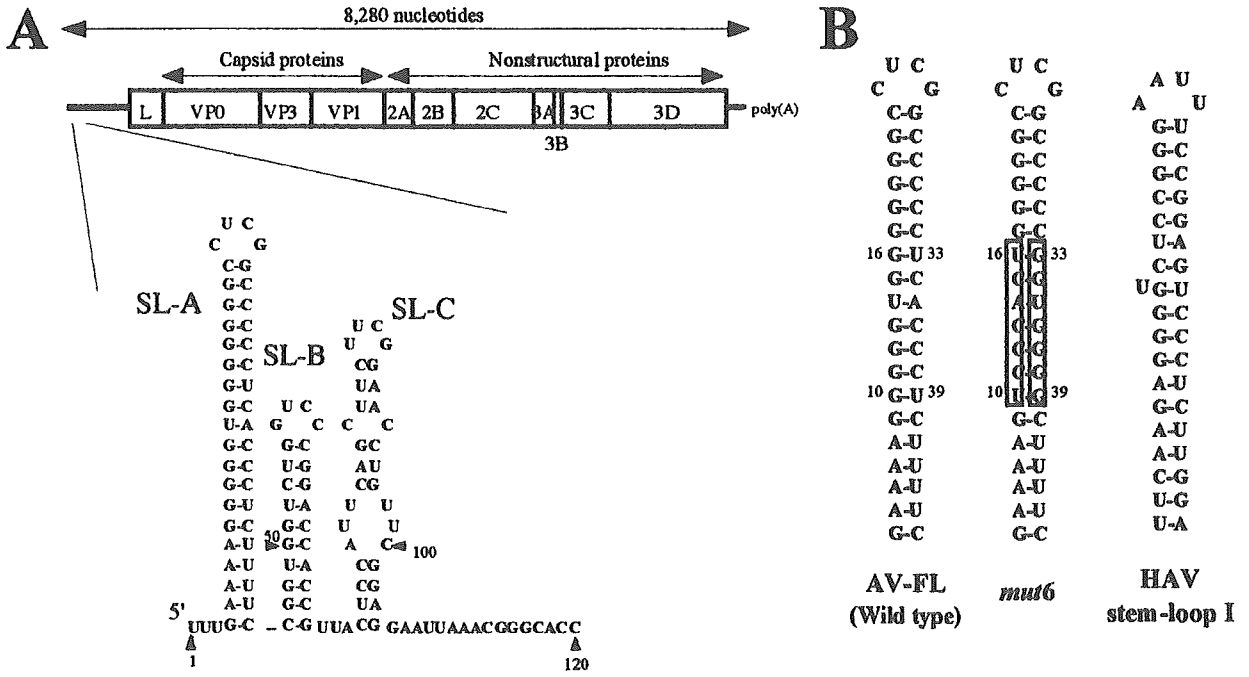

FIG. 1. (A) Schematic diagram of the Aichi virus genome and the predicted secondary structure of the 5′-end 120 nucleotides of the genome. The open box and bold lines indicate coding and noncoding regions, respectively. Vertical lines within the box represent putative cleavage sites for viral proteinase. The three stem-loop structures are termed SL-A, SL-B, and SL-C. (B) Diagrams of SL-A of AV-FL, *mut*6, and the stem-loop structure (stem-loop I) formed at the 5′ end of the HAV genome. Mutated nucleotides in *mut*6 are boxed.

wild-type Aichi virus, we used viruses derived from Vero cells transfected with in vitro transcripts synthesized from a full-length cDNA clone, pAV-FL (24). Poliovirus Sabin 1, as a reference virus, was propagated in Vero cells.

Mutants of pAV-FL. *mut*6 was constructed in the previous study (24). Other mutants were created by PCR-based site-directed mutagenesis as described previously (24). The sequences of the primers used were as follows: for 11-15/34-38, 5′-CGGCCCCCTGTGGGTCTTTTCCGGTGGTCT and 5′-AGGCCCCCCGTGGGCCTTTTCAAACCTATAGTGA; for 12-14/35-37, 5′-CGGCCCCCTCTGGCTCTTTTCCGGTGGTCT and 5′-AGGCCCCCCCTGGCCCTTTTCAAACCTATAGTGA; for 11-16/33-38, 5′-CGGCCCCCGGTGGGTCTTTTCCGGTGGTCT and 5′-AGGCCCCCAGTGGGCCTTTTCAAACCTATAGTGA; and for 10-15/34-39, 5′-CGGCCCCCTGTGGGGCTTTTCCGGTGGTCT and 5′-AGGCCCCCCGTGGGACTTTTCAAACCTATAGTGA.

In vitro transcription. pAV-FL and its mutants were linearized by digestion with *Hin*dIII, and RNA transcripts were synthesized with T7 RNA polymerase as described previously (24).

Electroporation. RNA transcripts (20 μg) were electroporated into 10^7 Vero cells by using a Gene Pulser (Bio-Rad) as described previously (24).

Preparation of radiolabeled viruses and sucrose gradient analysis. A Vero cell monolayer in a 100-mm plate was incubated with viruses at a multiplicity of infection of 5 to 10 for 1 h at 37°C. The inoculum was removed, and the cells were incubated in growth medium for an additional 2 h. For preparation of ^{35}S-labeled viruses from RNA transcripts, Vero cells were transfected with RNAs by electroporation, and then the cells were incubated with growth medium in a 100-mm plate for 2 h. The cells were then washed with phosphate-buffered saline (PBS) two times, and 3.7 MBq of L-[^{35}S]methionine (Amersham Pharmacia) in 2 ml of MEM without methionine was added. After incubation for 4 h, the cells were lysed with Nonidet P-40 and sodium deoxycholate at final concentrations of 1% (vol/vol) and 0.5% (wt/vol), respectively. Cell debris was pelleted, and the supernatant was centrifuged through a 30% sucrose cushion at 30,000 rpm for 3 h in a P40ST rotor (Hitachi). The pelleted viral particles were resuspended in PBS and sedimented through a 10 to 30% sucrose gradient at 39,000 rpm for 80 min in a P40ST rotor. The gradients were fractionated, and the radioactivity in each fraction was counted with a liquid scintillation counter.

For preparation of ^{3}H-labeled viruses, a Vero cell monolayer in a 100-mm plate was treated with 1.25 μg of actinomycin D/ml for 30 min before infection. Virus infection was performed as described above, followed by incubation in growth medium containing 1.25 μg of actinomycin D/ml for 2 h. After incubation, the medium was replaced with 0.5 ml of growth medium containing 1.25 μg of actinomycin D/ml and 3.7 MBq of [5,6-^{3}H]uridine (Amersham Pharmacia). After additional incubation for 4 h, the cells were lysed, and ^{3}H-labeled viruses were prepared by sucrose gradient centrifugation as described above.

Immunofluorescence staining. Three sets of serial dilutions of a lysate of cells harvested at 6 h after electroporation with AV-FL or *mut*6 RNAs were prepared. For one set of serial dilutions, RNase A was added at the concentration of 20 μg/ml. For the other two sets of serial dilutions, either guinea pig antiserum raised against purified Aichi virus particles or preimmune serum at a dilution of 1/30 and RNase A were added. These lysates were incubated at 37°C for 1 h. The treated lysates were added into Vero cells and, after incubation at 37°C for 1 h, the cells were washed with MEM two times and then cultured in growth medium. At 6 and 24 h after infection, the medium was discarded, and the cells were fixed with cold (−80°C) methanol for 10 min. After removal of the methanol, the cells were incubated with guinea pig antiserum raised against purified Aichi virus particles for 1 h at 37°C. The cells were washed with PBS and then incubated with fluorescein isothiocyanate-conjugated goat anti-guinea pig immunoglobulin G for 1 h at 37°C. The cells were washed with PBS and then viewed under a fluorescence microscope.

Dot blot hybridization. RNA transcripts were transfected into Vero cells by electroporation. Total RNA was extracted from the transfected cells by using Trizol reagent (Life Technologies, Inc.) at 3, 6, 9, 12, and 24 h after electroporation. Dot blot hybridization was carried out to detect plus-strand viral RNA as described in a previous study (24).

Western blotting. Vero cells electroporated with RNAs were lysed at 3 and 9 h after electroporation, and then the lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene difluoride membrane, and then immunoblot analysis with antiserum against virus particles was performed as described previously (24).

Titration of viable viruses in cells transfected with RNA transcripts. Vero cells electroporated with RNAs were frozen at 3, 6, 9, 12, and 24 h postelectroporation. The cells were disrupted by three consecutive freeze-thaw cycles, and the lysates were used for the plaque assay. The number of plaques was determined at 72 h after infection.

RESULTS

***mut6* has a defect in encapsidation.** We previously examined the function of SL-A by using various site-directed mutants derived from an infectious cDNA clone of Aichi virus, pAV-FL. *mut*6 was constructed by exchanging the seven-nucleotide stretches (nucleotide numbers 10 to 16 and 33 to 39) of the middle part of the stem of SL-A with each other to maintain the base pairings of the stem (Fig. 1B). It was found that *mut*6 exhibited efficient RNA replication ability in transfected cells but had lost the plaque-forming ability (24). We assumed two possible causes of the inability of *mut*6 to form plaques; one is that *mut*6 RNA is not encapsidated, and the other is that *mut*6 RNA can be encapsidated but the resultant viral particles have a defect in a certain early step of the infection cycle, e.g., the adsorption or uncoating step.

To determine whether *mut*6 RNA is encapsidated, we carried out sedimentation analysis of virus particles. We performed a preliminary experiment to compare the sedimentation profile of Aichi virus with that of poliovirus Sabin 1, whose sedimentation profile has been well analyzed. Analysis of [^{35}S]methionine- and [^{3}H]uridine-labeled viral and subviral particles showed that the sedimentation profiles of virions and empty capsids of Aichi virus were similar to those of poliovirus (data not shown).

We next analyzed the sedimentation profile of *mut*6. Vero cells were electroporated with *mut*6 and AV-FL RNAs, and then [^{35}S]methionine-labeled viral and subviral particles were prepared and sedimented through sucrose gradients. For AV-FL, well-separated peaks of virions and empty capsids were found, as expected (Fig. 2). In contrast, in *mut*6, empty capsids were accumulated, whereas few virions were formed, demonstrating that *mut*6 has a severe defect in encapsidation. Thus, a defect at the stage of encapsidation would be a primary cause of the inability of *mut*6 to form plaques.

***mut6* virions are infectious.** Although *mut*6 has a severe defect in encapsidation, a small portion of *mut*6 RNA appeared to be encapsidated (Fig. 2, fractions 18 and 19). This suggests that infectious *mut*6 virus particles are generated at very low efficiency.

To confirm this possibility, Vero cells were infected with serial dilutions of the lysates of the cells electroporated with AV-FL and *mut*6 RNAs, and then capsid proteins synthesized in the infected cells were detected at 6 and 24 h after infection by means of an immunofluorescence assay with antiserum raised against purified virions (Fig. 3). The cell lysates used for infection were pretreated with RNase A to rule out the possibility of infection by naked viral RNAs. At 6 h after infection, synthesis of capsid proteins was observed in cells infected with *mut*6, although the number of cells synthesizing capsid proteins was much lower than that of cells infected with AV-FL (Fig. 3). Infection of *mut*6, as well as AV-FL, was inhibited by incubation of the cell lysates with guinea pig antiserum against purified virions prior to infection; the number of the AV-FL-infected cells decreased to <1%, and infection by *mut*6 was almost perfectly blocked, whereas preimmune guinea pig serum exhibited no inhibitory effect (Fig. 3). These results indicate that infectious *mut*6 virions are produced at very low efficiency in *mut*6 RNA-transfected cells. At 24 h after infection, AV-FL viruses spread cell to cell and formed fluorescent

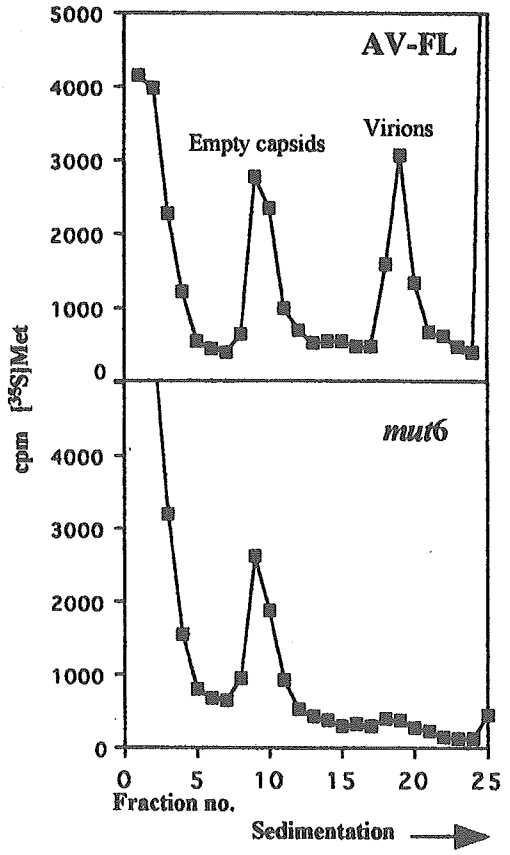

FIG. 2. *mut*6 has a defect in RNA encapsidation. Vero cells were electroporated with the AV-FL and *mut*6 RNAs, and then labeled with [^{35}S]methionine. At 6 h after electroporation, [^{35}S]methionine-labeled viral and subviral particles were collected and centrifuged through a 10 to 30% sucrose gradient. The gradient was fractionated, and the radioactivity in each fraction was counted with a liquid scintillation counter.

foci, whereas *mut*6 viruses hardly did so (Fig. 3). This is consistent with the observation that *mut*6 formed no plaques in the plaque assay (24). The encapsidation efficiency of *mut*6 RNA would be too low for the formation of plaques.

Further mapping of the region important for encapsidation. To further define the region important for encapsidation, we constructed several mutants in which the mutated region is reduced compared with *mut*6 (Fig. 4A). Vero cells were electroporated with the mutant RNAs, and then the accumulation of plus-strand viral RNA, synthesis of capsid proteins, and yield of viable viruses in the transfected cells were examined.

11-15/34-38 and 12-14/35-37 were constructed by exchanging the five- and three-nucleotide streches of the middle part of the stem, respectively, with each other to maintain the base pairings of the stem (Fig. 4A). Both mutant RNAs replicated and expressed capsid proteins as efficiently as AV-FL (Fig. 4B and C). In the plaque assay, 11-15/34-38 formed approximately 10-fold fewer plaques than AV-FL, and 12-14/35-37 produced a similar amount of viruses compared to the wild type (Fig. 4D). This indicates that as the mutated region is narrowed, the encapsidation efficiency is improved. Interestingly, only reduc-

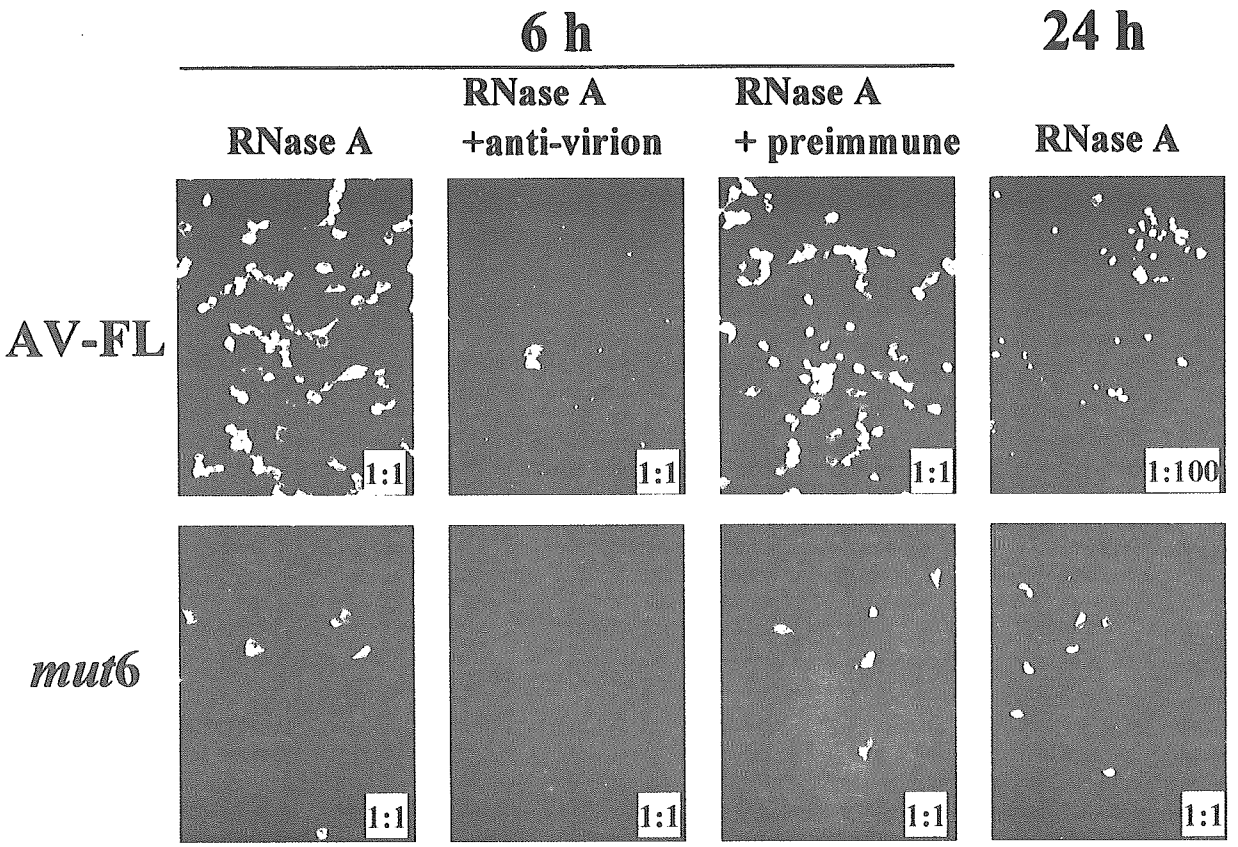

FIG. 3. *mut*6 virions are infectious. Serial dilutions of the lysates of cells harvested at 6 h after electroporation with the AV-FL and *mut*6 RNAs were prepared and then treated with RNase A alone or with RNase A and guinea pig antiserum raised against purified virus particles (RNase A + anti-virion) or preimmune guinea pig serum (RNase A + preimmune). Vero cells were infected with the treated lysates. At 6 and 24 h after infection, an immunofluorescence assay was performed with antiserum against purified virions. The dilution factor of the cell lysate is shown in each panel.

ing the mutated regions from 7 to 5 bp recovered the yield of viruses up to ca. 10% of that of wild type (Fig. 4D, compare *mut*6 and 11-15/34-38). To investigate which of the changes of the G_{10}-U_{39} and the G_{16}-U_{33} pairs affects the encapsidation efficiency more severely, we constructed two more mutants, 11-16/33-38 and 10-15/34-39 (Fig. 4A). These mutants also exhibited efficient RNA replication and translation abilities (Fig. 4B and C). 11-16/33-38 produced infectious viruses at almost the same efficiency as 11-15/34-38 (Fig. 4D), whereas 10-15/34-39 generated approximately 10-fold fewer viruses than 11-15/34-38 (Fig. 4D) and formed pinpoint plaques (data not shown). Thus, mutation of the G_{10}-U_{39} pair affected the encapsidation efficiency more severely than did mutation of the G_{16}-U_{33} pair. However, mutation of the 7 bp was responsible for abolishing the plaque-forming ability.

DISCUSSION

In the present study, we showed that the RNA sequence at the 5′ end of the Aichi virus genome, which folds into a stem-loop structure (SL-A), contains an element important for viral RNA encapsidation. The mutation introduced into both the seven-nucleotide stretches (nucleotides 10 to 16 and 33 to 39) in the middle part of the stem remarkably reduced the efficiency of RNA encapsidation (Fig. 2). As the mutated region is narrowed, the encapsidation efficiency was improved (Fig. 4). As a result, the mutation of the 7 bp was found to be required to abolish the plaque-forming ability.

This is the first detailed identification of a *cis*-acting element critical for encapsidation in picornaviruses. Chimeric poliovirus in which the internal ribosome entry site (IRES) within the 5′-UTR is replaced by the encephalomyocarditis virus (EMCV) IRES is viable, but the yield of the chimeric virus is slightly decreased compared to that of the wild-type virus (1, 23, 26). Johansen and Morrow (14) performed a *trans*-encapsidation assay using poliovirus replicons in which the capsid-coding region is replaced with a luciferase gene and a recombinant vaccinia virus expressing poliovirus capsid proteins. They showed that when the IRES of the replicon is replaced by the EMCV IRES, the efficiency of encapsidation is reduced. However, further mapping of the region within the poliovirus IRES responsible for encapsidation efficiency has not been carried out.

The 5′-end 90 nucleotides of the poliovirus genome fold into a cloverleaf-like structure, and this structure is known to be a *cis*-acting element important for viral RNA replication (3, 6, 12). Many site-directed mutants of the 5′ cloverleaf structure

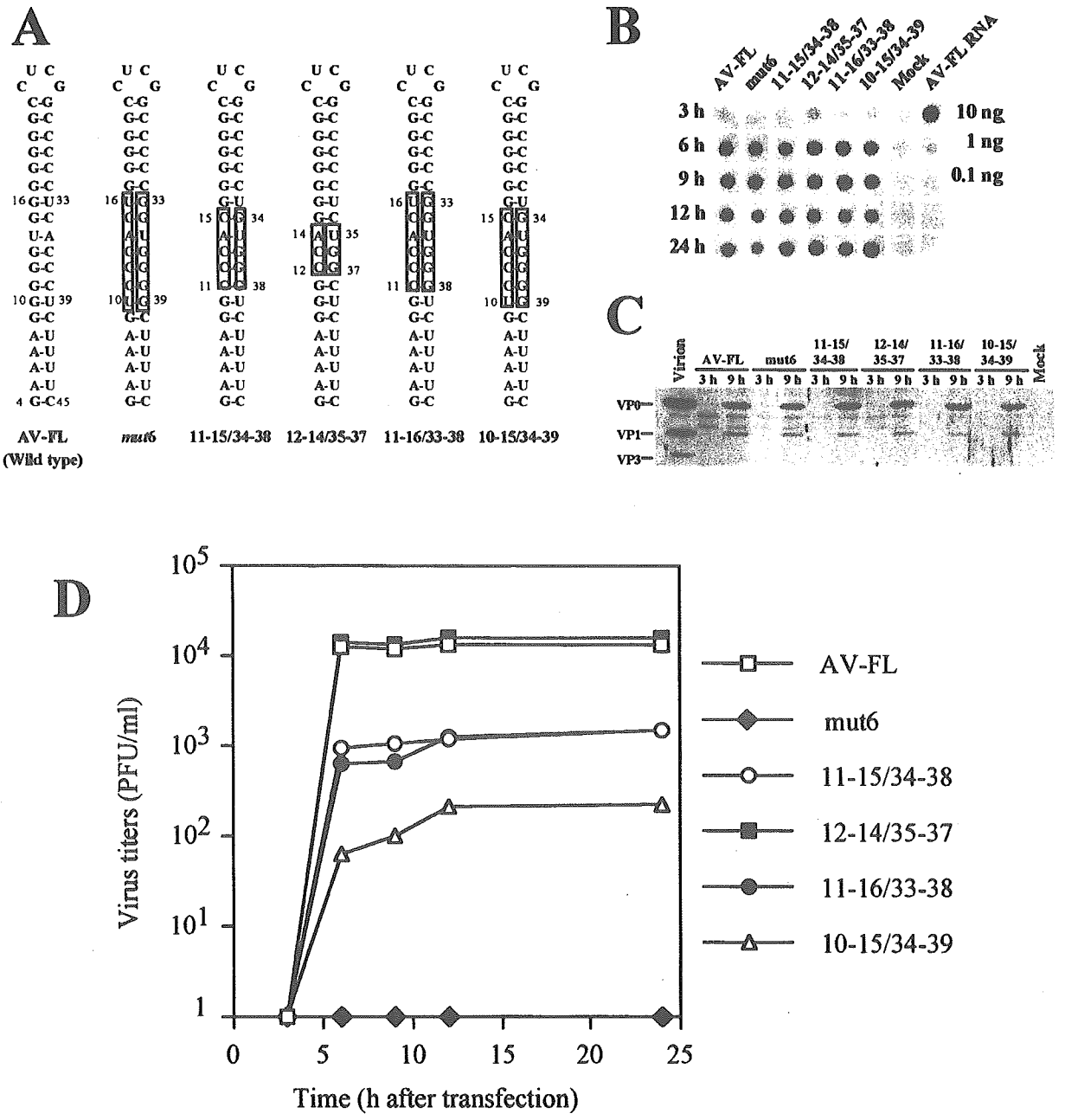

FIG. 4. Effects of mutations introduced into the middle part of the stem of SL-A on viral RNA replication, protein synthesis, and yields of viable viruses. (A) Diagram of SL-A of AV-FL and mutants. Mutated nucleotides in the mutants are boxed. (B) RNA replication of AV-FL and its mutants. Vero cells were electroporated with the AV-FL and mutant RNAs, and total RNAs were extracted from the cells at the indicated times after electroporation. The total RNA samples were dotted and probed with digoxigenin-labeled negative-sense viral RNA. As controls, 10-, 1-, and 0.1-ng portions of the AV-FL transcripts were dotted. (C) Accumulation of capsid proteins in the transfected cells. At the indicated times after electroporation, cell lysates were prepared and subjected to SDS–10% polyacrylamide gel electrophoresis, and capsid proteins were detected by Western blotting with antiserum raised against purified virus particles. As a control, the proteins of purified virions were analyzed. The position of each capsid protein is indicated on the left. (D) Virus yields in cells electroporated with the AV-FL and mutant RNAs. At the indicated times after electroporation, cells were harvested, and the virus titer was determined by a plaque assay. The number of plaques was determined at 72 h after infection.

in poliovirus have been constructed and characterized (2, 3, 18, 26), but there has been no report showing that the structure acts as a packaging signal. In some picornaviruses, including cardioviruses, parechoviruses, and HAV, a stem-loop structure is formed at the 5′ end of the genome (7, 8, 11, 15). The function of the stem-loop structure of these viruses has not been sufficiently analyzed. The Aichi virus genome also has a stem-loop structure at the 5′ end. We have previously shown

that SL-A is a structural element required for viral RNA replication (24). In addition, we elucidated in the present study that SL-A is critical for RNA encapsidation. Thus, SL-A has been shown to be bifunctional. It would be significant to investigate whether the 5′ end of other picornaviral genomes is involved in the encapsidation process because, in other groups of viruses, the location of the packaging signal on the genome is not necessarily conserved among related viruses. The region containing the packaging signal is different between two alphaviruses, Sindbis virus and Ross River virus (10).

The 5′ end of the HAV genome folds into three stem-loop structures. The most 5′-end stem-loop (stem-loop I) consists of 41 nucleotides including a 4-nucleotide loop and a single unpaired U residue in the middle part of the stem (7) (Fig. 1B). On the whole, SL-A is similar to HAV stem-loop I in size and shape, although its primary sequence is different. In a previous study (24), we characterized a mutant in which SL-A was replaced with the stem-loop I of HAV. The mutant had a severe defect in RNA replication but yielded viable viruses, albeit with low efficiency. This suggests that only SL-A is not sufficient for determining the specificity of encapsidation. Nugent et al. (17) showed the coupling of poliovirus RNA encapsidation to RNA replication and proposed a model for encapsidation. According to this model, specific interactions occur between capsid proteins and proteins in the viral RNA replication complex, and a newly synthesized positive-strand RNA emerging from the replication complex is encapsidated through interaction between capsid proteins and the viral RNA. The specificity of encapsidation has thus been proposed to be determined by specific interactions between capsid proteins and proteins constituting the RNA replication complex. This model may be applied to Aichi virus. Aichi virus SL-A may serve as a binding site for capsid proteins. Alternatively, it is possible that another region, in addition to SL-A, is required for determining the specificity of encapsidation. Further studies are needed to clarify the role of SL-A in encapsidation and the requirement for the specificity of encapsidation.

In various viruses, including Sindbis virus (25), a nodavirus (flock house virus) (29), a coronavirus (mouse hepatitis virus) (9), a tombusvirus (turnip crinkle virus) (21), a hepadnavirus (HBV) (19), and retroviruses (13), it has been reported that the region containing the RNA encapsidation signal folds into one or more stem-loop structures. In Aichi virus, the nucleotide sequence of the middle part of the stem of SL-A was shown to be critical for encapsidation. It remains to be elucidated whether the secondary structure of SL-A in addition to the primary sequence is important for encapsidation, although it is currently difficult to resolve this question. Since disruption of the secondary structure of SL-A abolished RNA replication (24), the site-directed mutations to be introduced into SL-A should be restricted to those that maintain the secondary structure. An assay system by which encapsidation can be evaluated independently of RNA replication is needed. For example, if encapsidation of *mut*6 RNA is restored by insertion of wild-type SL-A into another region of the RNA, the resultant mutant would be useful. We are trying to develop such a system.

This and the previous studies (24) showed that the 5′ end of the Aichi virus genome is involved in both viral RNA replication and encapsidation. The phenotypic property of *mut*6 indicates that the requirements in SL-A for viral RNA encapsidation and RNA replication are distinct. We presume that the 5′ end of the genome would interact with different factors during RNA replication and encapsidation. The 5′ end of the Aichi virus genome may play a role in determining which process the newly synthesized positive-strand RNAs are used for: RNA replication or encapsidation.

ACKNOWLEDGMENTS

This work was supported in part by a grant for the Human Science Research Foundation of Japan and a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture (Tokyo, Japan).

REFERENCES

1. **Alexander, L., H. H. Lu, and E. Wimmer.** 1994. Polioviruses containing picornavirus type 1 and/or type 2 internal ribosome entry site elements: genetic hybrids and the expression of a foreign gene. Proc. Natl. Acad. Sci. USA **91:**1406–1410.
2. **Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore.** 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. EMBO J. **12:**3587–3598.
3. **Andino, R., G. E. Rieckhof, and D. Baltimore.** 1990. A functional ribonucleoprotein complex forms around the 5′ end of poliovirus RNA. Cell **63:**369–380.
4. **Ansardi, D. C., Z. Moldoveane, D. C. Porter, D. E. Walker, R. M. Conry, A. F. LoBuglio, S. McPherson, and C. D. Morrow.** 1994. Characterization of poliovirus replicons encoding carcinoembryonic antigen. Cancer Res. **54:** 6359–6364.
5. **Barclay, W., Q. Li, G. Hutchinson, D. Moon, A. Richardson, N. Percy, J. W. Almond, and D. J. Evans.** 1998. Encapsidation studies of poliovirus subgenomic replicons. J. Gen. Virol. **79:**1725–1734.
6. **Barton, D. J., B. J. O'Donnell, and J. B. Flanegan.** 2001. 5′ cloverleaf in poliovirus RNA is a *cis*-acting replication element required for negative-strand synthesis. EMBO J. **20:**1439–1448.
7. **Brown, E. A., S. P. Day, R. W. Jansen, and S. M. Lemon.** 1991. The 5′ nontranslated region of hepatitis A virus RNA: secondary structure and elements required for translation in vitro. J. Virol. **65:**5828–5838.
8. **Duke, G. M., M. A. Hoffman, and A. C. Palmenberg.** 1992. Sequence and elements that contribute to efficient encephalomyocarditis virus RNA translation. J. Virol. **66:**1602–1609.
9. **Fosmire, J. A., K. Hwang, and S. Makino.** 1992. Identification and characterization of a coronavirus packaging signal. J. Virol. **66:**3522–3530.
10. **Frolova, E., I. Frolov, and S. Schlesinger.** 1997. Packaging signals in alphaviruses. J. Virol. **71:**248–258.
11. **Ghazi, F., P. J. Hughes, T. Hyypiä, and G. Stanway.** 1998. Molecular analysis of human parechovirus type 2 (formerly echovirus 23). J. Gen. Virol. **79:** 2641–2650.
12. **Herold, J., and R. Andino.** 2001. Poliovirus RNA replication requires genome circularization through a protein-protein bridge. Mol. Cell **7:**581–591.
13. **Jewell, N. A., and L. M. Mansky.** 2000. In the beginning: genome recognition, RNA encapsidation and the initiation of complex retrovirus assembly. J. Gen. Virol. **81:**1889–1899.
14. **Johansen, L. K., and C. D. Morrow.** 2000. The RNA encompassing the internal ribosome entry site in the poliovirus 5′ nontranslated region enhances the encapsidation of genomic RNA. Virology **273:**391–399.
15. **Le, S.-Y., J.-H. Chen, N. Sonenberg, and J. V. Maizel, Jr.** 1993. Conserved tertiary structural elements in the 5′ nontranslated region of cardiovirus, aphthovirus and hepatitis A virus RNAs. Nucleic Acids Res. **21:**2445–2451.
16. **Lu, H.-H., X. Li, A. Cuconati, and E. Wimmer.** 1995. Analysis of picornavirus $2A^{pro}$ proteins: separation of proteinase from translation and replication functions. J. Virol. **69:**7445–7452.
17. **Nugent, C. I., K. L. Johnson, P. Sarnow, and K. Kirkegaard.** 1999. Functional coupling between replication and packaging of poliovirus replicon RNA. J. Virol. **73:**427–435.
18. **Percy, N., W. S. Barclay, M. Sullivan, and J. F. Almond.** 1992. A poliovirus replicon containing the chloramphenicol acetyltransferase gene can be used to study the replication and encapsidation of poliovirus RNA. J. Virol. **66:**5040–5046.
19. **Pollack, J. R., and D. J. Ganem.** 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. J. Virol. **67:**3254–3263.
20. **Porter, D. C., D. C. Ansardi, and C. D. Morrow.** 1995. Encapsidation of poliovirus replicons encoding the complete human immunodeficiency virus type 1 *gag* gene by using a complementation system which provides the P1 capsid protein in *trans*. J. Virol. **69:**1548–1555.
21. **Qu, F., and J. Morris.** 1997. Encapsidation of turnip crinkle virus is defined by a specific packaging signal and RNA size. J. Virol. **71:**1428–1435.
22. **Racaniello, V. R.** 2001. Picornaviridae: the viruses and their replication, p. 685–722. *In* D. M. Knipe, P. M Howley, D. E. Griffin, R. A. Lamb, M. A.

Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott/The Williams & Wilkins Co., Philadelphia, Pa.

23. **Rohll, J. B., N. Percy, R. Ley, D. J. Evans, J. W. Almond, and W. S. Barclay.** 1994. The 5′-untranslated regions of picornavirus RNAs contain independent functional domains essential for RNA replication and translation. J. Virol. **68**:4384–4391.
24. **Sasaki, J., Y. Kusuhara, Y. Maeno, N. Kobayashi, T. Yamashita, K. Sakae, N. Takeda, and K. Taniguchi.** 2001. Construction of an infectious cDNA clone of Aichi virus (a new member of the family *Picornaviridae*) and mutational analysis of a stem-loop structure at the 5′ end of the genome. J. Virol. **75**:8021–8030.
25. **Weiss, B., U. Geigenmuller-Gnirke, and S. Schlesinger.** 1994. Interactions between Sindbis virus RNAs and a 68 amino acid derivative of the viral capsid protein further defines the capsid binding sites. Nucleic Acids Res. **22**:780–786.
26. **Xiang, W., K. S. Harris, L. Alexander, and E. Wimmer.** 1995. Interaction between the 5′-terminal cloverleaf and 3AB/3CD^{pro} of poliovirus is essential for RNA replication. J. Virol. **69**:3658–3667.
27. **Yamashita, T., S. Kobayashi, K. Sakae, S. Nakata, S. Chiba, Y. Ishihara, and S. Isomura.** 1991. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. J. Infect. Dis. **164**:954–957.
28. **Yamashita, T., K. Sakae, H. Tsuzuki, Y. Suzuki, N. Ishikawa, N. Takeda, T. Miyamura, and S. Yamazaki.** 1998. Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the *Picornaviridae* associated with acute gastroenteritis in humans. J. Virol. **72**:8408–8412.
29. **Zhong, W., R. Dasgupta, and R. Rueckert.** 1992. Evidence that the packaging signal for nodaviral RNA2 is a bulged stem-loop. Proc. Natl. Acad. Sci. USA **89**:11146–11150.

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2003, p. 5764–5769 Vol. 41, No. 12
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.12.5764–5769.2003

Serologic and Genomic Characterization of a G12 Human Rotavirus in Thailand

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Received 9 December 2002/Returned for modification 5 May 2003/Accepted 14 September 2003

The G and P type specificity of the human rotavirus strain T-152 (G12P[9]) isolated in Thailand was serologically confirmed with G12-specific monoclonal antibodies prepared in this study by using a reference G12 strain, L26, as an immunizing antigen and a P[9]-specific monoclonal antibody, respectively. The genomic relationship of strain T-152 with representative human rotavirus strains was examined by means of Northern blot analysis. The results showed that T152 is closely related to strain AU-1 (G3P[9]). Gene 5 (NSP1 gene) of T152, which did not hybridize with those of any other strains examined, was characterized by sequence determination. The T152 NSP1 gene is 1,652 nucleotides in length, encodes 493 amino acids, and exhibits low identity to those of representative human and animal rotaviruses.

Rotavirus, a member of the *Reoviridae* family, is the most common agent of severe, dehydrating gastroenteritis in infants and young children and in the young of most mammalian species (12). In developing countries, rotavirus infection results in high mortality, and an annual death rate of 500,000 to 600,000 persons has been estimated. Furthermore, in developed countries, rotavirus infection is a cause of high morbidity. Vaccination is thought to be the best way to reduce this significant mortality and morbidity worldwide.

Two rotavirus outer capsid proteins, a glycoprotein VP7 and a protease-sensitive VP4, have independent serotype specificities, G (VP7) serotype and P (VP4) serotype, respectively, and rotaviruses are classified by a binary system as are used for influenza viruses. For P typing, sequence analysis has been adopted due to the lack of readily available typing sera. A total of 15 G serotypes have been reported. Among them, 10 G serotypes have been detected in humans. G1 to G4 are the major G serotypes, with G5, G6, G8 to G10, and G12 being minor or unusual ones (5, 9, 12). In contrast, 22 P types have been recognized, with at least 10 P genotypes having been detected in humans (5, 8, 9, 12). Recently a number of human rotavirus strains with unusual G or P types and rare combinations of G and P types have been detected worldwide (1–9, 15–17, 20, 21, 27–29). For example, G5 was detected in almost half of the rotavirus-positive samples in Brazil (7). G9 is increasing rapidly (3, 17) and has become more common than G4 in many locations. Human G8 strains have been detected in Africa at a high frequency (1, 2, 4). P[8] is the most common, followed by P[4] and P[9]. Recently, P[6], which was first detected as an asymptomatic infection in neonates, has been increasing (1, 4, 9).

Since G12 was first detected in stool specimens collected from diarrheic children under 2 years of age between December 1987 and February 1988 in the Philippines (24, 28), no further report on the detection of G12 in humans or animals has appeared, although extensive surveys on the distribution of the G serotype worldwide have been conducted. In a previous study, however, members of our group detected a human G12P[9] rotavirus, T152, in Thailand and characterized it by means of reverse transcription-PCR and sequence determination (18). Also, in the United States, Griffin et al. (9) found a G12 strain with P[6] specificity. These results imply that the prevalence of G12 strains is expanding. In this study, we analyzed the Thai G12 strain T152 serologically using neutralizing monoclonal antibodies specific to G12 and P[9]. In addition, genomic relatedness between the T152 strain and representative human rotavirus strains was examined by Northern blot hybridization. Furthermore, we found the uniqueness of the NSP1 gene of strain T152.

TABLE 1. Reactivity patterns of monoclonal antibodies to human and animal rotaviruses

Strain	G, P type	Neutralizing titer of monoclonal antibody			
		1G11	3B5	3G9	4E7
KU	G1P[8]	<100	<100	100	<100
K8	G1P[9]	<100	<100	<100	<100
S2	G2P[4]	<100	<100	<100	<100
YO	G3P[8]	<100	<100	<100	<100
AU-1	G3P[9]	<100	<100	<100	<100
Hosokawa	G4P[8]	100	<100	<100	100
OSU	G5P[7]	<100	<100	<100	100
NCDV	G6P[1]	<100	400	200	200
Ty-1	G7P[17]	<100	<100	200	<100
69M	G8P[10]	<100	400	<100	<100
WI-61	G9P[8]	200	100	<100	200
B223	G10P[11]	<100	200	<100	<100
YM	G11P[7]	<100	100	<100	<100
L26	G12P[4]	3,200	102,400	51,200	102,400
L27	G12P[4]	3,200	102,400	51,200	25,600
T152	G12P[9]	3,200	102,400	12,800	12,800
L338	G13P[18]	<100	100	<100	<100
FI-23	G14P[12]	<100	100	<100	<100

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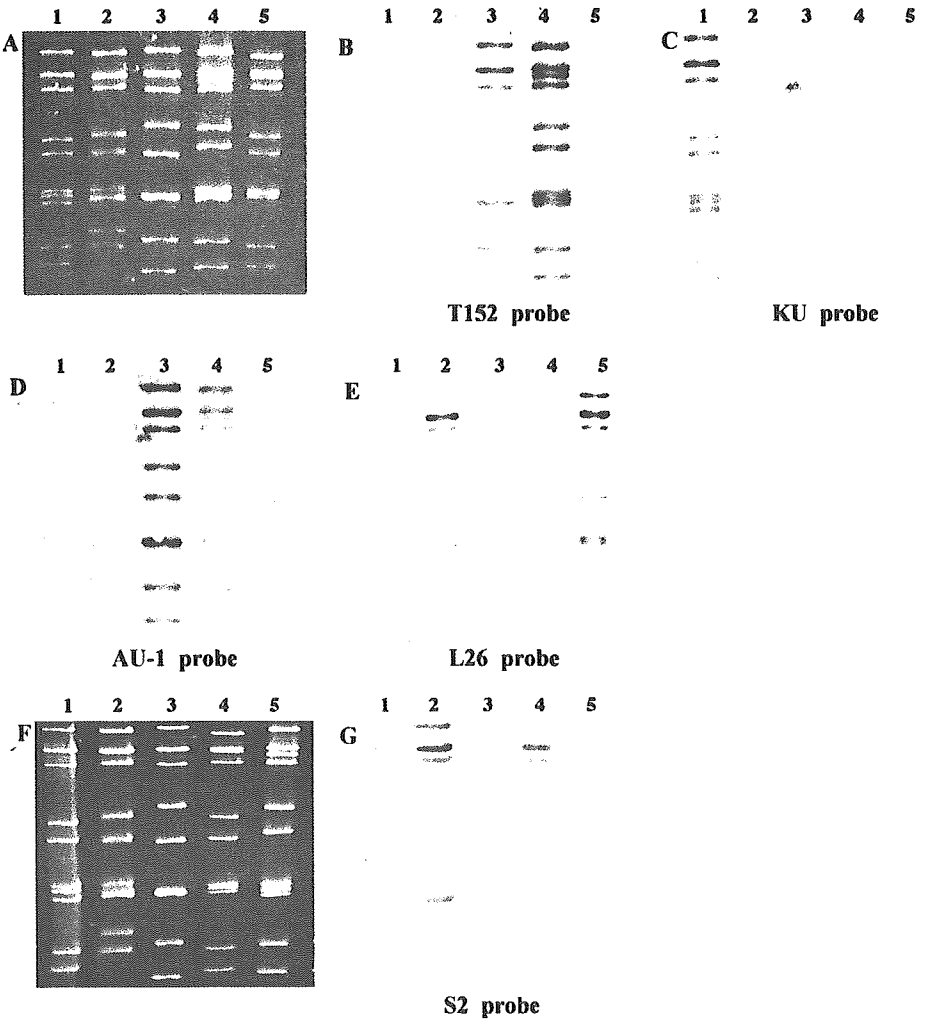

FIG. 1. Northern blot hybridization analysis of strain T152. (A) RNA profiles on PAGE. Lanes: 1, strain KU; 2, strain S2; 3, strain AU-1; 4, strain T152; 5, strain L26. (B) Northern blot analysis using the T152 probe. (C) Northern blot analysis using the KU probe. (D) Northern blot analysis using the AU-1 probe. (E) Northern blot analysis using the L26 probe. (F) RNA profiles on PAGE. Lanes: 1, strain KU; 2, strain S2; 3, strain AU-1; 4, strain L26; 5, strain T152. (G) Northern blot analysis using the S2 probe. Northern blot analyses in panels B to E and G were performed using the blot transferred from the polyacrylamide gel shown in panels A and F, respectively.

The following representative human and animal rotavirus strains were employed: KU (human, G1P[8]), K8 (human, G1P[9]), S2 (human, G2P[4]), YO (human, G3P[8]), AU-1 (human, G3P[9]), Hosokawa (human, G4P[8]), OSU (porcine, G5P[7]), NCDV (bovine, G6P[1]), Ty-1 (turkey, G7P[17]), 69 M (G8P[10]), WI-61 (human, G9P[8]), B223 (bovine, G10P[11]), YM (porcine, G11P[7]), L26 (human, G12P[4]), L27 (human, G12P[4]), T152 (human, G12P[9]), L338 (G13P[18]), and FI-23 (equine, G14P[12]). Each rotavirus strain was pretreated with 10 μg of trypsin (type IX, from porcine pancreas and crystallized; Sigma) per ml, inoculated onto MA-104 cells in the presence of trypsin (1 μg/ml), and then harvested 1 to 3 days after infection.

The purified L26 strain was used as an immunizing antigen. P3-X63-Ag8.653 mouse myeloma cells were fused with spleen cells from mice immunized intraperitoneally with L26 as described previously (23). To obtain ascitic fluid, 10^7 hybridoma cells were inoculated intraperitoneally into Pristane-primed BALB/c mice.

A 1:5 dilution (for hybridoma screening) of culture fluid or twofold serial dilutions (for determination of neutralizing antibody titers) of ascitic fluid was reacted with a virus suspension containing about 500 fluorescent cell-forming units/0.025 ml for 1 h. At 18 to 24 h postinfection, the infected cells were washed with phosphate-buffered saline, fixed with cold (−80°C) ethanol, and then reacted with a 1:30 dilution of anti-human rotavirus rabbit serum for 1 h. After an additional 1-h reaction with a 1:50 dilution of fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G goat serum (Seikagaku Kogyo, Tokyo, Japan), the number of fluorescent cells was determined by vertically illuminated fluorescence microscopy. The neutralization titer was expressed as the reciprocal of the highest serum dilution that reduced the fluorescent-cell count by more than 60%.

TABLE 2. Nucleotide and amino acid sequence homologies of NSP1 gene and protein from strain T152 with NSP1 genes and proteins from representative human and animal rotavirus strains

Strain	G type and P type	Species	Homology (%)	
			Nucleotide	Amino acid
KU	G1P[8]	Human	61.5	50.4
DS1	G2P[4]	Human	62.0	51.1
K8	G1P[9]	Human	62.5	51.5
69M	G8P[10]	Human	61.7	49.5
L26	G12P[9]	Human	61.3	51.6
A44	G10P[11]	Cow	63.5	55.8
A5-10	G8P[1]	Cow	60.8	*[a]
A5-13	G8P[1]	Cow	63.5	53.0
UK	G6P[5]	Cow	63.2	55.0
Gottfried	G4P[6]	Pig	62.1	50.1
OSU	G5P[7]	Pig	62.0	52.8
YM	G11P[7]	Pig	61.5	50.5
FI-14	G3P[12]	Horse	56.9	40.3
H2	G3P[12]	Horse	57.0	40.9
L338	G13P[18]	Horse	56.4	38.0
SA11	G3P[2]	Monkey	57.9	42.5
RRV	G3P[3]	Monkey	55.9	39.8
EW	G3P[16]	Mouse	53.6	39.9
PO-13	G7P[17]	Pigeon	46.8	17.5

[a] *, Since NSP1 of strain A5-10 is 40 amino acids in length due to the presence of a nonsense codon at nucleotides 153 to 155, the homology could not be determined.

An enzyme-linked immunosorbent assay with monoclonal antibodies was carried out as described previously (25). The following monoclonal antibodies were used: group A-common YO-156 (directed to VP6), subgroup I-specific S2-37 (VP6), subgroup II-specific YO-5 (VP6), G1-specific KU-4 (VP7), G2-specific S2-2G10 (VP7), G3-specific YO-1E2 (VP7), G4-specific ST-2G7 (VP7), and a group A-common YO-2C2 (VP4).

Rotavirus double-stranded RNA was extracted from stools and culture fluid with a disruption solution comprising 1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 50 mM EDTA and then with phenol and chloroform. The RNA was electrophoresed in 10% acrylamide gels (2 mm thick) for 16 h at 20 mA at room temperature. RNA segments were visualized by silver staining.

Full-length cDNA of the NSP1 gene of culture-adapted strain T152 was prepared by reverse transcription-PCR. PCR-amplified cDNA was ligated into the pCRII vector with a TA cloning kit (Invitrogen Corp). The PCR products and three cDNA clones were sequenced with the ABI PRISM BigDye terminator cycle sequencing ready reaction kits (PE Biosystems, Chiba, Japan) and an automated sequencer, the ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, Calif.). Nucleotide sequences were analyzed for construction of a phylogenetic tree using the Neighbor-Joining method.

Northern blot hybridization was carried out as previously described (19). Briefly, after polyacrylamide gel electrophoresis (PAGE) analysis, double-stranded RNA was denatured by soaking the gel in 0.1 N NaOH and 0.25 M NaCl for 20 min and was then neutralized in 4× Tris-acetate-EDTA for 20 min twice and in 1× Tris-acetate-EDTA for 20 min. Electrotransfer of rotavirus RNA to Hybond N+ (Amersham) was conducted at 0.2 mA overnight at 4°C. Hybridization was performed with an enhanced chemiluminescence direct nucleic acid labeling and detection system (Amersham) according to the instructions of the manufacturer. Stringency was regulated by changing the concentration of the SSC solution (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for primary and secondary wash buffers.

For serological characterization of strain T152, we prepared neutralizing monoclonal antibodies specific to G12 rotaviruses. Four hybridoma clones were established in two fusion experiments, using strain L26 (G12P[4]) as an immunizing antigen. In neutralization tests involving various human and animal rotavirus strains, the four monoclonal antibodies all reacted specifically with the Philippine G12 strains L26 and L27 (Table 1). The antibodies also neutralized strain T152 with similar efficiency (Table 1).

P9-specific K8-2C12 monoclonal antibody prepared previously (13) neutralized strain T152 as well as strains K8 and AU-1 with P[9] specificity (data not shown). Thus, we confirmed serologically that the G and P type specificity of strain T152 was G12P[9].

The overall genomic relatedness determined through RNA-RNA hybridization assays has revealed that there are three distinct human-specific genogroups that are almost not related to one another at all: the Wa, DS-1, and AU-1 genogroups. In this study, we performed Northern blot hybridization to examine the overall genomic relatedness of strain T152 with strains KU, S2, and AU-1, representing the Wa, DS-1, and AU-1 genogroups, respectively, since this assay can reveal the relatedness in a segment-to-segment manner and can permit the reuse of the blot with different probes.

The T152 probe reacted with nine RNA segments of strain AU-1, although the probe showed no reaction or only a faint reaction with a few RNA segments in the genome of strain KU, S2, or L26 (Fig. 1). A reciprocal assay with the AU-1 probe also showed the high relatedness between strains T152 and AU-1. In the assay with the L26 probe, the probe reacted only with RNA segment 7, which may be the VP7 gene, and segment 11 of T152 (Fig. 1). As described previously (14), L26 was found to be partly related to strains S2 and KU, members of the DS-1 genogroup and Wa genogroup, respectively. In contrast, the KU and S2 probes did not exhibit any significant relationship with T152 (Fig. 1). Thus, T152 was shown to be highly related to strain AU-1, except in genes 5 and 7.

With Northern blot hybridization, gene 5 of strain T152 did not react with any of the probes employed and vice versa. This finding prompted us to characterize the T152 gene 5 (the NSP1 gene) by sequencing. The NSP1 gene of strain T152 was found to be 1,652 nucleotides in length and to encode 493 amino acids. Comparison of its sequence with the published NSP1 sequences revealed the uniqueness of the T152 NSP1 gene; the nucleotide sequence identity ranged from 46.8 to 63.5% (Table 2). Phylogenetic analysis of the NSP1 genes showed the species relatedness, and the T152 NSP1 gene was found to be related to those of bovine rotaviruses (Fig. 2).

Although no reports have appeared on the detection of G12 rotaviruses since the detection of human G12 rotaviruses (prototype strain L26) in the Philippines in 1990 (24, 28), two human G12 strains, T152 and Se585, were very recently detected in Thailand and in the United States, respectively (9, 18). On comparison of the VP7 genes of strains L26, T152, and

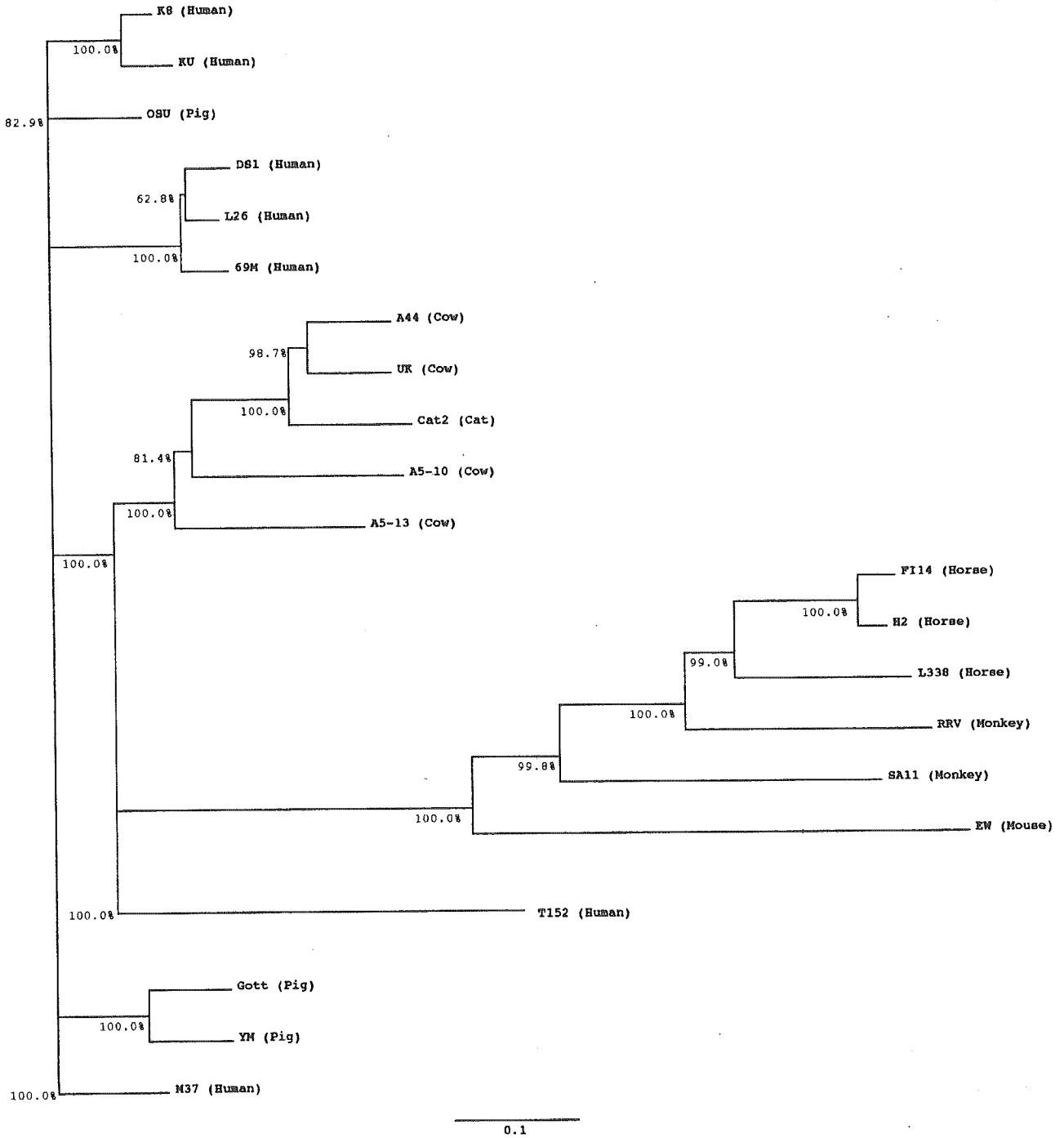

FIG. 2. Phylogenetic tree for the nucleotide sequences of the NSP1 genes of strain T152 and other representative human and animal rotaviruses. The bootstrap confidence levels obtained by 1,000 replicates are shown. The bar indicates the variation scale.

Se585, higher identity (97.8 and 98.5%, nucleotide and amino acid, respectively) was found between T152 and Se585 than that (90.2 or 91.0% and 92.9 or 94.2%, respectively) between L26 and T152 or Se585. The VP7 gene of strain L26 has accumulated point mutations, and the present G12 strains (strains T152 and Se585) with the VP7 gene may have evolved. However, the genome constellation of the two strains is quite different. Seven and 10 genes, respectively, of Se585 hybridized to those of strains L26 and US1205 of the DS-1 genogroup (9). Few or no hybrids were formed between the genomes of Se585 and Wa or AU-1 (9). Thus, strain Se585 was considered to be a natural single-gene reassortant between strain L26 and strain US1205 or a similar one of the DS1 genogroup. In contrast, the genes of T152 hybridized to nine genes of strain AU-1. The

VP7 gene is related to L26, and the origin of the NSP1 gene of T152 is unknown. This is a striking contrast with the finding that RNA segments of Se585 did not react to any of the genes of strain AU-1 (14). These results imply that T152 is also a natural reassortant between L26 and a strain in the AU-1 genogroup. A second reassortment step might have occurred for acquisition of the unusual NSP1 gene from an unknown third strain. To begin with, strain L26 is thought to be a reassortant. In our previous study involving RNA-RNA hybridization assaying, it was found that two or three genes were from the Wa genogroup and five or six were from the DS-1 genogroup (14). Thus, the reassortment of rotaviruses may occur through multiple steps between two reassortants or between a reassortant and a nonreassortant virus, such as strain Wa, DS-1, or AU-1, reference strains of the Wa, DS-1, and AU-1 genogroups, respectively.

In this study, we first prepared G12-specific neutralizing monoclonal antibodies. The G12-specific monoclonal antibodies were potent in neutralization tests and enzyme-linked immunosorbent assays. Although the protein specificity (VP7 or VP4) of the antibodies could not be determined, they may be directed to VP7, since they did not react with any strains of P[4], which is the P type of strain L26 employed as an immunizing antigen for hybridoma production. The use of reassortants or the preparation of mutants resistant to the antibodies is necessary for a final conclusion. These antibodies will be quite useful for large-scale epidemiological surveys for the detection of G12 rotaviruses.

In order to determine the overall genomic relatedness of rotavirus strains, Northern blot assays were performed in this study. Compared to the liquid RNA-RNA hybridization assays used more commonly, Northern blot assays have some advantages: the relatedness in a segment-to-segment manner can be determined, the reuse of blots for a different probe is possible, and there is no necessity for radioisotopes for the preparation of probes. However, depending on the lengths of RNA segments, the cutoff levels might be different; short RNA segments, such as segments 10 and 11, tend to react more readily with each other than longer RNA segments exhibiting the same homology. As a whole, segment-to-segment comparison is very useful. Indeed, we could detect the uniqueness of gene 5 of strain T152.

The gene 5 equivalent to the NSP1 gene (1,652 nucleotides) of T152 is much longer than those of other rotaviruses except for that (1,870 nucleotides) of pigeon strain PO-13 (11). It has been shown that NSP1 genes among rotaviruses exhibit great diversity (10, 26). Among the 11 genes, the NSP1 gene exhibits the least identity. In addition, several truncated NSP1 proteins have been reported for the rearranged NSP1 genes (1). Furthermore, we previously found strains (A5-10 and A5-16) with NSP1 proteins of only 40 or 50 amino acids (22). Some clones of these strains produced very large plaques and induced diarrhea in suckling mice as efficiently as the strains with the normal NSP1 genes (Taniguchi et al., unpublished data). Further characterization of a unique NSP1 gene of strain T152 in vitro and in vivo will provide more useful data.

Although G1 to G4 and P[8] or P[4] rotaviruses are common worldwide, strains with unusual properties appear to prevail more frequently than before. In particular, G9 strains are increasing (3, 17), and we also detected G9 strains at an extremely high frequency in a district in Japan (Taniguchi et al., unpublished data). The detection of G12 strains in Thailand and the United States implies expansion of the distribution of the G12 strains. For the development of an effective rotavirus vaccine and for the examination of rotavirus ecology, it is still necessary to continue to survey the G and P type distributions worldwide.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper for the NSP1 gene of strain T152 have been deposited with the DDBJ/EMBL/GenBank data libraries under accession no. AB097459.

This study was supported in part by a Grant-in-Aid from the Ministry of Education and Science, Japan, and the U.S.-Japan Cooperative Medical Science Program.

REFERENCES

1. **Adah, M. I., A. Wade, and K. Taniguchi.** 2001. Molecular epidemiology of rotaviruses in Nigeria: detection of unusual strains with G2P[6] and G8P[1] specificities. J. Clin. Microbiol. **39:**3969–3975.
2. **Armah, G. E., C. T. Pager, R. H. Asma, F. R. Anto, A. B. Oduro, F. Binka, and D. Steele.** 2001. Prevalence of unusual human rotavirus strains in Ghanaian children. J. Med. Virol. **63:**67–71.
3. **Cunliffe, N. A., W. Dove, J. E. G. Bunn, M. B. Ramadam, J. W. O. Nyangao, R. L. Riveron, L. E. Cuevas, and C. A. Hart.** 2001. Expanding global distribution of rotavirus serotype G9: detection in Libya, Kenya, and Cuba. Emerg. Infect. Dis. **7:**890–892.
4. **Cunliffe, N. A., J. S. Gondwe, R. L. Broadhead, M. E. Molyneux, P. A. Woods, J. S. Bresee, R. I. Glass, J. R. Gentsch, and C. A. Hart.** 1999. Rotavirus G and P types in children with acute diarrhea in Blantyre, Malawi, from 1997 to 1998: predominance of novel P[6]G8 strains. J. Med. Virol. **57:**308–312.
5. **Estes, M. K.** 2001. Rotaviruses and their replication, p. 1747–1785. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott-Raven Publishers, Philadelphia, Pa.
6. **Gentsch, J. R., P. A. Woods, M. Ramachandran, B. K. Das, J. P. Leite, A. Alfieri, R. Kumar, M. K. Bhan, and R. I. Glass.** 1996. Review of G and P typing results from a global collection of strains: implications for vaccine development. J. Infect. Dis. **174**(suppl. 1):S30–S36.
7. **Gouvea, V., and N. Santos.** 1999. Rotavirus serotype G5: an emerging cause of epidemic childhood diarrhea. Vaccine **17:**1291–1292.
8. **Griffin, D. D., C. D. Kirkwood, U. D. Parasgar, P. A. Woods, J. S. Bresee, R. I. Glass, J. R. Gentsch, and the National Rotavirus Strain Surveillance System Collaborating Laboratories.** 2000. Surveillance of rotavirus strains in the United States: identification of unusual strains. J. Clin. Microbiol. **38:** 2784–2787.
9. **Griffin, D. D., T. Nakagomi, Y. Hoshino, O. Nakagomi, C. D. Kirkwood, U. D. Parashar, R. I. Glass, J. R. Gentsch, and the National Rotavirus Strain Surveillance System.** 2002. Characterization of nontypeable rotavirus strains from the United States: identification of a new rotavirus reassortant (P2A[6],G12) and rare P3[9] strains related to bovine rotaviruses. Virology **294:**256–269.
10. **Hua, J., and J. T. Patton.** 1994. The carboxyl-half of the rotavirus nonstructural protein NS53 (NSP1) is not required for virus replication. Virology **198:**567–576.
11. **Ito, H., M. Sugiyama, K. Masubuchi, Y. Mori, and N. Minamoto.** 2001. Complete nucleotide sequence of a group A avian rotavirus genome and a comparison with its counterparts of mammalian rotaviruses. Virus Res. **75:** 123–138.
12. **Kapikian, A. Z., Y. Hoshino, and R. M. Chanock.** 2001. Rotaviruses. p. 1787–1833. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
13. **Kobayashi, N., K. Taniguchi, T. Urasawa, and S. Urasawa.** 1991. Preparation and characterization of a neutralizing monoclonal antibody directed to VP4 of rotavirus strain K8 which has unique VP4 neutralization epitopes. Arch. Virol. **121:**153–162.
14. **Kojima, K., K. Taniguchi, and N. Kobayashi.** 1996. Species-specific and interspecies relatedness of NSP1 sequences in human, porcine, bovine, feline, and equine rotavirus strains. Arch. Virol. **141:**1–12.
15. **O'Halloran, F., M. Lynch, B. Cryan, H. O'Shea, and S. Fanning.** 2000. Molecular characterization of rotavirus in Ireland: detection of novel strains circulating in the population. J. Clin. Microbiol. **38:**3370–3374.
16. **Okada, J., T. Urasawa, N. Kobayashi, K. Taniguchi, A. Hasegawa, K. Mise, and S. Urasawa.** 2000. New P serotype of group A human rotavirus closely related to that of a porcine rotavirus. J. Med. Virol. **60:**63–69.

17. **Palombo, E. A., P. J. Masendycz, H. C. Bugg, N. Bogdanovic-Sakran, G. L. Branes, and R. F. Bishop.** 2000. Emergence of G9 in Australia. J. Clin. Microbiol. **38:**1305–1306.
18. **Pongsuwanna, Y., R. Guntapong, M. Chiwakul, R. Tacharoenmuang, N. Onvimala, M. Wakuda, N. Kobayashi, and K. Taniguchi.** 2002. Detection of a human rotavirus with G12 and P[9] specificity in Thailand. J. Clin. Microbiol. **40:**1390–1394.
19. **Pongsuwanna, Y., K. Taniguchi, M. Chiwakul, T. Urasawa, F. Wakasugi, C. Jayavasu, and S. Urasawa.** 1996. Serological and genomic characterization of porcine rotaviruses in Thailand: detection of a G10 porcine rotavirus. J. Clin. Microbiol. **34:**1050–1057.
20. **Ramachandran, M., J. R. Gentsch, U. D. Parashar, S. Jin, P. A. Woods, J. L. Holmes, C. D. Kirkwood, R. F. Bishop, H. B. Greenberg, S. Urasawa, G. Gerna, B. S. Coulson, K. Taniguchi, J. S. Bresee, R. I. Glass, and the National Rotavirus Strain Surveillance System collaborating laboratories.** 1998. Detection and characterization of novel rotavirus strains in the United States. J. Clin. Microbiol. **36:**3223–3229.
21. **Santos, N., R. C. C. Lima, C. F. A. Pereiira, and V. Gouvea.** 1998. Detection of rotavirus types G8 and G10 among Brazilian children with diarrhoea. J. Clin. Microbiol. **36:**2727–2729.
22. **Taniguchi, K., K. Kojima, and S. Urasawa.** 1996. Nondefective rotavirus mutants with an NSP1 gene which has a deletion of 500 nucleotides, including a cysteine-rich zinc finger motif-encoding region (nucleotides 156 to 248), or which has a nonsense codon at nucleotides 153 to 155. J. Virol. **70:**4125–4130.
23. **Taniguchi, K., S. Urasawa, and T. Urasawa.** 1985. Preparation and characterization of neutralizing monoclonal antibodies with different reactivity patterns to human rotaviruses. J. Gen. Virol. **66:**1045–1053.
24. **Taniguchi, K., T. Urasawa, N. Kobayashi, M. Gorziglia, and S. Urasawa.** 1990. Nucleotide sequence of VP4 and VP7 genes of human rotaviruses with subgroup I specificity and long RNA pattern: implication for new G serotype specificity. J. Virol. **64:**5640–5644.
25. **Taniguchi, K., T. Urasawa, Y. Morita, H. B. Greenberg, and S. Urasawa.** 1987. Direct serotyping of human rotaviruses in stools by enzyme-linked immunosorbent assay using serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies to VP7. J. Infect. Dis. **155:**1159–1166.
26. **Tian, Y., O. Tarlow, A. Ballard, U. Desselberger, and M. A. McCrae.** 1993. Genomic concatemerization/deletion in rotaviruses: a new mechanism for generating rapid genetic change of potential epidemiological importance. J. Virol. **67:**6625–6632.
27. **Urasawa, S., A. Hasegawa, T. Urasawa, K. Taniguchi, F. Wakasugi, H. Suzuki, S. Inouye, B. Pongprot, J. Supawadee, S. Suprasert, P. Rangsiyanond, S. Tonusin, and Y. Yazaki.** 1992. Antigenic and genetic analysis of human rotaviruses prevailing in Chiang Mai, Thailand: evidence for a close relationship between human and animal rotaviruses. J. Infect. Dis. **166:**227–234.
28. **Urasawa, S., T. Urasawa, F. Wakasugi, N. Kobayashi, K. Taniguchi, I. C. Lintag, M. C. Saniel, and H. Goto.** 1990. Presumptive seventh serotype of human rotavirus. Arch. Virol. **113:**279–282.
29. **Urasawa, T., K. Taniguchi, N. Kobayashi, K. Mise, A. Hasegawa, Y. Yamazi, and S. Urasawa.** 1993. Nucleotide sequence of VP4 and VP7 genes of a unique human rotavirus strain Mc35 with subgroup I and serotype 10 specificity. Virology **195:**766–771.