the majority of Pakistani sequences belonged to two distinct sapovirus genogroups I and II. Of these, the positives were further classified into one sapovirus GI genotype and one sapovirus GII genotype according to the recent sapovirus capsid region classification scheme of Farkas et al. (2004). Moreover, other eight sapoviruses did not cluster with any the published references and presented four novel sapovirus genotypes tentatively called GI/4, GI/5, GI/6, GI/7 and GII/4, respectively. These sapoviruses had a low identity on the nucleotide as well as the amino acid with other reference strains in the same genogroup previously registered in the DDBJ DNA database. It was noteworthy that sapovirus could be classified into 7 GI and 4 GII genotypes.

Another interesting feature was a high identity at the nucleotide and the amino acid between Karachi/730/1992 and only one representative of a genogroup IV, Hou7-1181/90. Both of them, which revealed unique sequences distinct from known sapoviruses in the DDBJ, were recovered from fecal specimens over 10 years ago. Possibly, these special strains might be emerging in the past. Taken together, our findings clearly indicated that sapovirus strains co-circulating among infants and children with acute gastroenteritis in Karachi city, Pakistan demonstrated the great genetic diversity. Additionally, these data have described the molecular epidemiology as well as the importance of sapovirus causing acute gastroenteritis in Pakistan and increased the evidence for the worldwide distribution of this virus. This is the first indication on molecular epidemiology of sapovirus infection conducted in Karachi city, Pakistan, showing the genetic diversity among them.

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Author's address: Hiroshi Ushijima, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; e-mail: ushijima@m.u-tokyo.ac.jp

17 genotypes, respectively (21). ORF2 encodes the major structural capsid protein, including a shell domain (S) and a protruding (P) domain (31). Several reports suggested a good correlation between the clustering NV strains by the sequence of the 5′ end of ORF2 and antigenic grouping confirmed by patient immune response against NVs (4, 23, 24, 30). Therefore, it may be possible to predict antigenic types by phylogenetic analysis of the capsid region.

Previously, we described classification of NV strains detected in Osaka City, Japan by probe types, based on hybridization of the amplified viral genomes with six NV-specific DNA probes (13–15). However, we often came across NV strains to which none the probes would hybridize, making their classification difficult. In this report, we describe the characterization of the NVs in fecal specimens from 31 nonbacterial gastroenteritis outbreaks occurring in Osaka City, Japan, between April 2002 and March 2003.

Materials and Methods

Outbreaks and specimens. Fecal specimens were collected from 40 outbreaks of acute nonbacterial gastroenteritis, including 22 outbreaks associated with oysters, in Osaka City, Japan, between April 2002 and March 2003. A total of 111 fecal specimens were examined by real time RT-PCR.

RNA extraction. A 10% stool suspension was prepared as described previously (13). Viral RNA was extracted from 140 μl of the suspension with a QIAamp viral RNA Mini kit (Qiagen, Valencia, Calif., U.S.A.) according to the manufacturer's instructions. RNA was eluted with 60 μl of diethyl pyrocarbonate-treated water and kept at −80 C until use in RT-PCR.

Real time RT-PCR. Real time RT-PCR was carried out as described by Kageyama et al. (20). Viral RNA (15 μl) was added to 15 μl of the mixture containing 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 1 mM of each deoxynucleoside triphosphate, 10 mM dithiothreitol, 75 pmol of random hexamer (pdN6; Amersham Pharmacia Biotech, Piscataway, N.J., U.S.A.), 30 U of RNase inhibitor (TaKaRa Shuzo, Kyoto, Japan), and 7.5 U of avian myeloblastosis virus reverse transcriptase XL (Life Science Inc., St. Petersburg, Fla., U.S.A.). RT was performed at 42 C for 60 min, and the enzyme was inactivated at 70 C for 15 min. cDNA was stored at −20 C.

The real time quantitative PCR was carried out in 50 μl reactions containing 4 μl of cDNA, 25 μl of *Taq*Man Universal PCR Master Mix (Applied Biosystems, Branchburg, N.J., U.S.A.), 5 pmol each of primers COG1F and COG1R and 15 pmol of RING1 (a)-*Taq*Man probe (TP) and 5 pmol RING1 (b)-TP fluorogenic probe for GI NV detection, or 5 pmol each of primers COG2F and COG2R and 5 pmol RING2-TP for GII NV detection. PCR amplification was performed with an ABI7700 sequence detector (Applied Biosystems) under the following conditions: 2 min at 50 C and 10 min at 95 C, then 40 cycles of 95 C for 15 sec and 56 C for 1 min. Data were corrected by using internal standards as described by Kageyama et al. (20).

Sequencing of the N/S domain. When more than 2 samples from an outbreak appeared to be positive by real time RT-PCR, several samples, including these 2, were selected for sequencing analysis. To amplify the N-terminal/shell (N/S) domain of the capsid region, PCR was carried out with primers G1SKF and G1SKR for GI NV strains or G2SKF and G2SKR for GII NV strains as described by Kojima et al. (25). After purification of the amplicon with a QIAquick PCR purification kit (Qiagen), the nucleotide sequences were determined with the BigDye Terminator Cycle sequence kit and ABI 310 sequencer (Applied Biosystems).

Phylogenetic analysis. Capsid sequences of the reference strains of NV were obtained from GenBank. These strains and accession numbers are shown in Table 1. Phylogenetic analysis was performed as described by Katayama et al. (22). Briefly, the sequences of N/S domain (GI, 290 nt; GII, 278 nt) were aligned by using Clustal X (version 1.81) with parameters provided in Clustal W1.6. A phylogenetic tree was constructed by the neighbor-joining method, and the genetic distances were calculated according to the Kimura two-parameter method. Reliability of the tree was estimated by 1,000 bootstrap replications, and bootstrap values of 950 or higher were considered statistically significant for the grouping (7).

Probe typing. The polymerase region was amplified by RT-PCR, using SR primers (G1 sets: SR33, SR48, SR50, and SR52; G2 sets: SR33, SR46, OC0281B) as described in Ando et al. (1). PCR products were analyzed by Southern hybridization with probes P1-A, P1-B, P2-A, P2-B, SOV, and 96065 (13), and strains were classified according to the probe to which they hybridized.

Nucleotide sequences and accession numbers. The nucleotide sequences of the N/S shell domains determined in this study were submitted to DNA Databank of Japan with the accession numbers AB186057 to AB186107.

Results

Diagnosis and Epidemiology of NV Associated Outbreaks

Fecal samples from 40 outbreaks of acute nonbacte-

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Genotyping of *Norovirus* Strains Detected in Outbreaks between April 2002 and March 2003 in Osaka City, Japan

Yoshiyuki Seto*[,1], Nobuhiro Iritani[2], Hideyuki Kubo[2], Atsushi Kaida[2], Tsukasa Murakami[2], Kosuke Haruki[2], Osamu Nishio[3], Minoru Ayata[1], and Hisashi Ogura[1]

[1]*Department of Virology, Osaka City University Medical School, Osaka, Osaka 545–8585, Japan,* [2]*Department of Microbiology, Osaka City Institute of Public Health and Environmental Sciences, Osaka, Osaka 543–0026, Japan, and* [3]*Department of Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Musashi-murayama, Tokyo 208–0011, Japan*

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***Abstract*: *Noroviruses* (NVs) are the major cause of food- and waterborne nonbacterial gastroenteritis in Japan. Between April 2002 and March 2003, a total of 111 fecal specimens from 40 outbreaks of acute nonbacterial gastroenteritis in Osaka City, Japan were subject to NV detection. Seventy-two samples (64.9%) from 31 outbreaks (77.5%) were NV positive by a real time reverse transcription (RT)-PCR assay. To further determine the genotype of individual NV strains, we sequenced the capsid N-terminal/shell (N/S) domain of some representative strains from each outbreak. The 51 NV strains detected in this study were segregated into 15 genotypes (6 in genogroup I and 9 in genogroup II), and GII/5 genotype NV was a dominant outbreak genotype.**

***Key words*: *Norovirus*, N/S domain, Genotype, Epidemiology**

Norovirus (NV) is a genus within the family *Caliciviridae* (www.ictvdb.iacr.ac.uk/Ictv), which has been previously termed Norwalk-like virus or small round structured virus. The NV prototype strain, Norwalk/68/US, has been entirely sequenced from cDNA clones derived from stool specimens, and its genome is a single-stranded, positive-sense RNA molecule of 7.5 kb that comprises three open reading frames (ORFs) (17, 19). NVs are the major cause of acute nonbacterial gastroenteritis worldwide, and illness occurs in people of all ages. NVs are transmitted not only by a fecal-oral route but also by direct person-to-person contact (11). There have been numerous outbreaks due to NV-contaminated foods, such as shellfish, salads, and deli sandwiches (6, 8, 12, 13), and due to NV-contaminated water (5, 26).

Since NVs have not yet been cultivated *in vitro*, electron microscopy (EM) or immuno-EM had been routinely used to detect NV particles in stool specimens in the laboratory. After the cloning and sequencing of Norwalk/68/US (17) and Southampton/91/UK (27), a reverse transcription-PCR (RT-PCR) assay was developed to target the RNA-dependent RNA polymerase gene in ORF1 of the NV genome (18, 28). Using sequence information of additional NV strains, different primer sets targeting the polymerase region have been used for the diagnosis of NV in fecal specimens from both outbreaks and sporadic cases (2, 9, 34, 35). Based on the sequence information obtained from the polymerase region, the NV strains can be divided into two genogroups, genogroup I (GI) and genogroup II (GII), each comprising a large number of genetically diverse strains (1, 10, 30).

A classification system has been proposed for NVs, in which the sequence of ORF2 (the gene for the major capsid protein) of the strains is compared with that of reference strains (3, 11, 22, 33). A recent study indicated that NV GI and GII strains consist of at least 14 and

**Address correspondence to* Dr. Yoshiyuki Seto, Department of Virology, Osaka City University Medical School, Asahi-machi, Abeno-ku, Osaka, Osaka 545–8585, Japan. Fax: +81–6–6645–3912. E-mail: yoshi3486@med.osaka-cu.ac.jp

Abbreviations: EM, electron microscopy; G, genogroup; N/S, N-terminal/shell; NV, *Norovirus*; ORF, open reading frame; RT-PCR, reverse-transcription polymerase chain reaction.

Table 1. Reference strains and genetic classification of NVs

Genotype[a]	Reference strains (GenBank accession no.)	Genetic classification			
		Katayama[b]	Vinje[c]	Ando[d]	Green[e]
GI/1	Norwalk/68/US (M87661)	GI/1	GI/1	GI/1	GI/1
GI/2	Southampton/91/UK (L07418)	GI/4	GI/2	GI/2	GI/2
	Whiterose/96/UK (AJ277610)				
GI/3	DesertShield/90/SA (U04469)	GI/9	GI/3	GI/3A	GI/3
	Birmingham/93/UK (AJ277612)			GI/3B	
	LittleRocks/94/US (AF414405)				
	Stav/95/NO (AF145709)				
GI/4	Chiba/87/JP (AB022679)	GI/7	GI/4	GI/4	GI/4
	Valetta/95/MA (AJ277616)				
GI/5	Musgrove/89/UK (AJ277614)	GI/6	GI/6	GI/5	GI/5
	AppalachicolaBay/95/US (AF414406)				
	KU83aGI/99/JP (AB058545)				
GI/6	BS5/97/GE (AF093797)	GI/2	NA[f]	NA	GI/6
GI/7	Winchester/94/UK (AJ277809)	GI/8	GI/5	NA	GI/7
GI/8	Sindlesham/95/UK (AJ277615)	GI/3	GI/7	NA	GI/6
	WUG1/00/JP (AB081723)				
GI/9	SzUG1/99/JP (AB039774)	GI/5	NA	NA	NA
GII/1	Hawaii/71/US (U07611)	GII/7	GII/3	GII/1A	GII/1
	Girlington/93/UK (AJ277606)				
GII/2	Melksham/94/UK (X81879)	GII/4	GII/4	GII/2	GII/2
	SnowMountain/76/US (U70059, U75682)				
GII/3	Toronto/TV24/91/CA (U02030)	GII/2	GII/1	GII/3	GII/3
	Mexico/89/MX (U22498)				
	Arg320/95/AR (AF190817)				
GII/4	Bristol/93/UK (X76716)	GII/1	GII/2	GII/4	GII/4
	Lordsdale/93/UK (X86557)				
GII/5	Hillingdon/90/UK (AJ277607)	GII/5	GII/5	GII/5	GII/5
	WhiteRiver/290/94/US (AF414423)				
GII/6	Seacroft/90/UK (AJ277620)	GII/8	GII/7	GII/6	GII/6
	Miami/292/94/US (AF414410)				
GII/7	Leeds/90/UK (AJ277608)	GII/3	GII/6	GII/7	GII/7
	Gwynedd/273/94/US (AF414409)				
GII/8	Amsterdam/98/NL (AF195848)	GII/10	NA	GII/8	NA
GII/9	Idaho Falls/378/1996/US (AY054299)	NA	NA	GII/9	NA
	VA9207/97/US (AY038599)				
GII/10	Erfurt/00/DE (AF427118)	NA	NA	NA	NA
	KU5GII/00/JP (AB058575)				
GII/12	Chitta/96/JP (AB032758)	GII/6	GII/8	GII/1B	GII/1
	Wortley/90/UK (AJ277618)				
GII/14	Fayetteville/1998/US (AY113106)	NA	NA	NA	NA
	Kashiwa47/00/JP (AB078334)				
GII/15	Saitama KU82GII/99/JP (AB058588)	NA	NA	NA	NA
GII/17	Alphatron/98/NL (AF195847)	GII/9	NA	NA	NA
	Fort Lauderdale/560/98/US (AF414426)				

[a] Kageyama et al. (21).
[b] Katayama et al. (22).
[c] Vinje et al. (33).
[d] Ando et al. (3).
[e] Green et al. (11).
[f] Not assigned.

Table 2. Description of outbreaks in which NVs were detected in Osaka City, Japan, between April 2002 and March 2003

Outbreak no.	Mo/yr	Source	Attack rate ill/risk	No. of specimens	No. of NV-positive	Probe type	Genotype
02065	Apr/02	UK[a]	2/5	2	1 (GI)	P1A	GI/7
02172	Sep/02	Oyster	5/28	5	5 (GI, GII)	P1A	ND[b]
						P2B	GII/5, GII/3, GII/12
02189	Nov/02	UK	2/3	2	2 (GII)	P2B	GII/12
02198	Dec/02	UK	UK/25	3	3 (GII)	P2B	GII/4
02202	Dec/02	UK	4/5	1	1 (GII)	P2B	GII/4
03006	Jan/03	Oyster	1/1	1	1 (GII)	P2B	GII/3
03008	Jan/03	Oyster	28/35	1	1 (GII)	—	GII/5
03009	Jan/03	Oyster	3/3	2	2 (GI, GII)	P1A	GI/7
						P1B	ND
						P2B	GII/5
03011	Jan/03	Oyster	1/1	1	1 (GI)	—	GI/7
03012	Jan/03	Oyster	77/295	12	9 (G1, GII)	—	GI/7
						P1B	ND
						P2B	GII/5, GII/3, GII/15
03017	Jan/03	UK	10/13	9	8 (GII)	P1B	GII/6
03020	Feb/03	Oyster	3/3	1	1 (GI)	P1A	GI/4
						SOV	ND
03021	Feb/03	Oyster	2/2	2	2 (GII)	P2B	GII/3, GII/1
03022	Feb/03	Oyster	5/5	4	4 (GI, GII)	—	GI/7
						SOV	GI/2
						P2B	GII/3, GII/15
03024	Feb/03	UK	6/15	5	3 (GII)	P2B	GII/5
03026	Feb/03	Oyster	26/331	2	2 (GI, GII)	P1A	GI/4
						SOV	ND
						P2B	GII/5
03027	Feb/03	Oyster	2/2	1	1 (GII)	—	GII/5
03028	Feb/03	Oyster	3/3	1	1 (GII)	P2B	GII/5
03034	Feb/03	Oyster	3/3	3	3 (GI, GII)	P1A	GI/7, GI/4
						SOV	ND
						UT[c]/GI	GI/8
						P2B	GII/5, GII/3
03035	Feb/03	Oyster	6/6	2	1 (GII)	P2B	GII/15
03036	Feb/03	Oyster	3/5	1	1 (GII)	P2B	GII/3
03037	Feb/03	UK	3/15	3	2 (GI)	P1A	GI/9
03039	Feb/03	Oyster	5/9	4	3 (GI, GII)	P1A	GI/4
						P2B	GII/14, GII/8
03040	Mar/03	UK	5/9	4	2 (GII)	P2B	GII/8
03042	Mar/03	UK	20/47	1	1 (GII)	—	GII/8
03047	Mar/03	UK	2/8	1	1 (GI, GII)	P1A	GI/1
						P2B	GII/8
03048	Mar/03	UK	UK	1	1 (GII)	—	GII/6
03050	Mar/03	UK	27/62	7	4 (GII)	P2B	GII/15
03053	Mar/03	UK	11/50	3	1 (GII)	P2B	GII/3
03054	Mar/03	Oyster	3/6	2	2 (GII)	P2B	GII/5
03055	Mar/03	UK	157/283	2	2 (GII)	P1B	GII/6

[a] Unknown.
[b] Not determined.
[c] Untype.

rial gastroenteritis were tested for NV by real time RT-PCR. Seventy-two of 111 fecal specimens (64.9%) from 31 outbreaks (77.5%) were positive for NV (Table 2). GII NV was detected from 28 outbreaks, including 8 outbreaks also positive for GI. The 31 NV-positive outbreaks occurred in different settings, including restaurant, party, hotel, and home. The most common viral transmission mode in these outbreaks was ingestion of

contaminated oysters (54.8%). The NV-positive gastroenteritis outbreaks in Osaka City occurred mostly between January 2003 and May 2003 (83.9%).

All NV-positive specimens were tested for the probe type. Twenty-six of the 31 NV-positive outbreaks could be classified as 2 P1A, 2 P1B, 13 P2B, and 9 mixed probe types (Table 2). P2B strains were detected in 8 of 9 mixed probe-type outbreaks. In total, the P2B type was detected in 21 outbreaks (67.7%) and was, therefore, a predominant probe type during the 2002–03 seasons in Osaka City. The probe type could not be determined for 5 outbreaks (03008, 03011, 03027, 03042, and 03048), nor for one specimen each in outbreaks 03012 and 03022; all of these specimens were NV-positive by real time RT-PCR, but could not be amplified by RT-PCR using G1 or G2 primer sets. In addition, a single specimen from outbreak 03034, which was positive by RT-PCR using the G1 primer set, did not react with any probes (represented as UT/GI in Table 2).

Phylogenetic Analysis and Genotyping of NVs

To analyze the genetic relationships among the NV strains from the 31 outbreaks, the nucleotide sequence encoding the N/S domain of the capsid protein was determined. A total of 58 NV-positive specimens, including 18 GI and 51 GII NV-positive, were sequenced. Any strains from a single outbreak having identical nucleotide sequence in this region were considered to be identical strains.

The 51 nucleotide sequences (14 GI, 37 GII) of the N/S domain were aligned with the reference strains described in Table 1, and the genotype of each strain was determined. Phylogenetic trees based on the N/S domain were constructed by the neighbor-joining method for GI and GII NVs (Fig. 1, A and B). The bootstrap values of each genetic cluster was greater than 999, except for the GI/6 (Hesse cluster) type in GI NVs. The 51 NV strains were classified into 15 genotypes (6 GI and 9 GII genotypes) based on reference strains, and the number of each genotype was according to Kageyama's report (21). The genotypes of the 31 outbreaks strains were classified as follows: 2 GI/7, 1 GI/9, 5 GII/5, 3 GII/3, 3 GII/6, 2 GII/4, 2 GII/8, 2 GII/15, 1 GII/12, and 10 mixed genotype outbreaks.

In the outbreaks caused by mixed genotype NVs, GI/7 NV was detected in 4 outbreaks, and GII/5 NV in 5 outbreaks. Therefore, GI/7 NV was detected in a total of 6 outbreaks (19.4%), making it a dominant outbreak genotype in GI NV, and GII/5 NV was detected in 10 outbreaks (32.3%) and a dominant outbreak genotype in GII NV. In 9 of the outbreaks involving the GII/5 NV, oyster was the causal food.

In 7 outbreaks, strains of a single probe type were classified into multiple genotypes: 03021/P2B strains were of the GII/1 and GII/3, 03022/P2B strains were GII/3 and GII/15, 03034/P1A strains were GI/4 and GI/7, 03039/P2B strains were GII/8 and GII/14, and 02172/P2B, 03012/P2B, and 03034/P2B strains were of the GII/3 and GII/5 genotypes. The strain 03034-2/GI, which did not hybridize with any probes, was classified into the GI/8 genotype. Within each outbreak, the NV strains classified into the same genotype shared identical nucleotide sequence, except for NVs from outbreaks 02172 (02172-1 and 02172-2 in the GII/3 genotype) and 02198 (02198-1 and 02198-2 in the GII/4 genotype).

The relationship between probe types and genotypes of the NV outbreak strains detected in this study is as follows: P1A probe reacted with 4 genotypes (GI/1, GI/4, GI/7, and GI/9), SOV probe reacted with the GI/2 genotype, P1B reacted with the GII/6 genotype, and P2B reacted with 8 genotypes (GII/1, GII/3, GII/4, GII/5, GII/8, GII/12, GII/4, and GII/15).

Discussion

Molecular epidemiological studies of NV infections have been based on the phylogenetic analysis of the polymerase and capsid regions. The RNA polymerase region, which is relatively conserved among NV strains, has been used for detection of a wide variety of field strains, and most epidemiological studies of NV infection have been based on the sequence in this region (1, 28, 34, 35). We also reported epidemiological studies of NV infection in Osaka City, Japan targeting the polymerase region (13–15).

In general, good correlation has been reported between phylogenetic analyses of the polymerase region and capsid region (30, 33). However, recent studies indicated that phylogenetic analysis of the polymerase region sequence did not facilitate the classification of strains into genotypes (22), and a system has been proposed for the identification of NVs in which the capsid sequences are compared to those of reference strains. Ando et al. (3) used sequences encoding the capsid N-terminal 94 amino acids to divide G1 NVs into 5 "genetic clusters" and GII NVs into 10 clusters. Vinje et al. (33) demonstrated that the NVs could be divided into 7 "phylogenetic groups" within GI and 5 within GII using the capsid N-terminal region sequence (GI; 278 nt, GII; 249 nt). Katayama et al. (22) demonstrated that the NVs could be divided 9 "genotypes" within GI and 10 within GII using the capsid N/S domain. Furthermore, Green et al. (11) demonstrated that the NVs could be divided 7 "genetic clusters" within GI and 7 within GII using the complete capsid

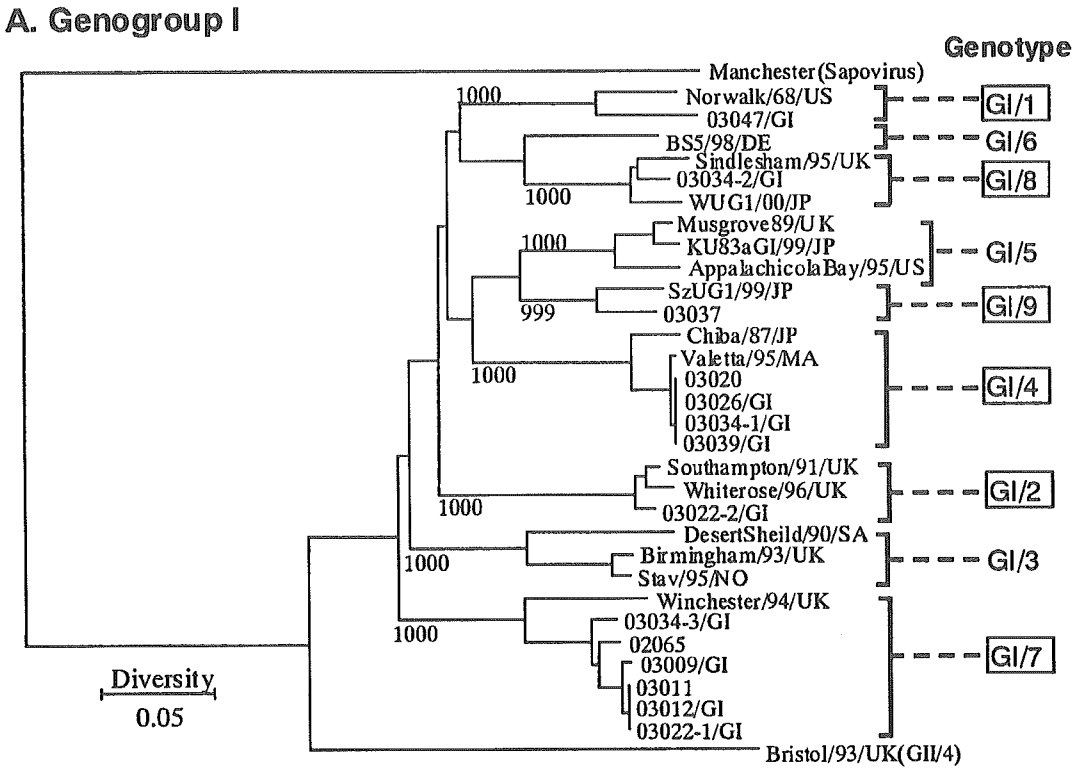

Fig. 1. Phylogenetic trees of published NV sequences and outbreak strains from this study based on the N/S domain region (GI, 290 nt; GII, 278 nt). Genogroup I NVs and Bristol/93/UK(GII/4) (A), genogroup II NVs and Norwalk/68/US(GI/1) (B) are reconstructed using Manchester *sapovirus* as the outgroup. The numbers on each branch indicate the bootstrap value for the clusters supported by that branch. The genotypes that include an outbreak strain are boxed.

region. The genetic classification described in these four reports is summarized in Table 1. The comparison indicates that their constellations of genotypes or genetic clusters are almost identical except for the names. The phylogeny based on the ORF2 region is related to antigenic types, as indicated by solid phase immuno-EM for 8 genetic types (33). The phylogenetic analysis of the N/S domain (22) was well correlated with antigenic analysis using recombinant virus-like particles and their antisera (23, 24). Therefore, the 5′ end of ORF 2 region (N/S domain) may be suitable for the molecular typing of NV strains.

In this study, a phylogenetic analysis of the N/S domain incorporating new outbreak strains, NV reference strains from previous reports, and additional reference strains, GI NVs were segregated into 9 genotypes, as reported by Katayama et al. (22), but GII NVs were segregated into 14 genotypes (Fig. 1, A and B). Kageyama et al. reported that the NV could be divided into 14 genotypes within GI and 17 genotypes within GII based on the capsid N/S domain (21).

The P2B outbreak strains, the predominant probe type in this season, were characterized into 8 genotypes (Table 2). However, there was no predominant genotype of NV outbreak strains; multiple genotypes of NV were prevalent in Osaka City, Japan. The outbreaks in which mixed NV genotypes were detected mainly occurred by consumption of oysters. It may be that concurrent infections with more than one strain occurred by ingesting the contaminated oysters. Similar results of coinfection have been reported previously (21, 32), and various types of NVs have been detected from oysters in Japan (29). Ninety percent of GII/5 NV outbreaks were associated with consumption of oysters, indicating that the GII/5 NV was closely related to oysters in this season. The 9 GII/3 NV strains, classified as P2B, detected in this study, in Fig. 1B, were closely related to Arg-320/95/AR which might be occurred a genetic recombination between ORF1 and ORF2 (15, 16, 33).

In summary, we applied a recently developed quantitative real time PCR a method (20) to detect NV genomes from stool specimens in Osaka City, Japan. This method is useful for routine diagnosis, because of

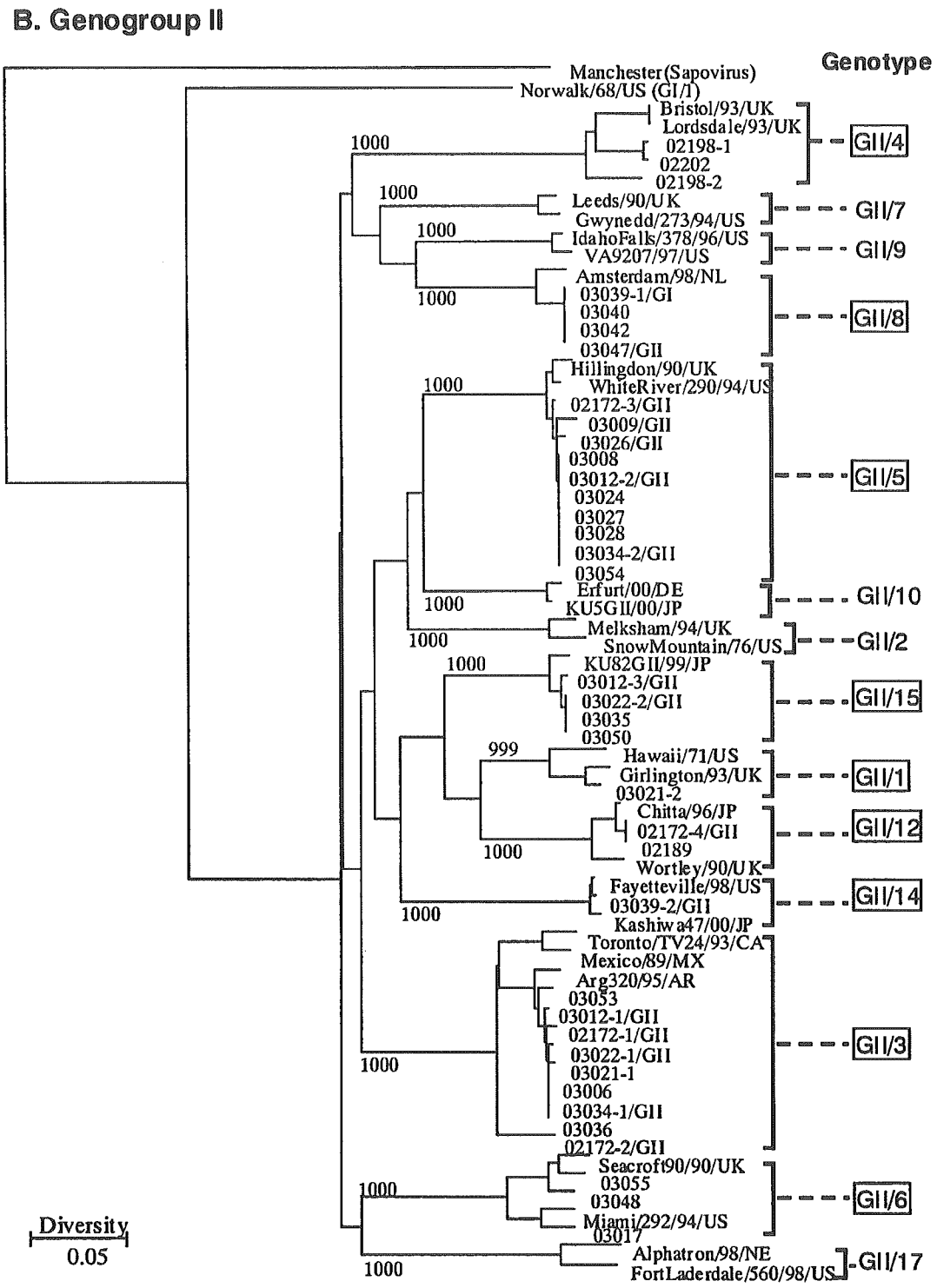

Fig. 1. B

its broad reactivity and high sensitivity compared to our previous diagnostic method using RT-PCR and hybridization. Application of genotyping methods has provided information on disease transmission for epidemiological investigations of public health significance. Further molecular phylogenetic studies of NVs will contribute to an understanding of the epidemiology of NV infection.

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Hepatitis E Virus Transmission from Wild Boar Meat

Tian-Cheng Li,* Katsumi Chijiwa,† Nobuyuki Sera,† Tetsuya Ishibashi,† Yoshiki Etoh,† Yuji Shinohara,‡ Yasuo Kurata,‡ Miki Ishida,§ Shigeru Sakamoto,¶ Naokazu Takeda,* and Tatsuo Miyamura*

We investigated a case of hepatitis E acquired after persons ate wild boar meat. Genotype 3 hepatitis E virus (HEV) RNA was detected in both patient serum and wild boar meat. These findings provided direct evidence of zoonotic foodborne transmission of HEV from a wild boar to a human.

Hepatitis E virus (HEV), a causative agent of human hepatitis E, is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (1,2). HEV is transmitted primarily by the fecal-oral route through contaminated drinking water. However, recent studies have demonstrated that various animal species have serum antibodies to HEV, suggesting that hepatitis E is a zoonotic disease (3). In Japan, 4 hepatitis E cases have been linked directly to eating raw deer meat (4), and several cases of acute hepatitis E have been epidemiologically linked to eating undercooked pork liver or wild boar meat (5,6). These cases provide convincing evidence of zoonotic food-borne HEV transmission. We report direct evidence of HEV transmission from a wild boar to a human.

The Study

A 57-year-old woman came to Iizuka Hospital on March 12, 2005, with malaise and anorexia. Although she was a healthy hepatitis B virus carrier and negative for serologic markers of hepatitis A and C, testing upon admission showed elevated levels of liver enzymes (alanine aminotranferase 752 IU/L, aspartate aminotransferase 507 IU/L, and γ-glutamyl transpeptidase 225U/L). A serum sample collected on March 16 was positive for both immunoglobulin M (IgM) and IgG antibodies to HEV when tested by an antibody enzyme-linked immunosorbent assay using recombinant viruslike particles (7). This led to the diagnosis of hepatitis E. The hepatitis was typical, acute, and self-limiting, and the patient recovered by the end of March.

The patient's husband traditionally hunted boar for food 3 or 4 times a year, and she had eaten boar meat on 2 occasions. With her husband, she ate the meat as part of a hot pot on December 28, 2004, 11 weeks before her illness, and again, grilled, on January 19, 2005, along with 10 other people (including her husband) 8 weeks before her illness. Disease did not develop in the other 10 people. Except for this wild boar meat, the patient had not eaten meat or liver from other wild animals. Since she had not traveled abroad in the past 30 years, transmission must have occurred in Japan. Two portions of meat from the wild boar (meats 1 and 2) eaten on December 28, 2004, and 1 portion from the other wild boar (meat 3) eaten on January 19, 2005, remained and were frozen.

Juice was obtained from the sliced meat by centrifugation at 10,000 × *g* for 15 min. The supernatant was used for RNA extraction. A nested reverse transcription–polymerase chain reaction (RT-PCR) was conducted to amplify part of open reading frame 2 (ORF2), which corresponds to nucleotides (nt) 5939–6297 of the genotype 1 HEV genome (GenBank D10330), with external sense primer HEV-F1 (5′-TAYCGHAAYCAAGGHTGGCG-3′) and antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′). A nested PCR was conducted with internal sense primer HEV-F2 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′). This procedure allows amplification of HEV 1, 3, and 4 genotypes. A PCR product of 359 bp including the primer sequences was obtained from meat 3 by nested PCR. However, meats 1 and 2 were negative. HEV RNA was not detected in the patient's serum by the same amplification method. This may have resulted from an extremely small amount of RNA.

New primers for the nested RT-PCR were designed for a region within the 359 base region based on the meat 3 sequences, which corresponded to nt 5983–6243. The first PCR was performed with external sense primer HEV-WB-F1 (5′-ACCTCTGGCCTGGTAATGCT-3′) and antisense primer HEV-WB-R2 (5′-GAGAAGCGTATCAGCAAGGT-3′). The nested PCR was performed with internal sense primer HEV-WB-F2 (5′-TATTCATGGCTCTCCTGTCA-3′) and internal antisense primer HEV-WB-R1 (5′-ACAGTGTCAGAGTAATGCCT-3′). These primers allowed amplification of 281 nt, including the primer sequences from the patient serum collected on March 16, 2005. In contrast, meats 1 and 2 were negative with these new primers.

To further analyze the RNA in the patient serum and meat 3, RNA genomes encoding an entire ORF2 were

*National Institute of Infectious Diseases, Tokyo, Japan; †Fukuoka Institute of Health and Environmental Sciences, Fukuoka, Japan; ‡Tagawa Health, Welfare, and Environment Office, Fukuoka, Japan; §Fukuoka Prefectual Government, Fukuoka, Japan; and ¶Iizuka Hospital, Fukuoka, Japan

amplified as overlapping segments, nucleotide sequences were determined, and phylogenetic analysis was carried out with avian HEV as an outgroup. Avian HEV is a causative agent of chicken hepatitis-splenomegaly syndrome (8). Two sequences, 1 from the patient (DQ079629) and the other from meat 3 (DQ079630), were classified into genotype 3 (Figure). Only 1 nt difference was observed in the 1,980 nt of the entire ORF2; the nucleotide sequence identity was 99.95%. The difference was not accompanied by any amino acid changes. These data demonstrated that HEV infection was transmitted from the wild boar meat to the patient on January 19, 2005.

Conclusions

Currently, deer, pig, and wild boar are suspected sources of foodborne zoonotic transmission of HEV in Japan, and genotypes 3 and 4 of HEV are believed to be indigenous (4–6,9,10). Direct evidence for transmission of genotype 3 HEV from animals to humans was observed in acute hepatitis in 4 persons who had eaten uncooked deer meat that contained $\approx 10^7$ copies of HEV RNA (4). However, the rare finding of HEV antibody-positive deer in Japan suggest that deer are not the major zoonotic reservoir of HEV in this country (11). In contrast, high antibody-positive rates in domestic pig and wild boar, including HEV genotypes 3 and 4, have been frequently detected, suggesting that persons who eat uncooked meat are at risk for infection with HEV (12,13). This report is the first to provide direct evidence of zoonotic foodborne genotype 3 HEV transmission from wild boar to a human.

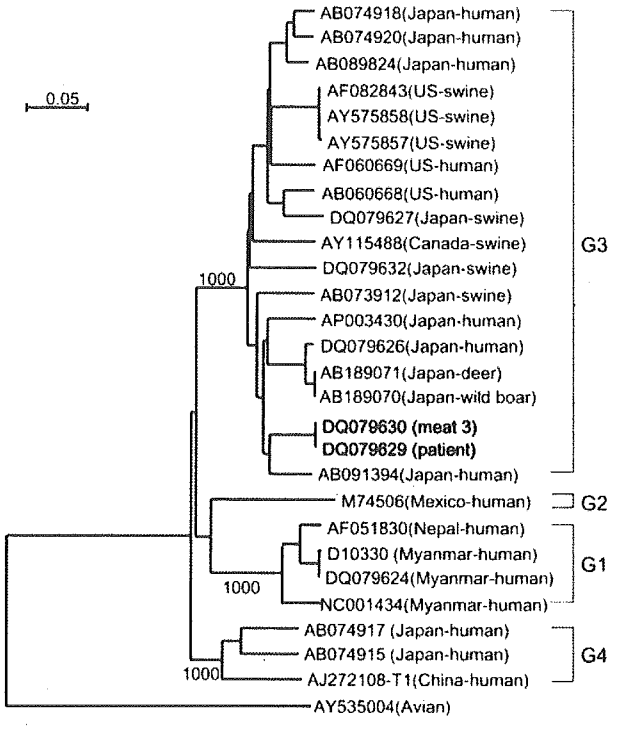

Figure. Phylogenetic tree of hepatitis E virus (HEV) reconstructed with avian HEV as an outgroup. Nucleotide sequences of the entire open reading frame 2 were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. The 2 nucleotide sequences characterized in this study are shown in **bold**. The horizontal scale bar at the top left indicates nucleotide substitutions per site.

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Dr Li is a senior researcher at National Institute of Infectious Diseases in Tokyo, Japan. His research focuses on epidemiology, expression of viral proteins, and the three-dimensional structure of hepatitis E virus.

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Address for correspondence: Tatsuo Miyamura, Department of Virology II, National Institute of Infectious Diseases. 4-7-1 Gakuen, Musashimurayama, Tokyo, 208-0011, Japan; fax: 81-42-565-4279; email: tmiyam@nih.go.jp

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Essential Elements of the Capsid Protein for Self-Assembly into Empty Virus-Like Particles of Hepatitis E Virus

Tian-Cheng Li,[1]* Naokazu Takeda,[1] Tatsuo Miyamura,[1] Yoshiharu Matsuura,[2] Joseph C. Y. Wang,[3] Helena Engvall,[3] Lena Hammar,[3] Li Xing,[3] and R. Holland Cheng[3,4]

Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011,[1] and Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Suita-shi, Osaka 565-0871,[2] Japan; Karolinska Institute, Department of Biosciences, 141 57 Huddinge, Sweden[3]; and Department of Molecular and Cellular Biology, University of California, Davis, California 95616[4]

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Hepatitis E virus (HEV) is a noncultivable virus that causes acute liver failure in humans. The virus's major capsid protein is encoded by an open reading frame 2 (ORF2) gene. When the recombinant protein consisting of amino acid (aa) residues 112 to 660 of ORF2 is expressed with a recombinant baculovirus, the protein self-assembles into virus-like particles (VLPs) (T.-C. Li, Y. Yamakawa, K. Suzuki, M. Tatsumi, M. A. Razak, T. Uchida, N. Takeda, and T. Miyamura, J. Virol. 71:7207–7213, 1997). VLPs can be found in the culture medium of infected Tn5 cells but not in that of Sf9 cells, and the major VLPs have lost the C-terminal 52 aa. To investigate the protein requirement for HEV VLP formation, we prepared 14 baculovirus recombinants to express the capsid proteins truncated at the N terminus, the C terminus, or both. The capsid protein consisting of aa residues 112 to 608 formed VLPs in Sf9 cells, suggesting that particle formation is dependent on the modification process of the ORF2 protein. In the present study, electron cryomicroscopy and image processing of VLPs produced in Sf9 and Tn5 cells indicated that they possess the same configurations and structures. Empty VLPs were found in both Tn5 and Sf9 cells infected with the recombinant containing an N-terminal truncation up to aa residue 125 and C-terminal to aa residue 601, demonstrating that the aa residues 126 to 601 are the essential elements required for the initiation of VLP assembly. The recombinant HEV VLPs are potential mucosal vaccine carrier vehicles for the presentation of foreign antigenic epitopes and may also serve as vectors for the delivery of genes to mucosal tissue for DNA vaccination and gene therapy. The results of the present study provide useful information for constructing recombinant HEV VLPs having novel functions.

Hepatitis E virus (HEV), which causes severe acute liver failure, belongs to the genus *Hepevirus* in the family *Hepeviridae* (22). HEV contains an approximately 7.2-kb single-stranded positive-sense RNA molecule (21). The RNA is 3′ polyadenylated and includes three open reading frames (ORF). ORF1, mapped in the 5′ half of the genome, encodes viral nonstructural proteins (7, 12). ORF2, located at the 3′ terminus of the genome, encodes a protein-forming viral capsid (11, 25). ORF3, mapped between ORF1 and ORF2, encodes a 13.5-kDa protein that is associated with the membrane as well as with the cytoskeleton fraction (27). This protein is shown to be phosphorylated by the cellular mitogen-activated protein kinase (6, 8). The ORF3 protein may have a regulatory function (6, 8). Ever since HEV was first discovered in 1980 and visualized by immune electron microscopy in 1983 (2), many efforts have been made, using different expression systems, to express the structural protein (5, 11, 17, 26). It is particularly important to characterize the viral protein because so far no practical cell culture system for growing HEV is available. Only one neutralization epitope has been identified; it maps between amino acids 578 and 607 of the ORF2 protein (pORF2) (18).

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: (81)-42-561-0771. Fax: (81)-42-561-4729. E-mail: litc@nih.go.jp.

The expression of foreign proteins in baculovirus systems opens the prospect of studying HEV capsid assembly, since virus-like particles (VLPs) of pronounced spikes on the surface can be formed with the recombinant protein expressed with this system (11, 25). This VLP is capable of inducing systemic and mucosal immune responses in experimental animals (9). With an oral inoculation of 10 mg of recombinant HEV VLPs, cynomolgus monkeys can develop anti-HEV immunoglobulin M (IgM), IgG, and IgA responses and protect against HEV infection (10). All these data suggest that VLPs are a candidate HEV vaccine.

The VLPs produced from Tn5 cells appear as T=1 icosahedral particles, which are composed of 60 copies of truncated pORF2 (25). The protein contains two distinctive domains: the shell (S) domain forms the semiclosed icosahedral shell, while the protrusion (P) domain interacts with the neighboring proteins to form the protrusion. The projection of T=1 recombinant HEV VLPs appears as spikes decorated with spherical rings (25), which fits with the morphology obtained from negatively stained HEV native virions. The diameter of these VLPs, 27 nm, is less than that reported for partially purified native virions (16). However, VLPs retain the antigenicity of the native HEV virion by designated antigenic sites at the P domain and by the capsid connection at the S domain. The particles appear empty, with no significant RNA-like density inside. The N-terminal region of pORF2 is rich in positively charged amino acid residues and may interact with RNA mol-

TABLE 1. Oligonucleotides used in the construction of baculovirus recombinants

Recombinant baculovirus	Forward primer[a]	Reverse primer[b]
Ac[n111]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n111c52]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**TGCTAGCGCAGAGTGGGGGGCTAAAA
Ac[n111c58]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**GGCTAAAACAGCAACCGCAGAGATGG
Ac[n111c59]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**TAAAACAGCAACCGCAGAGATGGAGA
Ac[n111c60]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**AACAGCAACCGCAGAGATGGAGACGG
Ac[n111c64]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**AGAGATGGAGACGGGACCAGCACCCA
Ac[n111c72]	AAGGATCC**ATG**GCGGTCGCTCCAGCCCATGACACCCCGCCAGT	AATCTAGA**CTA**ACCCAGGCTAGTGGTGTAAGTGGAAA
Ac[c52]	CAGGATCC**ATG**CGCCCTCGGCCTATTTTGTTGCTGCT	AATCTAGA**CTA**TGCTAGCGCAGAGTGGGGGGCTAAAA
Ac[n123]	AAGGATCC**ATG**GATGTCGACTCTCGCGGCGCCATCTT	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n124]	AAGGATCC**ATG**GTCGACTCTCGCGGCGCCATCTT	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n125]	AAGGATCC**ATG**GACTCTCGCGGCGCCATCTTGCG	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n126]	AAGGATCC**ATG**TCTCGCGGCGCCATCTTGCGCCG	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n130]	CAGGATCC**ATG**ATCTTGCGCCGGCAGTATAATCTATC	GGTCTAGA**CTA**TAACTCCCGAGTTTTACCCACCTTCTACTT
Ac[n125c59]	AAGGATCC**ATG**GACTCTCGCGGCGCCATCTTGCG	AATCTAGA**CTA**TAAAACAGCAACCGCAGAGATGGAGA

[a] BamHI (underlined) and an initiation codon (bold) are indicated.
[b] XbaI (underlined) and a stop codon (bold) are indicated.

ecules (21). Thus, the deletion of the N-terminal 111 amino acid (aa) residues and the insufficient volume of the central cavity may lead to the failure of RNA encapsidation (25).

Cell type dependence in the VLP formation of the recombinant capsid protein was observed when aa residues 112 to 660 of ORF2 were expressed with a recombinant baculovirus in two insect cell lines, Tn5 and Sf9. In Tn5 cells, two major bands, having molecular masses of 58 kDa (58K) and 53 kDa (53K), were found in the cell lysate, while a peptide in the VLPs comprising a 53K protein was found in the culture medium. The 53K protein has been designated as either the 50K or 54K protein in previous studies (9, 11). In Sf9 cells, an additional peptide with a size between that of 58K and that of 53K was found in the cell lysate. However, no VLP was recovered from the culture medium. In Tn5 cells, terminal sequencing revealed that 58K and 53K proteins have the same first 15 aa in the N terminus and that a posttranslation cleavage by cellular protease(s) occurred at the pORF2 C termini and converted 58K into 53K. An independent but similar observation was obtained when pORF2 of the Pakistani strain was expressed in Sf9 cells (17) where several immunoactive proteins were detected in the cell lysate, and a 53K protein was secreted into the culture medium, but no VLP was found. Further investigation of pORF2 expression in Sf9 and Tn5 cells may allow us to understand the mechanism underlying the subunit assembly and particle formation of the recombinant HEV capsid.

We analyzed particle formation with pORF2 containing a series of truncated deletions at the N- and/or C-terminal region. In both Sf9 and Tn5 cells, amino acid residues 126 to 601 appeared to form the pORF2 core structure and were capable of self-assembling into VLPs. These results indicated that the cell dependence on particle formation is due to the difference between Sf9 and Tn5 cells in the modification process of pORF2.

MATERIALS AND METHODS

Generation of recombinant baculoviruses and expression of capsid proteins. DNA fragments encoding the N- and/or C-terminal aa-truncated pORF2 were amplified by PCR using plasmid pHEV5134/7161 as a template. Plasmid pHEV5134/7161 containing a full-length genotype I (G1) HEV pORF2 was described previously (11). The primers used in the construction of baculovirus recombinants are shown in Table 1. Amplified DNA fragments were purified by using a QIAGEN PCR purification kit (QIAGEN, Valencia, CA), digested with restriction enzymes, and ligated with baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA). An insect cell line derived from *Spodoptera frugiperda* (Sf9) (19) (Riken Cell Bank, Tsukuba, Japan) was cotransfected with a linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Pharmingen), and the transfer vectors were cotransfected by the Lipofectin-mediated method as specified by the manufacturer (Gibco BRL, Gaithersburg, MD). The cells were incubated at 26.5°C in TC-100 medium (Gibco BRL) supplemented with 8% fetal bovine serum and 0.26% Bacto tryptose phosphate broth (Difco Laboratories, Detroit, MI). The proteins in the culture medium and cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot assay using serum from a patient with acute hepatitis E (11). Each recombinant virus was plaque purified three times. The baculovirus recombinants thus obtained were designated as Ac[n111], Ac[n111c52], Ac[n111c58], Ac[n111c59], Ac[n111c60], Ac[n111c64], Ac[n111c72], Ac[c52], Ac[n123], Ac[n124], Ac[n125], Ac[n126], Ac[n130], and Ac[n125c59]; a schematic diagram is shown in Fig. 1. Both insect Sf9 and Tn5 cells, the latter from a *Trichoplusia ni* insect cell line, BTI-Tn-5B1-4 (Invitrogen, San Diego, CA), were infected with recombinant baculoviruses at a multiplicity of infection of 10 and incubated for 5 days at 26.5°C as previously described (11, 23).

Purification of VLPs. The culture medium was harvested on day 5 after infection. The intact cells, cell debris, and progeny baculoviruses were removed by centrifugation at 10,000 × *g* for 90 min. The supernatant was then spun at 25,000 rpm for 2 h in a Beckman SW28 rotor. The resulting pellet was resuspended in 4.5 ml EX-CELL 405 at 4°C overnight. After mixing with 1.96 g of CsCl, the sample was centrifuged at 35,000 rpm for 24 h at 4°C in a Beckman SW50.1 rotor. The visible white band (at a density of 1.285 g/ml) was harvested by puncturing the tubes with a 21-gauge needle, diluted with EX-CELL 405 medium, and then centrifuged again in a Beckman TLA45 rotor at 45,000 rpm (125,000 × *g*) for 2 h to remove CsCl. The VLPs were placed on a carbon-coated grid, and the proteins were allowed to be absorbed into the grid for 5 min. After being rinsed with distilled water, the sample was stained with a 1% aqueous uranyl acetate solution and examined with a Hitachi H-7000 electron microscope operating at 75 kV.

Terminal amino acid sequence analysis. The VLPs were further purified by 5 to ~30% sucrose gradient centrifugation at 35,000 rpm for 2 h in a Beckman SW50.1 rotor. The visible white band was harvested as described above, diluted with EX-CELL 405, and again centrifuged at 45,000 rpm for 2 h in a Beckman TLA55 rotor to precipitate the VLPs. N-terminal aa microsequencing was carried out using 100 pmol of the protein by Edman automated degradation on an Applied Biosystems model 477 protein sequencer, and C-terminal aa sequencing was performed by Applied Biosystems.

SDS-PAGE and Western blot analysis. Dispersed insect cells were incubated for 20 min at room temperature to allow the cells to attach to culture flasks in TC-100 (Sf9 cells) or EX-CELL 405 (Tn5 cells) medium. The culture medium was removed, and the cells were infected with the recombinant baculoviruses at

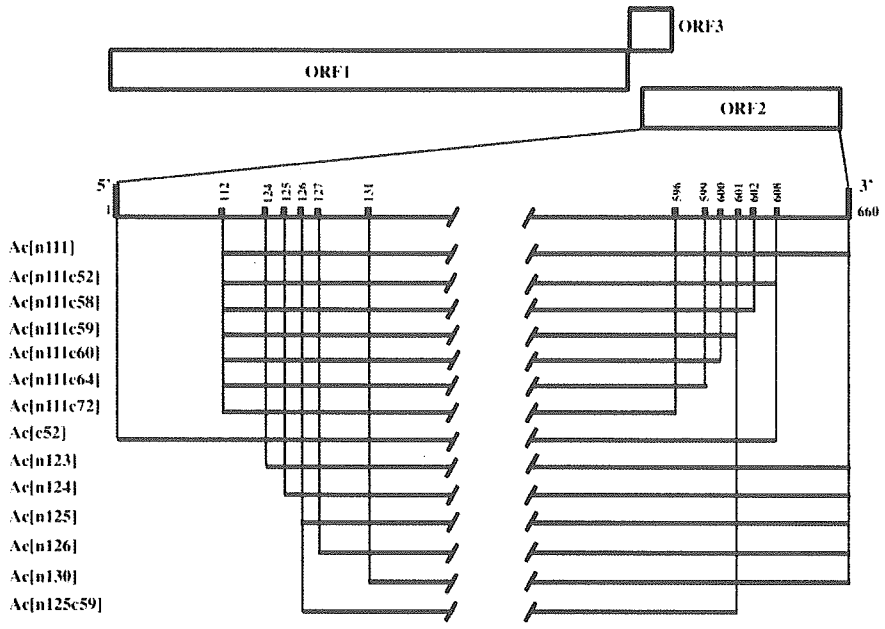

FIG. 1. Genome organization of HEV and schematic diagram of recombinant baculovirus vectors. DNA fragments encoding N- and C-terminal aa-truncated ORF2 were prepared by PCR with the primers listed in Table 1 and were used to construct 14 recombinant baculoviruses. Full-length pORF2 consisted of 660 aa. The N- and C-terminal aa numbers of the truncated protein are indicated.

a multiplicity of infection of 10. Virus adsorption was carried out for 1 h at room temperature, and then the cells were incubated at 26.5°C. The proteins in the cell lysate and in the culture medium were separated by 10% SDS-PAGE and stained with Coomassie blue. For Western blotting, the proteins in the SDS-PAGE gel were electrophoretically transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in 50 mM Tris-HCl (pH 7.4)–150 mM NaCl and reacted with a patient's serum from an acute phase. Human IgG antibody was detected by using alkaline phosphatase-conjugated goat anti-human immunoglobulin (1:1,000 dilution) (DAKO A/S, Copenhagen, Denmark). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as coloring agents (Bio-Rad Laboratories).

Cryo-electron microscopy (cryo-EM) and image reconstruction. A 3-μl drop of purified HEV VLP (~1 mg/ml) was applied onto holey carbon film. After extra solution was wiped away with filter paper, the grid was rapidly plunged into liquid ethane surrounded by liquid nitrogen. Thus embedded in a thin layer of vitrified ice, the specimen was then transferred via a Gatan 626 cryo-transfer system to a Philips CM120 microscope. The specimen was observed at liquid nitrogen temperature and photographed at a magnification of 45,000. Each area was photographed twice, with defocus levels of 1 μm and 3 μm, respectively. The electron dose of each exposure was less than 10 electrons/Å^2. The selected electron micrographs were digitized with a Zeiss scanner at a step size of 14 μm, corresponding to 3.1 Å at the specimen. The images were reconstructed according to icosahedral symmetry with Fourier-Basel procedures (4, 28). Briefly, the particle orientation and center of each image were estimated with the EMPFT program, where the structure of Tn5-produced HEV VLP was used as the initial model (1). The first reconstruction was generated from selected images and used as a model to refine the orientation and center parameters. After itinerant runs of EMPFT, the parameters were stable and appeared unchanged from one EMPFT run to another. The final reconstruction was computed by combining 353 images at a resolution of 23 Å. The surface-rendering map was generated with the NAG Explorer program combined with custom-created modules.

Mass spectrometry. The mass spectrometry experiment was done with a Reflex III mass spectrometer from Bruker, equipped with gridless delayed extraction. The samples were mixed with an equal volume of a saturated solution of sinapinic acid (Sigma Chemical Co., St. Louis, MO) in 33% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid. On the target plate, a thin layer was prepared with a saturated solution of sinapinic acid in ethanol. A sample volume of 0.5 μl was applied to a thin layer of sinapinic acid and allowed to crystallize. Data were acquired in the linear instrument mode. Data were processed and evaluated by XMASS software from Bruker.

RESULTS

C-terminal 52-amino-acid deletion is necessary for formation of VLPs in Sf9 cells. To understand the mechanism underlying VLP formation in Sf9 and Tn5 cells, we prepared a series of baculovirus recombinants expressing pORF2 with different deletions at the N- and/or C-terminal region (Table 1 and Fig. 1). The cell lysate and culture medium of infected insect cells were analyzed by Western blotting. In a previous study, the N-terminal 111 aa-truncated HEV pORF2 was expressed by a recombinant baculovirus, Ac[n111], in both insect cells (11). Two major proteins, ~58K and ~53K, were detected in both cell lysates. The 53K protein was released into cell culture medium and assembled into VLPs in Tn5 cells but not in Sf9 cells (11).

Analysis of the N- and C-terminal aa sequences of the VLPs revealed that the N terminus was at aa residue 112 and the C terminus ended at aa residue 608, indicating that the C-terminal 52 aa of ORF2 were deleted. The protein that forms VLPs contains 497 amino acids (112 to ~608), and its molecular mass was about 53K. An N-terminal 111 aa- and C-terminal 52 aa-truncated construct, Ac[n111c52], was generated, and the protein was expressed in both Sf9 and Tn5 cells. As expected, a single 53K protein was found in both Sf9 and Tn5 cell lysates (Fig. 2, Ac[n111c52] lanes in Sf9 and Tn5). Interestingly, these 53K proteins were released into both culture media as VLPs, as observed by electron microscopy (Fig. 3). The particle appeared empty and homogenous in size. Therefore, C-terminal truncation to aa residue 608 is crucial for particle formation and release into Sf9 cells.

Ac[n111c58] and Ac[n111c59] encode truncated pORF2s with an N-terminal 111-aa deletion and respective C-terminal deletions of 58 and 59 aa. The expressed proteins migrated to

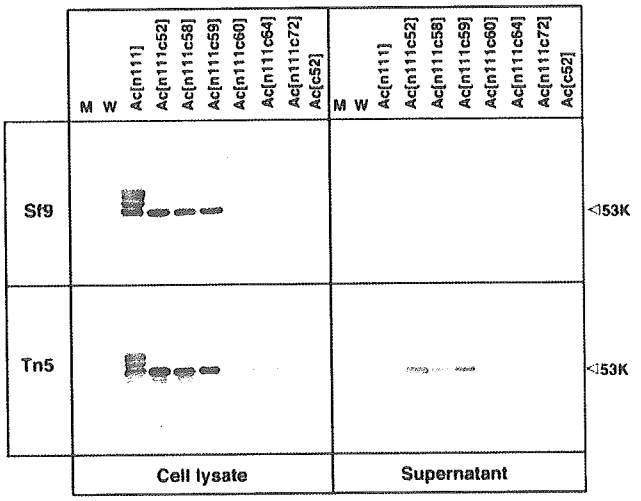

FIG. 2. Western blot assay of truncated pORF2 expressed in Sf9 and Tn5 cells. Eight recombinant baculoviruses, Ac[n111], Ac[n111c52], Ac[n111c58], Ac[n111c59], Ac[n111c60], Ac[n111c64], Ac[n111c72], and Ac[c52], were used to infect the insect cells. Ten microliters of the culture medium (right column) and 5 μl of the cell lysate (left column) were separated by 10% SDS-PAGE, and HEV-specific proteins were detected by Western blot analysis using the serum of a patient with acute hepatitis E. M, molecular weight markers; W, wild-type baculovirus-infected cells.

a position similar to that of 53K and appeared in both cell lysates as well as in the culture medium (Fig. 2); both were also assembled into VLPs (data not shown). In contrast, truncated pORF2 from Ac[n111c60], Ac[n111c64], and Ac[n111c72] was not released into the culture medium to detectable levels, and VLP was not formed even though protein expression remained similar to those of the other constructs (Fig. 2). Instead, a protein with a molecular mass of 42 kDa was detected in both of the cell lysates as well as in the culture medium by Western blot analysis. When pORF2 with a C-terminal 52-aa deletion was expressed with a recombinant baculovirus, Ac[c52], two major proteins, 65K and ~53K, were observed in infected Tn5 and Sf9 cell lysates 5 days postinfection (p.i.). However, these two proteins were not detected in their culture media (Fig. 2, Ac[c52] lanes in Sf9 and Tn5). These results indicated that aa residues before 601 were essential to the formation of VLPs.

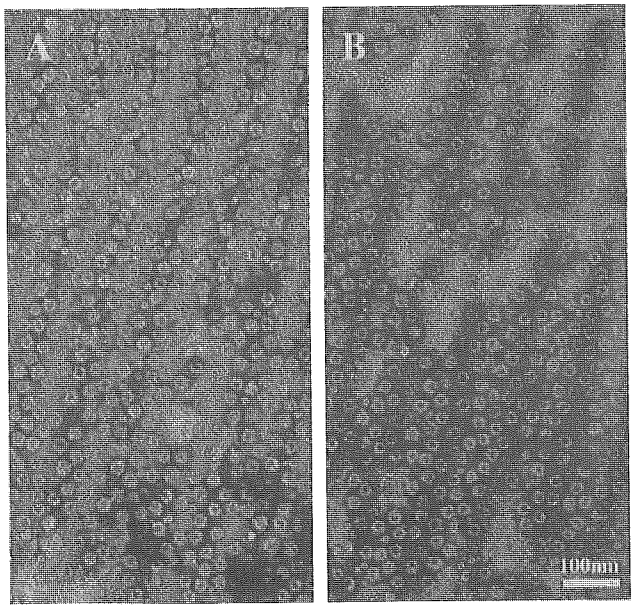

FIG. 3. EM of the HEV VLPs generated in Tn5 (A) and Sf9 (B) cells with recombinant baculovirus Ac[n111c52]. The VLPs were stained with 2% uranyl acetate. Bar, 100 nm.

VLPs produced in Sf9 and Tn5 cells possess the same configurations and structures. The morphology of the VLPs generated in Sf9 cells appeared to be similar to that generated in Tn5 cells, as observed in the negatively stained particles (Fig. 3). To investigate the structural properties of these two released VLPs, we performed cryo-electron microscopy and image processing using VLPs produced in Tn5 cells. The electron cryomicrographs showed that the particle projected as a spiky hollow sphere, indicating that no RNA-like density was packed inside the capsid (Fig. 4A). The image processing was done according to the icosahedral procedure. The rotational symmetry of 522 was applied to reconstruct the final three-dimensional structure. The reconstructed VLP displayed a T=1 surface lattice with protruding density located at each of 30 twofold axes (Fig. 4B). The VLP was composed of 60 copies of pORF2, and the protruding density consisted of dimeric, projecting domains from twofold-related peptides. The particle diameter was 270 Å, measured from the three-dimensional reconstruction. The protein shell was 85 Å thick at the twofold axes. A channel can be observed under each protruding density. The protruding density was about 43 Å high, and the twofold platform was 56 Å in the long axes (data not shown). The threefold-related dimers formed a regular triangle, and the dimer-dimer distance was 76 Å measured from center to center (Fig. 4B). Molecular interactions at the icosahedral threefold region appeared much stronger than those at the fivefold region. There was no significant difference in radial density distribution between Tn5- and Sf9-produced VLPs (Fig. 4C).

We further determined the composition of the particles obtained from Sf9 and Tn5 cells using mass spectrometry (Fig. 5). HEV VLPs produced from Tn5 and Sf9 cells with recombinant baculovirus Ac[n111c52] were analyzed. In both cases, the major density peak was monitored at the position corresponding to a mass of 53 kDa. The peak was symmetrically distributed, and a shoulder tip can be found in both cases. The shoulder tip was about 1 kDa larger than the main density peak. The signals further confirmed that the molecular mass of truncated pORF2 was 53 kDa, disregarding the production cell lines.

Essential N-terminal amino acids for VLP formation. Deletion of the N-terminal 111 residues is necessary for particle formation, which is consistent with our previous observation (11). The subsequent question is how many amino acids can be removed from pORF2 N termini without changing its capability to form VLPs. We made five constructs to express proteins with 123-, 124-, 125-, 126-, and 130-aa deletions at the N terminus by using five recombinant baculoviruses: Ac[n123], Ac[n124], Ac[n125], Ac[n126], and Ac[n130], respectively. As shown in Fig. 6, three proteins, having molecular masses of 58 to 51 kDa, were detected by Western blotting in both cell lysates at 5 days p.i., and the largest bands (58 to ~57K) were

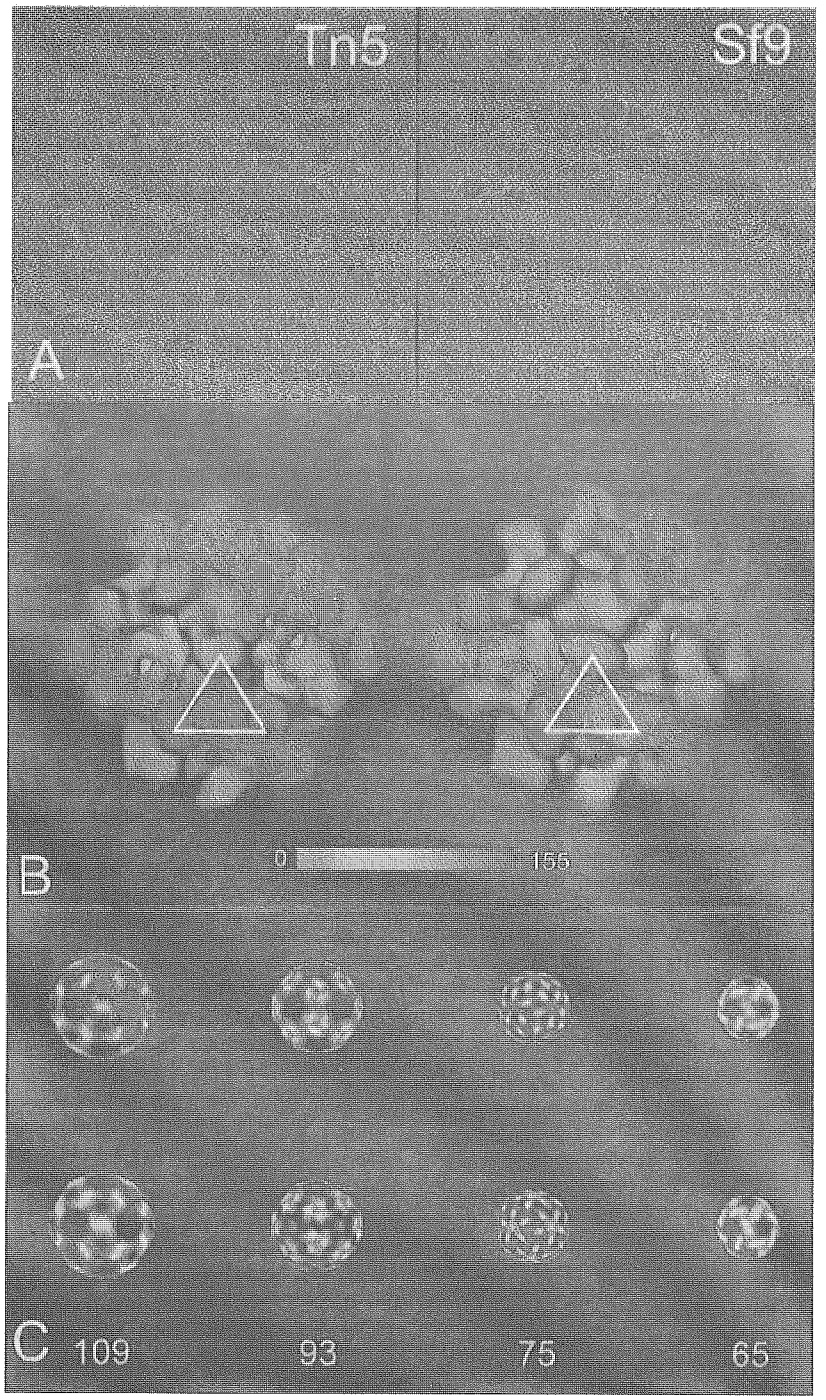

FIG. 4. HEV VLP structures determined by cryo-electron microscopy and image reconstruction. (A) Cryo-electron micrograph of ice-embedded HEV VLPs produced from Tn5 and Sf9 cells. The bar corresponds to 100 nm. (B) Surface-shaded representation of HEV VLP structures viewed along icosahedral twofold axes. VLPs from both Tn5 (left panel) and Sf9 (right panel) cells were color coded according to the radius, as indicated in the scale bar. The adjacent protruding spikes remain at equal distances of 76Å (white lines). The asterisks mark the positions of three adjacent icosahedral fivefold axes. (C) Sequential radial density projections generated from the twofold-oriented density map at corresponding radii. The protein density appears as the light color, while the background density is black.

thought to be the primary translation products encoded by N-terminal 123, 124, 125, 126, and 130 aa-truncated ORF2. In Tn5 cells, a C-terminal 52-aa-deleted product, about 51K protein, was the major protein to be efficiently released into the culture medium, where VLP formation occurred in Ac[n123]-, Ac[n124]-, and Ac[n125]-infected Tn5 cells (data not shown). Although the 51K protein was released into the culture medium, no VLP formation occurred in Ac[n126]- or Ac[n130]-

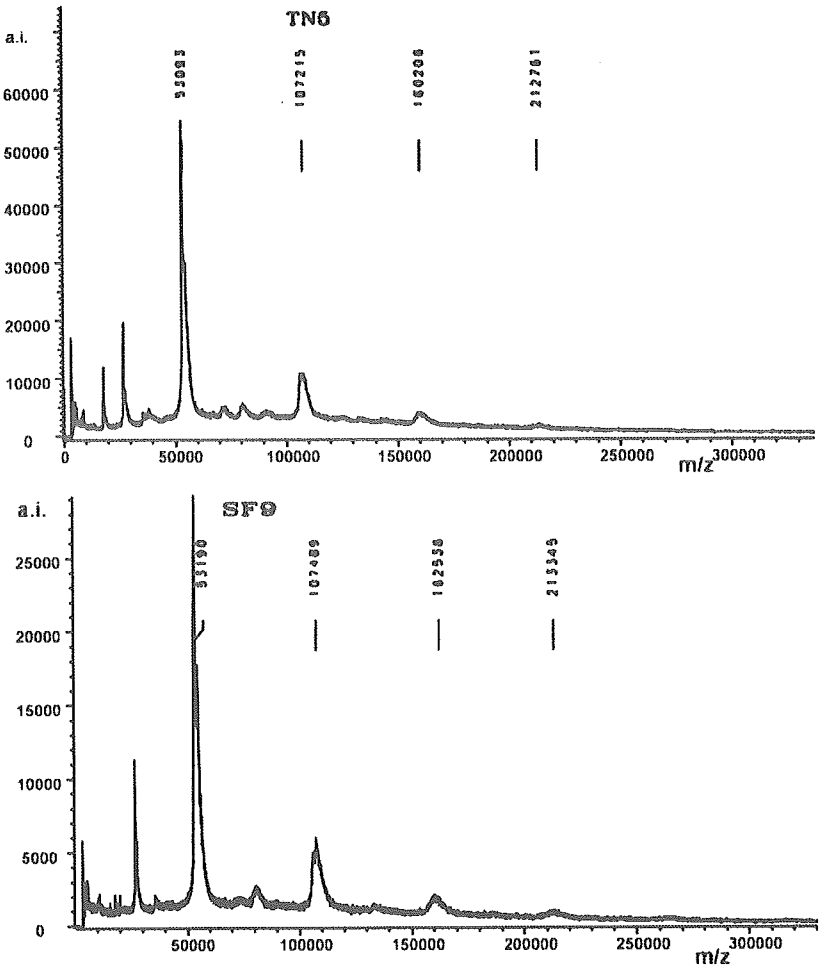

FIG. 5. Mass spectra from purified HEV VLPs displayed as the counts of isotope abundance (a.i.) versus mass/charge values (*m/z*). HEV VLPs produced from Tn5 (top panel) and Sf9 (bottom panel) cells with recombinant baculovirus Ac[n111c52] gave consistent mass spectra in which the abundant elements show similar *m/z* values at 53,000, 107,000, and 160,000.

infected Tn5 cells. In contrast, the 51K protein was not released into the culture medium in infected Sf9 cells (Fig. 6). These results demonstrated that aa residues after 125 were essential to the formation of VLPs.

When Ac[n125c59], an N-terminal 125 aa- and C-terminal 59 aa-truncated recombinant baculovirus, was expressed in Sf9 and Tn5 cells, the 51K protein was detected in both cell lysates and the culture media, where VLP formation occurred in both insect cell types (Fig. 6). This confirmed our observation that a C-terminal deletion of 52 to 59 amino acids was required for particle formation when Sf9 cells were used.

DISCUSSION

HEV is enigmatic due to the virus's inability to grow in conventional cell culture. Large quantities of the HEV capsid protein carrying antigenicity and immunogenicity comparable to those of the native virion have been generated for a long time, because the capsid protein is a key molecule for the diagnosis of hepatitis E as well as for vaccine development.

We previously found that when an N-terminal 111 aa-truncated ORF2 protein was expressed in Tn5 and Sf9 cells, two major peptides, having molecular masses of 58 and 53 kDa, were generated in both cells, and only the 53-kDa protein generated in Tn5 cells was released into culture medium and self-assembled into VLPs (11). The 58K protein presented the primary translation product, and the 53K protein is a processing product from the 58K protein. In this study, we examined the difference between Tn5 and Sf9 cells in HEV ORF2 gene expression and found that when a recombinant baculovirus (Ac[n111c52]) harboring a construct of the C-terminal 52-aa deletion was used, no difference between Sf9 and Tn5 cells in protein translation and particle formation was found. The observation that Ac[n111] failed to produce VLPs in Sf9 cells raised a question about the posttranslation modification in insect cells. In Tn5 cells, the levels of protein expression by Ac[n111] and Ac[n111c52] appeared to be similar. Therefore, it is likely that the 58K protein was incorrectly processed in Sf9 cells, thus affecting VLP assembly.

In addition, when Sf9 insect cells were infected with Ac[n111], the expressed proteins were localized in the cyto-