cell lysate at 12 hpi and was constantly synthesized thereafter. The rVP1 was only detected in the culture medium at 72 hpi, at which time most of the cells appeared broken. The rVP2 was similarly observed in the cell lysate and culture medium, but the exposure time was 15 min for rVP2 compared to 15 s for rVP1. This result indicated that the translation of rVP1 was faster and more efficient than that of rVP2.

Northern blot analysis

Total RNA was extracted from the cells and analyzed by Northern blotting. A negative-strand genomic-like RNA band (7.6 kb) was detected with the full-length construct (Fig. 4a). In contrast, a negative-strand genomic-like RNA band was not detected with the construct that had a disrupted RdRp (pT7U201F-ORF1/IGFP). A negative-strand genomic-like RNA band was not detected with any of the control constructs. These data indicated that functional RdRp was generated, and the negative-strand genomic-like RNA was synthesized from the positive-strand genomic-like RNA. A positive-strand subgenomic-like RNA band (2.6 kb) was detected with the subgenomic-like construct (pT7U201-ORF23) and was similar in size to that of the positive-strand subgenomic-like RNA band (2.6 kb) derived with the full-length construct (Fig. 4b). A positive-strand genomic-like RNA band (7.6 kb) was also detected with the pT7U201F-ORF1/IGFP construct. The 2.6-kb band was not detected with the pT7U201F-ORF1/IGFP construct. These results indicated that the viral RdRp produced the subgenomic-like RNA. The positive-strand genomic-like RNA of the pT7U201F construct was not only synthesized from vTF7, providing T7 RNA polymerase, but was also synthesized by RdRp, which was provided from pT7U201F. The ORF3 construct (pT7U201-ORF3) produced a band of 0.8 kb (Fig. 4b), i.e. the expected size of the VP2 gene. All signals disappeared when treated with DNase-free RNase A before Northern blotting.

Immunofluorescence analysis

The time-course expression of both the pT7U201F and pT7U201-ORF23 constructs was analyzed by immunofluorescence using anti-VPg MoAb and anti-VP1 purified rabbit IgG. Samples were stained at 6, 12, 24, 48, and 72 hpi. For the pT7U201-ORF23 construct, green rVP1 signals were detected in the cytoplasm from 6 to 72 hpi. The intensity of green signals became stronger over time (Fig. 5a). For the pT7U201F construct, only red rVPg signals near the nucleus were detected, and the intensity of these signals increased over time. We did not detect any green rVP1 signals for the pT7U201F construct (Fig. 5b). The time-course expression of co-transfected pT7U201F and pT7U201-ORF23 constructs was also examined with anti-VP1 and -VP2 purified rabbit IgG (Fig. 6). Samples were stained at 24, 48, and 72 hpi. The rVP1 and rVP2 were detected as green and red signals, respectively. The results suggested that expression of rVP1 was faster than that of rVP2. VP1 appeared to localize within the cytoplasm at 24, 48, and 72 hpi. At 48 hpi, rVP2 aggregated and appeared in granule-like forms, whereas at 72 hpi, rVP2 spread and co-localized with rVP1 within the cytoplasm.

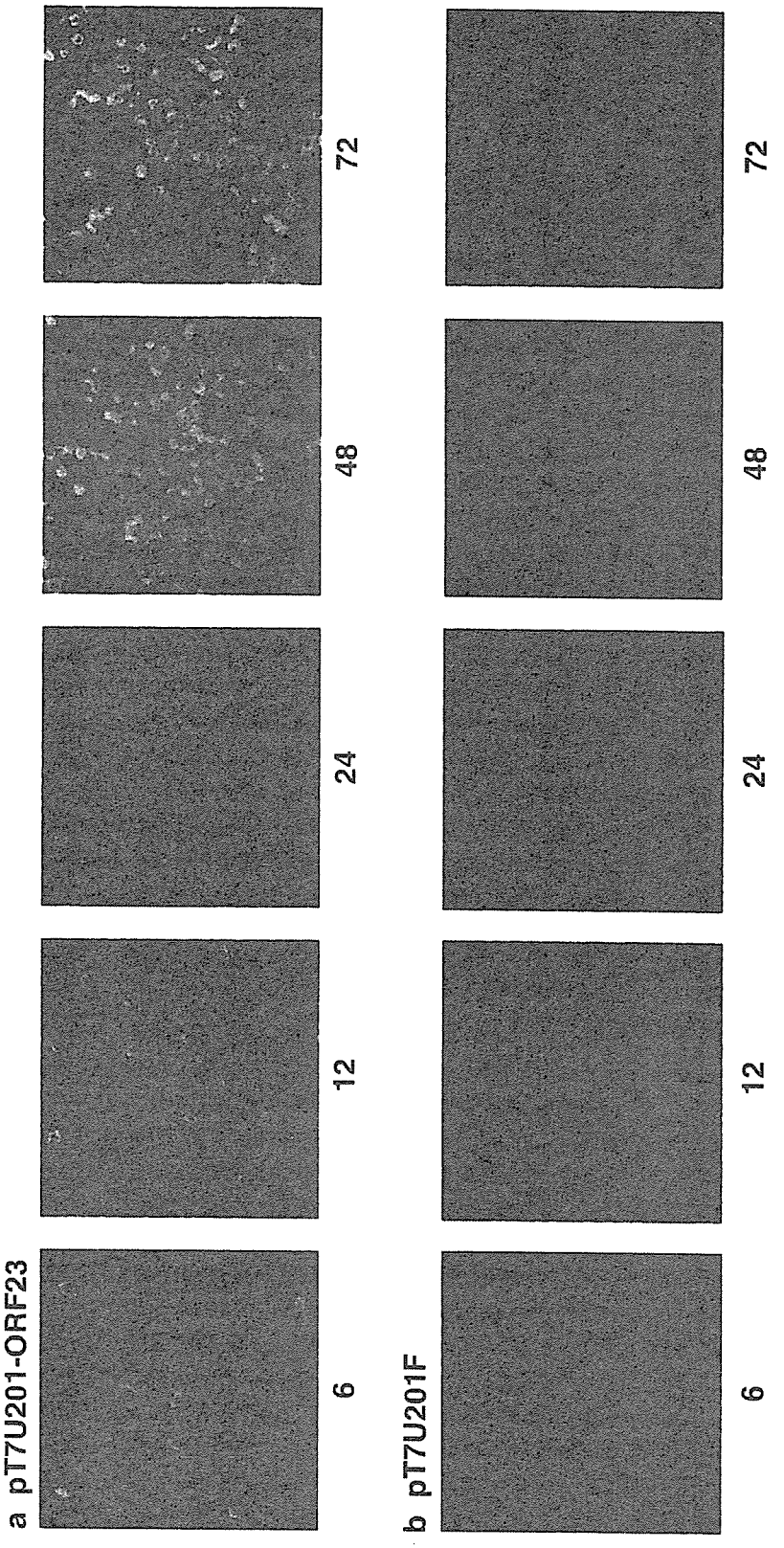

Fig. 5. The time-course expression of (**a**) pT7U201-ORF23 and (**b**) pT7U201F was analyzed by immunofluorescence with anti-VPg MoAb and anti-VP1 purified rabbit IgG. Samples were stained at 6, 12, 24, 48, and 72 hpi. For the pT7U201-ORF23 construct, green VP1 signals were detected in the cytoplasm from 6 to 72 hpi, and became stronger over time. For the pT7U201F construct, only red VPg signals near the nucleus were detected, and the intensity of these signals increased over time

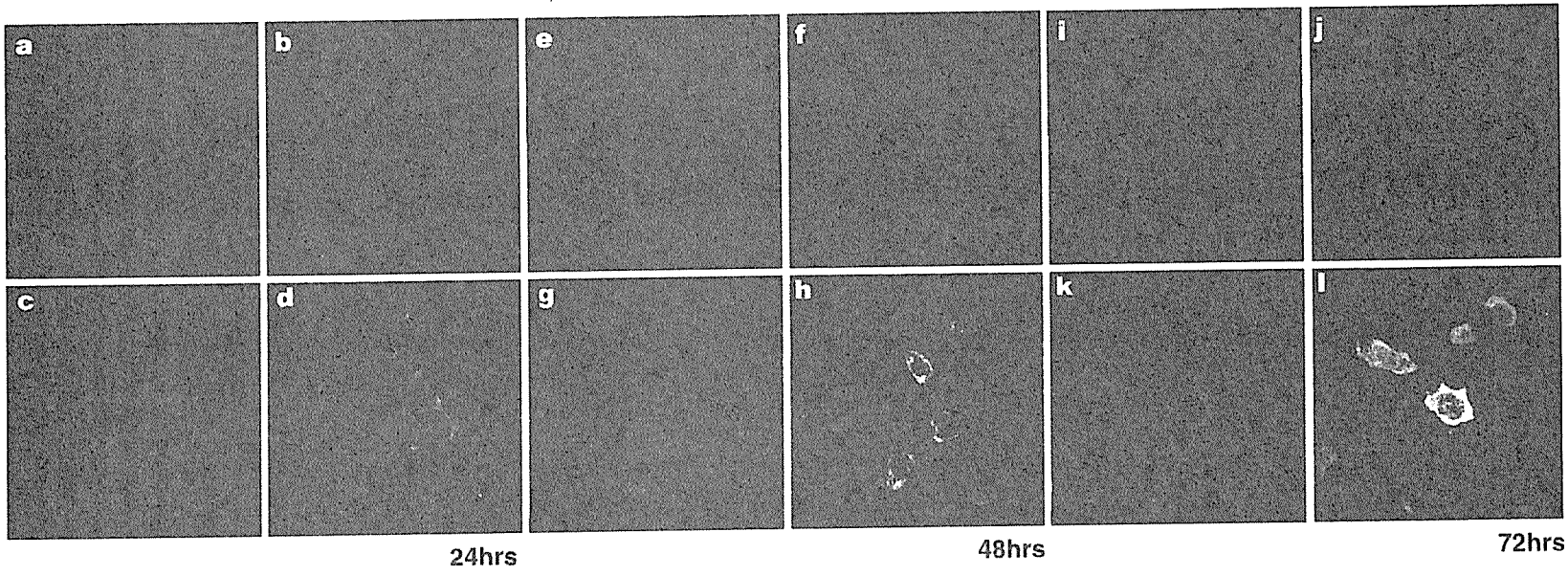

Fig. 6. The time-course expression of co-transfected pT7U201F and pT7U201-ORF23 constructs was examined with anti-VP1 and -VP2 purified rabbit IgG. Samples were stained at 24, 48, and 72 hpi. VP1 (green) and VP2 (red). A control was used throughout the experiment, i.e. no antibody (**a**, **e**, and **i**); only VP2 antibody (**b**, **f**, and **j**); only VP1 antibody (**c**, **g**, and **k**); and both VP1 and VP2 antibodies (**d**, **h**, and **l**). Expression of VP1 appeared more quickly than that of VP2. VP1 appeared to localize within the cytoplasm at 24, 48, and 72 hpi, whereas VP2 aggregated and appeared in granule-like forms at 48 hpi, and then spread and co-localized with VP1 within the cytoplasm at 72 hpi

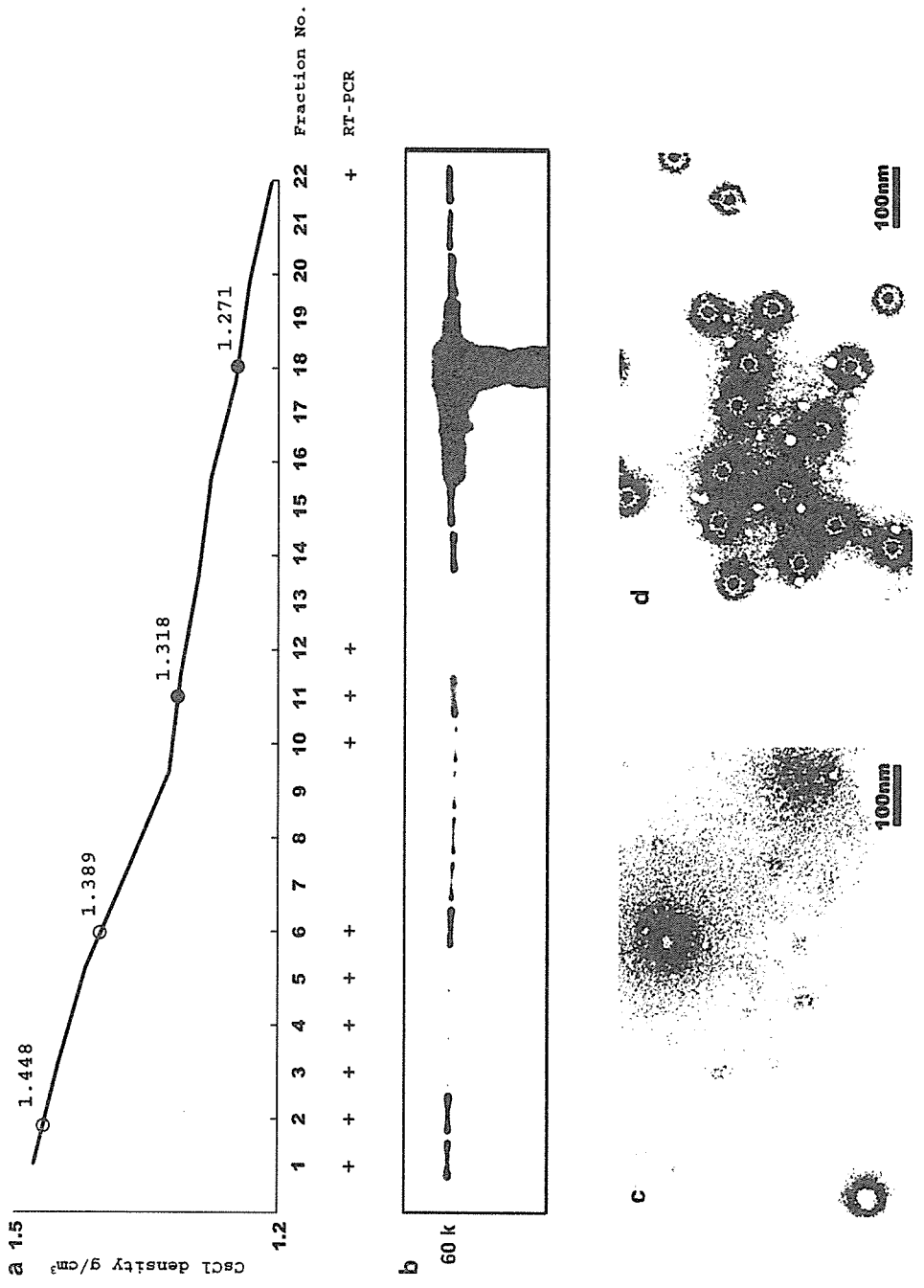

Fig. 7. Twenty-two fractions were collected from CsCl buoyant density centrifugation as described in Materials and methods, Large-scale expression of VP1 section. **a** The densities of the fractions were plotted. The open circles indicate the density of native NoV, the closed circles indicate the density of the VLPs detected in this study, and the plus signs represent positive RT-PCR fractions. **b** Western blotting of the 22 fractions using anti-VLP antisera. **c** The EM photo of fraction 11 (1.318 g/cm^3). **d** The EM photo of fraction 18 (1.271 g/cm^3). The solid black line indicates 100 nm

EM, RT-PCR, CsCl sedimentation, and Western blotting

As described above, the pT7U201F construct could provide the 7.6-kb negative strand genomic-like RNA, the 7.6-kb positive-strand genomic-like RNA, and the 2.6-kb positive subgenomic-like RNA. This construct could also express ORF1 non-structural proteins. However, our results showed that the full-length construct failed to express rVP1 and rVP2 proteins. In order to overcome this problem and to see whether VLP formation occurred, we co-transfected pT7U201F and pT7U201-ORF23 constructs, since the pT7U201-ORF23 construct was found to express rVP1 and rVP2 proteins and form VLPs (data not shown). The cell lysate was purified and analyzed by CsCl buoyant density centrifugation (Fig. 7a). Twenty-two fractions were collected and examined by single-round RT-PCR using U201 capsid region-specific primers, Western blotting, and EM (Fig. 7). Fractions 1 to 6 and 10 to 12 were positive by RT-PCR (Fig. 7a). Fraction 22 was also positive by RT-PCR, but likely included cell-associated or lipid-associated NoV RNA. Fractions 1 to 12 and 14 to 22 were positive for the 60-kDa rVP1 proteins by Western blotting (Fig. 7b). Fraction 18 (1.271 g/cm^3) showed the greatest band intensity but also included many degraded proteins. This density was slightly less than that of U201 VLPs expressed in insect cells (1.30 g/cm^3; and data not shown). Fractions 1, 2, 6 (less than 1.389 g/cm^3), and 11 (1.318 g/cm^3) had intermediate-strength rVP1 protein band intensities but were also positive by RT-PCR. Interestingly, fractions between 2 and 6 corresponded to the native NoV density. However, fractions 1, 2, and 6 contained only aggregated matter (data not shown). Fraction 11 (1.318 g/cm^3) contained round particles that ranged in size between 20 and 80 nm (Fig. 7c). Fraction 18 contained many VLPs that were morphologically similar to insect cell-expressed NoV VLPs (Fig. 7d).

Discussion

In order to better understand host infection factors, a model that closely mimics the natural host infection is required. To date, all laboratory efforts to cultivate human NoV strains have failed [7]. Nevertheless, expression of human NoV in insect or mammalian cells results in the formation of empty VLPs (i.e., without NoV nucleic acid) that are antigenically and morphologically similar to native NoV. Development of a self-replicating complete NoV VLP (i.e., containing RNA) would greatly enhance our understanding of NoV infectivity, antigenicity, and binding factors. The purpose of this study was to investigate NoV replication in a human cell line and produce an infectious artificial NoV particle.

The human NoV U201 strain was expressed in human 293T cells using a number of different constructs. Our results showed that ORF1-encoded proteins, i.e., rN-terminal protein (p37), rNTPase (p38), r3C-like protease (p18), and rRdRp (p56), were cleaved by the viral protease. These proteins were not detected with a mutated-protease construct (Fig. 2). The cleavage of the 189-kDa precursor protein was observed at 6 hpi, except in the case of r3A-VPg, which was detected at 24 hpi (Fig. 2). A time-course analysis of the proteolytic processing of the NoV MD145-12 strain's ORF1 polyprotein in an *in vitro* coupled transcription and

translation assay identified stable precursors r3A + VPg and rPro + RdRp [4]. In our system, rPro and rRdRp cleavage were fast and efficient, which was in contrast to the results obtained from the *in vitro* translation system [4]. This difference may be due to the lack of cell membranes and/or cell membrane-associated host factors accelerating the cleavage process, although we have no direct proof. Our results indicated that rRdRp was cleaved rapidly inside infected human cells and provided mature functional rRdRp (Fig. 3). We have previously shown that the NoV U201 strain's rRdRp expressed in a recombinant baculovirus expression system (insect cells) had negative-strand synthesis activity [9].

In FCV, the proteolytic cleavage sites, recognized by the virus-encoded protease, that define the borders of the nonstructural proteins were found as E/A, E/D, E/N, E/S. This protease cleaved the p38 rN-terminal precursor protein into p5.6 and p32 at E/A. The U201 rN-terminal protein had rN-terminal protein and rNTPase precursor 73-kDa and 37-kDa bands, respectively, as well as a 33-kDa minor band. The U201 strain had an ED motif at amino acids 79 and 80 from N-terminal methionine. The eighteen GII strains in the database had a PPXPXXED motif at amino acid positions 72 to 80 from U201 N-terminal methionine. This suggested that the NoV GII N-terminal protein could be cleaved by the virus protease. However, U201 proteins were expressed by a vTF7 system that also expressed vaccinia virus protease. Further investigations need to be done to investigate this possibility.

When we transfected with the subgenomic-like RNA construct (pT7U201-ORF23) we observed that rVP1 as well as rVP2 were translated (Fig. 2), suggesting that a re-initiation mechanism may occur, such as that reported for lagoviruses [23]. In contrast, neither rVP1 nor rVP2 was expressed when the full-length construct was used for transfection (Fig. 2), despite the fact that a 2.6-kb subgenomic-like RNA band was detected (Fig. 4b). This result indicates that ORF1 proteins were translated from U201 genomic RNA that was capped by the capping enzyme of the vTF7. However, the subgenomic RNA derived with the pT7U201F construct was not translated for unknown reason(s). The details of the calicivirus genome replication and transcription/translation mechanism still remain unclear, but the translation might require a cap or a cap-like structure, or VPg attached to the 5′ end of the genome and subgenome as reported for FCV [15, 30]. Recently, the interaction of NoV VPg with the translation initiation factor eIF3 has been reported [6], and thus it is possible that the NoV VPg regulates transcription and translation initiation events. In this study, a small amount of the rVPg protein (20 kDa) was detected at 24 hpi. However, a 36-kDa r3A-like + VPg precursor, analogous to the picornavirus 3AB (3A protein + VPg), remained a major product at 24 hpi, and still remained at 48 hpi (data not shown), which was similar to the 3A-like + VPg precursor identified in cell culture studies of FCV and RHDV. Interestingly, poliovirus 3A and its putative precursor 3AB are membrane-associated in infected cells, and cleavage of 3AB to 3A and 3B (VPg) by 3CD *in vitro* requires this membrane environment [32]. VPg is a strongly basic peptide predicted not to bind to the membranes of the RNA replication complex; thus, it was postulated that VPg is delivered to the replication complex in the form of a precursor molecule. In

this study, incomplete digestion between r3A-like and rVPg may have affected the translation efficiency from U201 genomic and subgenomic RNA. This suggested that r3A-like + VPg precursor proteins and cleavage events were important in NoV replication. We have not yet determined whether the rVPg was covalently attached at the 5′ end of the 2.6-kb subgenomic-like RNA and 7.6-kb genomic-like RNA. In addition, we could only investigate the maturation of ORF1 proteins until 72 hpi because of the cytopathic effect of the vTF7. Further studies will be needed to investigate NoV replication, including studies using expression systems without the vaccinia virus.

We found that the 7.6-kb negative-strand genomic-like RNA and the 2.6-kb subgenomic-like RNA were transcribed by the functional viral rRdRp. This was evident from the fact that a disrupted RdRp failed to produce genomic and subgenomic RNA. However, rVP1 and rVP2 were not expressed with the full-length construct but were expressed with a subgenomic-like RNA construct. Following this result, we co-transfected the full-length construct and subgenomic-like RNA construct for providing rVP1 and rVP2 proteins in cells. This resulted in the formation of VLPs morphologically similar to NoV with a buoyant density of 1.271 g/cm^3. EM analysis of a heavier fraction, 1.318 g/cm^3, showed round particles, 20 to 80 nm in diameter. Our results indicated that NoV RNA was incorporated into these round particles but not the VLPs. However, the density of these round particles was lighter than norovirus virions purified from stool, which have a density of 1.39–1.40 g/cm^3 in CsCl. Further studies are needed in order to investigate the possible infectivity of these round particles and to determine if they represent undeveloped VLPs.

Acknowledgements

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Genetic analysis of noroviruses associated with fatalities in healthcare facilities

Brief Report

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Summary. Norovirus outbreaks occurred in 236 healthcare facilities for the elderly in Japan during the winter of 2004–2005. Three norovirus strains associated with three fatal clinical courses were isolated from geographically separate facilities and genetically analyzed along with three strains from non-fatal cases in the same season. All six isolates were classified as the GII-4 genotype. No new variant strains like those observed in Europe in 2002 and 2004 were found in fatal cases, and the three outbreaks were deemed to have been caused by genetically close conventional norovirus GII-4 strains.

*

Norovirus (NoV) is a leading cause of acute gastroenteritis in humans and animals [10, 14], causing worldwide outbreaks in various epidemiological settings including hospitals, nursing homes, schools and restaurants [4, 8, 9, 15]. Transmission of NoV occurs via the faecal-oral route, food-borne route, person-to-person contact, and environmental contamination, and infection occurs in all age groups [4, 8, 9]. Human NoV is divided into two genogroups, genogroup I (GI) and GII [1], which are further classified into 15 and 18 genotypes, respectively, based on the capsid protein [12]. The GII-4 genotype, represented by Lordsdale virus isolated in the United Kingdom in 1993 [13], is a dominant genotype worldwide [7, 11, 12].

Although NoV causes relatively mild gastroenteritis in healthy individuals [10] with few fatal cases, elderly and immunocompromised patients can suffer from severe gastroenteritis, sometimes resulting in death [2, 8]. Fatality rates

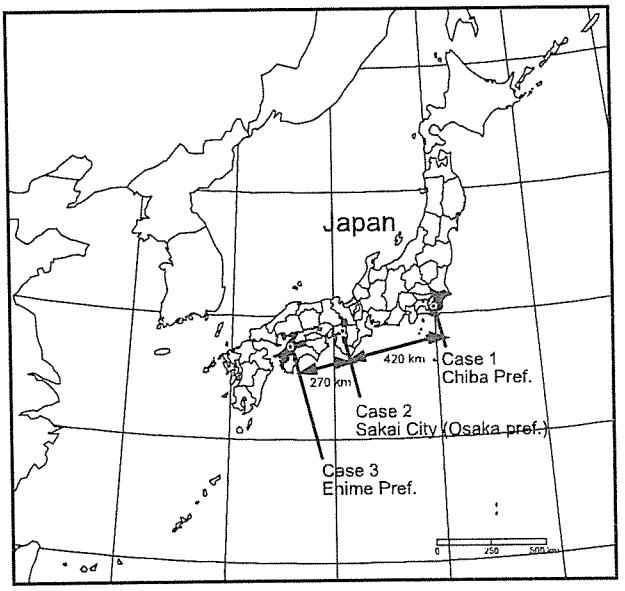

Fig. 1. Geographic relationships between three independent NoV outbreaks in Japan analyzed in this study. The geographic locations of the three outbreaks are shown in the map

associated with NoV outbreaks are reportedly 0.075 and 0.087% in England, Wales and the United States, respectively [8, 10]. In England and Wales, 43 fatal cases were observed in 38 outbreaks in hospitals and residential care facilities between 1992 and 2000. Recently, five fatal cases (fatality rate: 2.0%) associated with a large-scale gastroenteritis outbreak in nursing homes in Israel were also reported [2].

NoV outbreaks occurred in 236 healthcare facilities for the elderly in Japan in 2004–2005 with 12 fatal cases reported in six prefectures (http://www.mhlw.go.jp/houdou/2005/01/h0112-3.html). NoV samples were obtained from three independent fatal cases from geographically separate facilities in Chiba, Sakai and Ehime, respectively. The geographic locations of the three outbreaks analyzed in this study are shown in Fig. 1, and the epidemiological findings of the above three outbreaks are summarized in Table 1. Three NoV strains, Chiba/04-1050/2005 (Chiba/04-1050), Sakai/04-179/2005 (Sakai/04-179) and Ehime/05-30/2005 (Ehime/05-30), from these three fatal cases were analyzed. RNA extraction and RT-PCR targeting the 5′ end of open reading frame (ORF) 2 followed by genetic analysis were performed as described previously [12]. Comparisons of the nucleotide sequences demonstrated that these three strains had high nucleotide identities (approximately 99%), and these strains were classified into genotype GII-4 (data not shown). For further genome analysis, the NoV genome was amplified as three separate overlapping segments. The amplified products were directly sequenced as previously described [12], and the complete nucleotide sequence of Chiba/04-1050 and nearly complete nucleotide sequences minus the 5′ terminus of Sakai/04-179 and Ehime/05-30 were determined. Nucleotide sequences determined in this study were submitted to DDBJ with accession numbers AB220921 to AB220926.

Table 1. Summary of the three independent NoV outbreaks in healthcare facilities for the elderly analyzed in this study

	Case 1	Case 2	Case 3
Location Facility	Chiba Prefecture special nursing home for the elderly	Sakai City (Osaka Prefecture) hospital and healthcare facility for the elderly	Ehime Prefecture healthcare facility for the elderly
Total number of individuals			
residents	78	484 (in total)	97
workers	60		69
Affected individuals			
residents	43 (55.1%)	68 (14.0)%	35 (36.1)%
workers	20 (33.3%)		15 (21.7)%
Duration period	1st to 16th of January, 2005	3rd to 28th of January, 2005	2nd to 15th of January, 2005
Major symptoms	diarrhea, vomiting, fever, abdominal pain	diarrhea, vomiting, fever, abdominal pain	diarrhea, vomiting, fever
Number of death	1	1	1
Fatality rate	1.59%	1.47%	2.0%
NV testing methods	RT-PCR, electron microscopy	RT-PCR	RT-PCR, electron microscopy
Tested samples			
residents	10 stools & 1 vomitus	9 stools	12 stools & 2 vomitus
workers	3 stools	ND	2 stools
Positivity for NV[a]			
residents	7 [5] stools & 1[0] vomitus	7 stools	9 [6] & 2 [2] vomitus
workers	0 [0] stools	ND	2 [0] stools
Rate of positive samples[a]	57.1 [35.7]%	77.8%	81.3 [50.0]%
Enteric bacterial pathogen	not detected	not detected	not detected
Fatal cases			
age, sex	82 years, female	95 years, female	90 years, male
onset	8th January	9th January	7th January
death	10th January	17th January	10th January
Cause of death	suffocation as a result of vomiting	septicemia	Acute bleeding in the gastrointestinal tract
Sample ID	Chiba/04-1050/2005	Sakai/04-179/2005	Ehime/05-30/2005
Source of NV detection	stool on 9th January	stool on 17th January	vomitus on 10th January
NV genotype (ORF2)	GII-4	GII-4	GII-4

[a]Values in brackets show positivity with electron microscopy; other value show positivity with RT-PCR

Chiba/04-1050 was composed of 7,559 nucleotides without a poly-A tail, while the other two strains from Sakai and Ehime comprised 7,533 nucleotides lacking the 5′ terminus. The average nucleotide identities among the three strains were 99.2% in ORF 1, 98.6% in ORF 2, and 98.8% in ORF 3. In addition, 10, 4

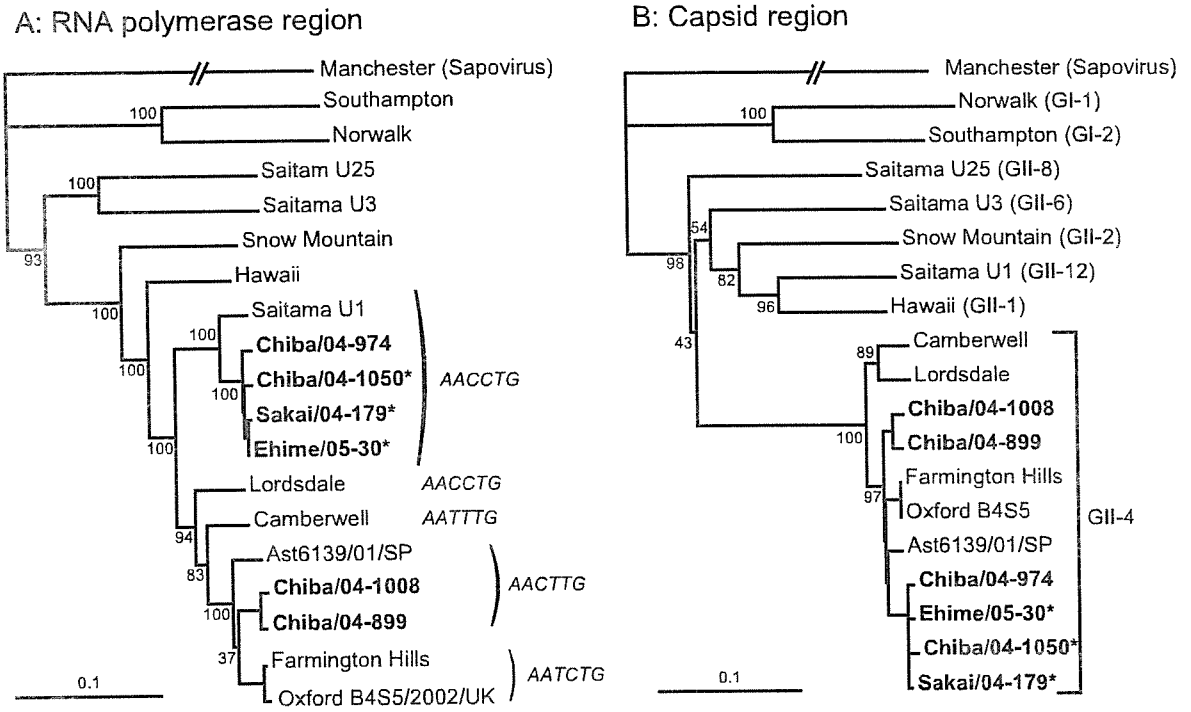

Fig. 2. Phylogenetic trees were constructed using the neighbor-joining method based on part of the RdRp region corresponding to 4307–5017 (A) and the capsid region corresponding to 5085–5509 (B) of Lordsdale virus. A sapovirus, Manchester strain, was used as the out-group. The six strains examined are shown in bold. Three strains from fatal cases are shown by asterisks, in which the complete and nearly complete genomes were amplified by RT-PCR with the following primers: NV5END (GAATGAAGATG GCGTCTAACGACG) and NV2690R (TGAGACCTTTGCTTGAGAAGGCTGT) for the 5′ genome region, NV2570F (CCAAAACCCAAAGATGATGAGGAGT) and NV5550R (GGTAAGGGGATCAACACAGGTTCCA) for the central region, and G2F1 and dT25VN [(T)25V(A/G/C)N(A/G/C/T)] for the 3′ genome region [12]. The 5′ end of the genome was amplified with the 5′ RACE Amplification System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Nucleotide sequences characterized as GII-4 variants reported by Lopman et al. [7] are also indicated in italic on the tree (A). Reference strains were Manchester virus (X86560), Norwalk virus (M87661), Southampton virus (L07418), Snow Mountain virus (AY134748), Lordsdale virus (X86557), Camberwell virus (AF145896), Farmington Hills (AY502023), Ast6139/01/SP (AJ583672), Oxford B4S5 (AY587984), Saitama U25 (AB067543), SaitamaU3 (AB039776) and Saitama U1 (AB039775)

and 6 amino acid substitutions were identified in each ORF. Phylogenetic trees based on the partial RNA-dependent RNA polymerase (RdRp) region (ORF1) and partial capsid region (ORF2) are shown in Fig. 2A and B. Phylogenetic analysis based on ORF2 indicated that the three strains were genetically close and clustered together with known GII-4 strains (Fig. 2B). These strains were also clustered with GII-4 strains when the RdRp region was compared (Fig. 2A). The Saitama

U1 strain is a recombinant strain between a GII-4-like (ORF 1) and GII-12 (ORF 2) strain [5]. These results clearly indicate that the three strains isolated from fatal cases were genetically close and indistinguishable from known GII-4 strains.

To further investigate the RdRp and capsid regions, an additional three strains detected in Chiba prefecture in the same season, Chiba/04-899/2004 (Chiba/04-899; outbreak in a nursery school), Chiba/04-974/2004 (Chiba/04-974, sporadic gastroenteritis patient) and Chiba/04-1008/2004 (Chiba/04-1008, outbreak in a healthcare facility) were similarly analyzed. Based on the capsid protein, these three strains were also grouped into GII-4 and shown to be closely related to the three strains from the fatal cases (Fig. 2B). When the RdRp region was compared, the three fatal case strains and Chiba/04-974 were closely related and grouped into a cluster including the Saitama U1 strain (Fig. 2A). In contrast, Chiba/04-899 and Chiba/04-1008 were closely related to other GII-4 strains including Lordsdale virus. Therefore, the six strains analyzed in this study were deemed conventional GII-4 strains widely circulating in this season. In addition, at least two genetically distinct GII-4 strains with different ORF1 sequences were shown to be co-circulating at the same time in Chiba prefecture.

Lopman et al. reported an increase in NoV-associated gastroenteritis in European countries due to emergence of new genetic variants of the GII-4 strain [7]. GII-4 strains detected before 2002 have an "AACTTG" sequence in the RdRp region while those detected in 2002 (new variants) show "AATCTG" [7]. Intermediate sequences have also been observed [7, 13]. Of the six GII-4 strains analyzed in this study, the three fatal strains and Chiba/040974 showed an intermediate sequence, "AACCTG" (Fig. 2A). The other two strains, Chiba/040899 and Chiba/041008, had 10 nucleotide substitutions in the RdRp region, which were observed in GII-4 2004 variant strains [6]. Therefore, no new variant strains like those isolated in 2002 and 2004 were identified in fatal cases in this study.

Previous studies have described the GII-4 genotype as the dominant genotype of NoV-associated gastroenteritis worldwide [7, 12, 15]. Furthermore, GII-4 strains are mainly detected in outbreaks in healthcare facilities such as nursing homes and hospitals [4, 9]. Lopman et al. reported that outbreaks in healthcare facilities showed a higher death rate and prolonged duration when compared to other outbreak settings [8]. Recently, fatal cases associated with GII-4 NoV outbreaks in nursing homes have been reported in Israel [2]. We detected genetically similar GII-4 strains from three independent outbreaks in geographically isolated healthcare facilities in Japan. Sequence analysis comparisons with an additional three strains from Chiba prefecture clearly indicated that these strains were not specific to these outbreaks. Although NoV infection is not likely the principal cause of death in most cases, NoV-associated outbreaks occurring in healthcare facilities for the elderly might constitute an additional burden. As neither common food nor food stuff was identified in the three fatal cases presented here, person-to-person transmission by either direct contact with stool or vomitus or through the caregiver was considered the most likely mode of transmission. These findings

suggest that we need to pay more attention to the activity of NoV, especially that of the GII-4 genotype, in outbreaks in healthcare facilities.

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Genetic and antigenic diversity among noroviruses

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Human norovirus (NoV) strains cause a considerable number of outbreaks of gastroenteritis worldwide. Based on their capsid gene (VP1) sequence, human NoV strains can be grouped into two genogroups (GI and GII) and at least 14 GI and 17 GII genotypes (GI/1–14 and GII/1–17). Human NoV strains cannot be propagated in cell-culture systems, but expression of recombinant VP1 in insect cells results in the formation of virus-like particles (VLPs). In order to understand NoV antigenic relationships better, cross-reactivity among 26 different NoV VLPs was analysed. Phylogenetic analyses grouped these NoV strains into six GI and 12 GII genotypes. An antibody ELISA using polyclonal antisera raised against these VLPs was used to determine cross-reactivity. Antisera reacted strongly with homologous VLPs; however, a number of novel cross-reactivities among different genotypes was observed. For example, GI/11 antiserum showed a broad-range cross-reactivity, detecting two GI and 10 GII genotypes. Likewise, GII/1, GII/10 and GII/12 antisera showed a broad-range cross-reactivity, detecting several other distinct GII genotypes. Alignment of VP1 amino acid sequences suggested that these broad-range cross-reactivities were due to conserved amino acid residues located within the shell and/or P1-1 domains. However, unusual cross-reactivities among different GII/3 antisera were found, with the results indicating that both conserved amino acid residues and VP1 secondary structures influence antigenicity.

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INTRODUCTION

Norovirus (NoV) strains are a leading cause of gastroenteritis worldwide and cause outbreaks in various epidemiological settings including hospitals, cruise ships, schools and restaurants (Beuret *et al.*, 2003; Inouye *et al.*,

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are AB081723, AB058547, DQ093065, AB195225, DQ093062, DQ093066, DQ093063, AB195226, DQ093064, AB039780, AY237414 and DQ093067.

2000; Johansson *et al.*, 2002; Kapikian *et al.*, 1972; McEvoy *et al.*, 1996; McIntyre *et al.*, 2002; Russo *et al.*, 1997). Numerous molecular epidemiological studies have revealed a global distribution of these viruses (Nakata *et al.*, 1998; Noel *et al.*, 1999; White *et al.*, 2002). Transmission routes include food-borne, person-to-person contact and environmental contamination. Human NoV can be divided into two genetically distinct genogroups, GI and GII. Recently, NoV strains were subdivided into at least 14 GI and 17 GII genotypes (GI/1–14 and GII/1–17) (Kageyama *et al.*, 2004). These viruses cannot be grown in culture and their antigenic relationships are not completely understood. Nevertheless, expression of the major capsid protein (VP1), which usually results in the formation of virus-like particles (VLPs) that are morphologically similar to the native virus, has permitted a better understanding of antigenicity in these viruses (Hansman *et al.*, 2004). Two types of assay have been used to examine cross-reactivity among these VLPs: antibody ELISA and antigen ELISA (Gray *et al.*, 1993; Jiang *et al.*, 1995a, b; Kageyama *et al.*, 2004; Kamata *et al.*, 2005; Kobayashi *et al.*, 2000a, b, c). The antibody ELISA is broadly reactive, but the antigen ELISA is highly specific, only detecting strains that are closely related (>95 % identity in the RNA polymerase region). However, detailed information on the cross-reactivity among many of the genetically distinct NoV strains is limited.

NoVs are small round viruses approximately 38 nm in diameter and possess a single-stranded, positive-sense RNA genome of 7·5–7·7 kb. The NoV genome contains three open reading frames (ORFs). ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase, ORF2 encodes VP1 and ORF3 encodes a minor capsid protein (VP2) (Jiang *et al.*, 1990). Cryo-electron microscopy (cryo-EM) and X-ray crystallography analyses of NoV VLPs have determined the shell and protruding domains (subdomains P1-1, P1-2 and P2) of the capsid protein (Prasad *et al.*, 1999). Chen *et al.* (2004) also described strictly and moderately conserved amino acid residues in the capsid protein among the four genera in the family *Caliciviridae*.

The aim of this study was to analyse cross-reactivity among 26 different NoV VLPs in order to understand NoV genetic and antigenic relationships in more detail. An antibody ELISA using polyclonal antisera raised against the VLPs was used to determine cross-reactivities. Our results found broad-range cross-reactivities with antisera raised against a number of distinct NoV strains.

METHODS

Specimens. Positive stool specimens were collected from a number of different sources (see GenBank accession numbers) and RNA was extracted as described previously (Katayama *et al.*, 2002). PCR-generated amplicons or plasmids were excised from the gel and purified by using the QIAquick Gel Extraction kit and Plasmid Purification kit (Qiagen). Nucleotide sequences were prepared with the BigDye Terminator Cycle Sequence kit (version 3.1) (Applied Biosystems) and determined by using the ABI 3100 Avant sequencer (Perkin-Elmer ABI). Nucleotide sequences were aligned with CLUSTAL_X and distances were calculated using Kimura's two-parameter method. Phylogenetic trees with bootstrap analysis from 1000 replicas were generated by the neighbour-joining method as described previously (Kageyama *et al.*, 2004). Amino acid VP1 secondary structure predictions were made using the PSIPRED secondary structure prediction software (McGuffin *et al.*, 2000).

Expression of VLPs. Previously, we expressed four GI NoV strains: GI/1 (strain SeV), GI/2 (strain 258), GI/3 (strain 645) and GI/4 (strain CV), and nine GII NoV strains: GII/3 (strain 809), GII/4 (strain 104), GII/5 (strain 754), GII/6 (strain 7k), GII/7 (strain 10-25), GII/10 (strain 026), GII/12 (strains CHV and 9912-02F; in this study 9912-02F was termed Hiro) and GII/14 (strain 47) (Hansman *et al.*, 2004; Kamata *et al.*, 2005; Kitamoto *et al.*, 2002; Kobayashi *et al.*, 2000a, b, c). Dr Kim Green provided us with the Hawaii virus recombinant baculovirus GII/1 (strain HV) (Green *et al.*, 1997). In this study, we expressed an additional 12 VLPs: GI/8 strain WUG1 (using primers G1SKF and TX30SXN; see Table 1 for primer sequences); GI/11 strain

Table 1. Primer sequences used for expression of VLPs

Primer	Sense/antisense	Sequence (5′–3′)*
G1SKF	Sense	CTGCCCGAATTYGTAAATGA
TX30SXN	Antisense	GACTAGTTCTAGATCGCGAGCGGCCGCCC$(T)_{30}$
G2/F3	Sense	TTGTGAATGAAGATGGCGTCGA
G2R0	Antisense	CCATTACTGAACCCTTCTACGCC
G2Fb	Sense	TGGGAGGGCGATCGCAATCT
G2R04	Antisense	GGCGTAGAAGGYTTCAYTAAGTC
MVR1	Antisense	AATTATTGAATCCTTCTACGCCCG
G2F2	Sense	GTGAATGAAGATGGCGTCGA
G2R03	Antisense	GGCGTAGAAGGATTCAATAATGG
G2F02	Sense	GTGAATGAAGATGGCGTCGAATGA
G2SKF	Sense	CNTGGGAGGGCGATCGCAA
NAL13	Sense	GATCTCGCTCCCGATTTTTGTGA
N235R	Antisense	ATGGCWGGAGCTTTRATAGC

*Y=C or T; N=A, C, G or T; W=A or T.

#8 (primers G1SKF and TX30SXN); GII/1 strain 485 (primers G2/F3 and G2R0); GII/2 strain Ina (primers G2Fb and G2R04); GII/3 strain 18-3 (primers G2/F3 and MVR1); GII/3 strain 1152 (primers G2F2 and G2R03); GII/3 strain 336 [primers G2/F3 and Oligo-$(dT)_{33}$]; GII/3 strain Sh5 (primers G2F02 and G2R03); GII/6 strain 445 [primers G2/F3 and Oligo-$(dT)_{33}$]; GII/8 strains Mc24 and U25 (primers G2SKF and TX30SXN); and GII/17 strain Alph23 (primers NAL13 and N235R). For expression of the recombinant VP1 in insect cells, all of the NoV constructs were designed to begin from the predicted VP1 AUG start codon. For six of the 12 constructs, the VP2 and poly(A) sequences were included by using either the TX30SXN or Oligo-$(dT)_{33}$ reverse primers (strains WUG1, #8, 336, 445 and Mc24). One construct (strain 485) excluded the poly(A) sequence, whereas the remaining five constructs excluded both the VP2 and poly(A) sequences (strains Alph23, Sh5, 1152 and 18-3 and Ina). Four constructs that were amplified with the TX30SXN reverse primer were expressed using the Gateway expression system (strains WUG1, #8, U25 and Mc24) (Hansman *et al.*, 2004), whilst the other eight constructs were expressed in a baculovirus expression system as described previously (Kamata *et al.*, 2005).

VLP purification and electron microscopy (EM). Recombinant baculovirus shuttle vectors (bacmids) were transfected into Sf9 cells using Effectene according to the manufacturer's instructions (Qiagen). Sf9 cells were incubated for 5–6 days at 26 °C, after which the culture medium was clarified by low-speed centrifugation and the supernatant was stored as the seed baculovirus. Tn5 cells were infected with the seed baculovirus at 26 °C and harvested 5–6 days post-infection. VLPs secreted into the cell medium were separated from cells by low-speed centrifugation, concentrated by ultracentrifugation at 30 000 r.p.m. at 4 °C for 2 h (Beckman SW-32 rotor) and then resuspended in 100 µl Grace's medium. VLPs were purified by CsCl equilibrium gradient ultracentrifugation at 45 000 r.p.m. at 15 °C for 18 h (Beckman SW-55 rotor). The harvested culture medium was examined for VLPs by negative-staining EM. Briefly, the samples (diluted 1 : 10 in distilled water) were applied to a carbon-coated 300-mesh EM grid and stained with 2 % uranyl acetate (pH 4). Grids were examined under an electron microscope (JEM-1220; JEOL) operating at 80 kV.

Antibody production and ELISA. Hyperimmune sera to newly developed VLPs were prepared in rabbits. The first subcutaneous injection was performed with purified VLPs (between 10 and 500 µg) in Freund's complete adjuvant. After 3 weeks, the animals received two or three booster injections of the same amount of VLPs in Freund's incomplete adjuvant at intervals of 1 week. The animals were bled 1 week after the last booster injection. An antibody ELISA was used to compare cross-reactivities among the VLPs. Then wells of 96-well microtitre plates (Maxisorp; Nunc) were each coated with 100 µl purified VLPs (1·0 µg ml^{-1} in carbonate/bicarbonate buffer, pH 9·6; Sigma) and incubated overnight at 4 °C. The wells were washed twice with PBS containing 0·1 % (v/v) Tween 20 (PBS-T)

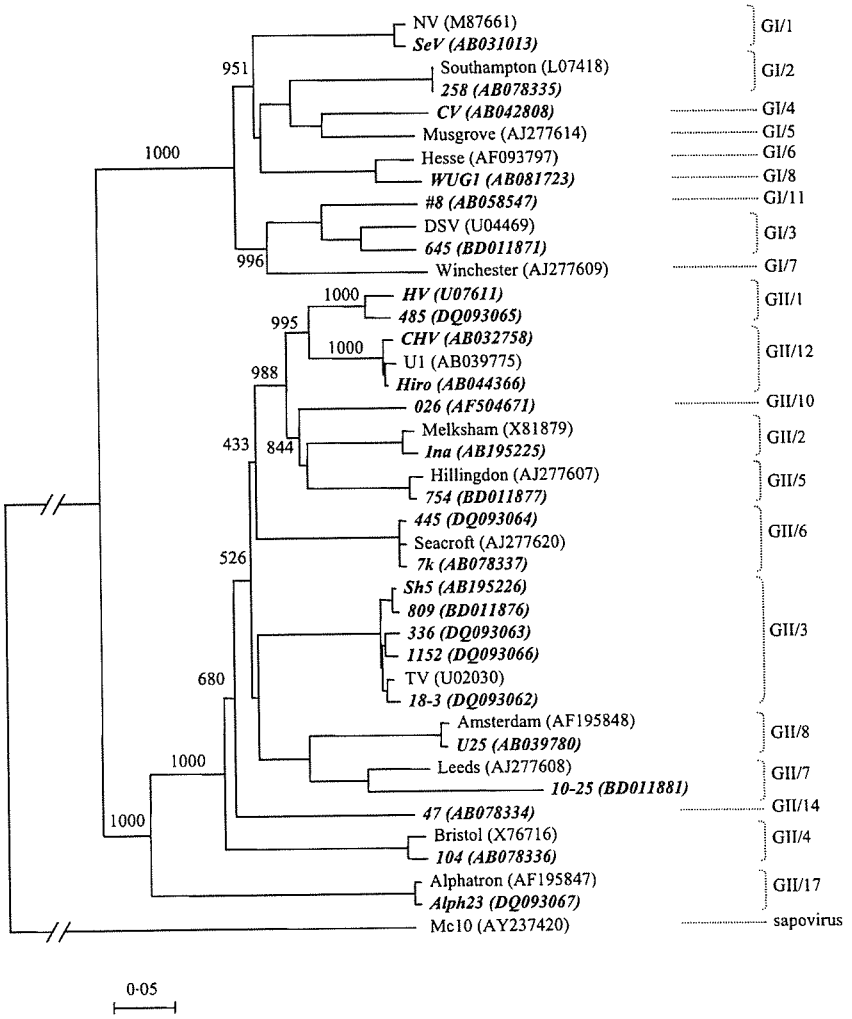

Fig. 1. Phylogenetic tree of NoV sequences examined in this study (shown in bold italic). NoV amino acid sequences were constructed using the entire VP1 sequence (the complete sequence for Mc24 was unavailable). Numbers on branches indicate bootstrap values for the clusters; values of 950 or higher were considered statistically significant for the grouping (Katayama *et al.*, 2002). Reference sequences have been reported previously (Kageyama *et al.*, 2004).

and then blocked with PBS containing 5% (w/v) skimmed milk (PBS-SM) for 1 h at room temperature. After the wells had been washed twice with PBS-T, 100 µl twofold serially diluted hyperimmune rabbit antiserum from a starting dilution of 1:2000 in PBS-T-SM was added to each well and the plates were incubated for 1 h at 37 °C. The wells were washed six times with PBS-T and 100 µl horseradish peroxidase-conjugated anti-rabbit IgG (1:1000 dilution in PBS-T-SM) was added to each well. The plates were incubated for 1 h at 37 °C. The wells were washed six times with PBS-T and 100 µl *o*-phenylenediamine substrate and H_2O_2 was added to each well. The plates were left in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 µl 1 M H_2SO_4 to each well and the absorbance was measured at 492 nm. ELISA titres were expressed as the reciprocal of the highest dilution of antiserum giving a value of $A_{492} > 0{\cdot}2$.

RESULTS

Sequence analysis

Nucleotide and amino acid sequences were aligned using CLUSTAL_X and distances were calculated using Kimura's two-parameter method. We divided the 26 strains used in this study into six GI and 12 GII genotypes using partial N-terminal VP1 nucleotide sequences (data not shown). These genotypes were maintained when we grouped the complete VP1 amino acid sequences (Fig. 1). Mc24 was excluded from the amino acid analysis since the full-length capsid sequence was unavailable. Nevertheless, using the partial N-terminal VP1 nucleotide sequence (GenBank accession no. AY237414), Mc24 clustered in GII/8 and was closely related to strain U25. Of the recently described NoV strains (Kageyama *et al.*, 2004), the GI and GII genotypes used in this study represented 43% (6/14) and 76% (13/17), respectively. For several GII genotypes, we used two or more VLPs in order to clarify antigenicity, including GII/1 (strains HV and 485), GII/3 (strains 809, Sh5, 336, 1152 and 18-3), GII/6 (strains 7k and 445) and GII/12 (strains CHV and Hiro).

Expression of VP1

EM confirmed that all strains formed VLPs with morphological features similar to native NoV (Fig. 2), despite the fact that different constructs and expression systems were used to express the recombinant VP1. The VLPs retained their morphological features, even when stored for >6 months at −20 °C (data not shown).

Homologous antigenic analysis

An antibody ELISA, which uses polyclonal antiserum raised against one type of VLP, was used to determine cross-reactivity among the 26 different NoV VLPs. ELISA titres were expressed as the reciprocal of the highest dilution of antiserum giving a value of $A_{492} > 0{\cdot}2$. A negative control (baculovirus-infected Tn5 cell lysate) was used for all experiments and found to be negligible [i.e. $A_{492} < 0{\cdot}05$, using up to 0·5 µg (ml lysate)$^{-1}$]. Antisera reacted strongly against homologous VLPs, with titres ranging from 102 400 to 1 638 400 (Table 2).

Heterologous antigenic analysis

We observed a number of novel cross-reactivities among different genotypes. For example, Fig. 3(a) shows the strong cross-reactivity of GI/11 #8 antiserum with both GII/6 7k and GII/6 445 VLPs. We found that GI/11 #8 antiserum cross-reacted with these GII/6 VLPs at titres of 102 400,

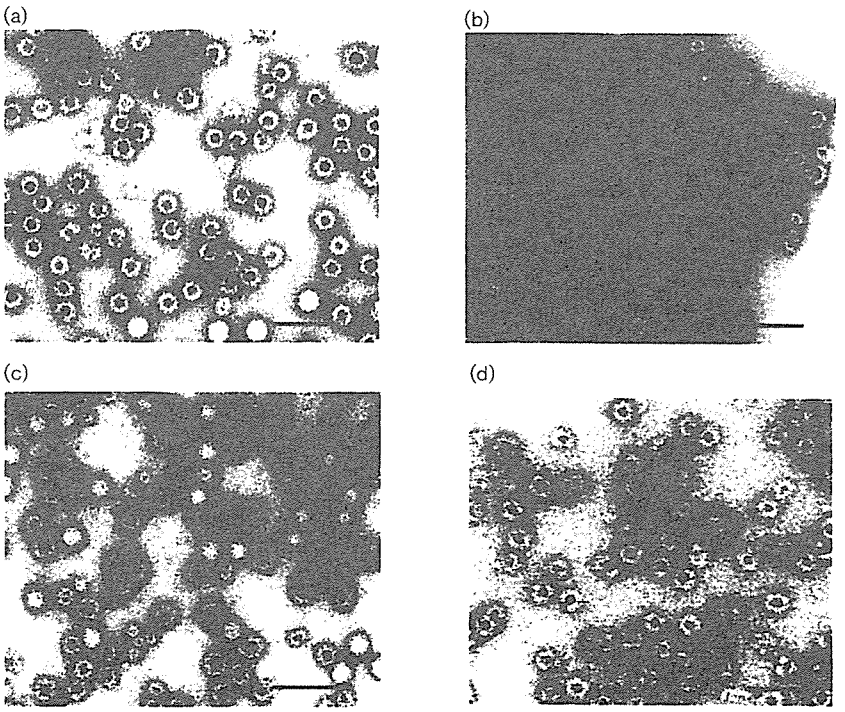

Fig. 2. EM images of CsCl-purified NoV VLPs negatively stained with 2% uranyl acetate (pH 4). (a) Strain 7k, (b) strain 485, (c) strain 445 and (d) strain 645. Bar, 100 nm.