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Intergenogroup Recombination in Sapoviruses

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Sapovirus, a member of the family *Caliciviridae*, is an etiologic agent of gastroenteritis in humans and pigs. Analyses of the complete genome sequences led us to identify the first sapovirus intergenogroup recombinant strain. Phylogenetic analysis of the nonstructural region (i.e., genome start to capsid start) grouped this strain into genogroup II, whereas the structural region (i.e., capsid start to genome end) grouped this strain into genogroup IV. We found that a recombination event occurred at the polymerase and capsid junction. This is the first report of intergenogroup recombination for any calicivirus and highlights a possible route of zoonoses because sapovirus strains that infect pig species belong to genogroup III.

The family *Caliciviridae* contains 4 genera, *Sapovirus*, *Norovirus*, *Lagovirus*, and *Vesivirus*. The sapovirus (SaV) and norovirus (NoV) strains are etiologic agents of gastroenteritis in humans, although animals such as pigs, cows, and mice can also be infected. SaV strains were originally detected by using electron microscopy, but today the most widely used method is reverse transcription–polymerase chain reaction (RT-PCR), which has a high sensitivity (1). Based on the capsid gene sequence, SaV can be grouped into 5 distinct genogroups (GI to GV) (2). Human SaV belong to GI, GII, GIV, and GV, whereas pig SaV belongs to GIII. The SaV GI, GIV, and GV genomes are believed to each contain 3 main open reading frames (ORFs), whereas the SaV GII and GIII genomes each have only 2 main ORFs (2). ORF1 encodes nonstructural proteins and the capsid protein, while ORF2 and ORF3 encode proteins of yet-unknown functions. Using complete genome sequence analysis, we recently identified the first recombinant (intragenogroup) SaV strains (3). Two SaV strains, Mc10 and C12, both belonging to GII, were identified as recombinants. Phylogenetic analysis of the nonstructural region (i.e., genome start to capsid start) grouped Mc10 and C12 together in 1 GII cluster (or genotype), while the structural region (i.e., capsid start to genome end) grouped Mc10 and C12 into distinct GII genotypes. Evidence suggested that the recombination site occurred at the polymerase and capsid junction on ORF1. This site is highly conserved among SaV strains, which suggests that the recombination event occurs when nucleic acids of parental strains come into physical contact in infected cells, e.g., during copy choice recombination (4), as we have recently described with recombinant NoV strains (5).

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Materials and Methods

We compared the complete genome sequences of 11 SaV strains to analyze suspected novel recombinant SaV strains. For this study, we sequenced the complete genomes of 4 SaV strains (Mc2, SK15, Ehime1107, and SW278). The Mc2 strain was isolated from a child with gastroenteritis in Chiang Mai, Thailand, in 2000 (6); SK15 was isolated from an adult with gastroenteritis in Sakai, Japan, in 2001 (unpub. data); Ehime1107 was isolated from an adult with gastroenteritis in Matsuyama, Japan, in 2002 (unpub. data); and SW278 was isolated from an adult with gastroenteritis in Solna, Sweden, in 2003 (7). The complete genome sequences were amplified and sequenced as described earlier (3). Phylogenetic analysis was performed by using the Genetyx program (Genetyx for the Macintosh version 13.0.5, Genetyx Corp., Tokyo, Japan) and ClustalX (Version 1.82; available from http://www.embl.de/~chenna/clustal/darwin/). Trees were drawn by using njplot (for the Macintosh; available from http://pbil.univ-lyon1.fr/software/njplot.html).

Results

Based on the classification scheme of either the partial or complete capsid sequences in our previous studies, we grouped Manchester into GI; Bristol, Mc2, Mc10, C12, and SK15 into GII; PEC into GIII; and NK24 into GV (6,8,9). For this study and on the basis of the structural region (i.e., capsid start to genome end), we grouped Manchester into GI; Mc2, Bristol, Mc10, C12; and SK15 into GII; PEC into GIII; SW278 and Ehime1107 into GIV, and NK24 into GV (Figure 1). These genogroups were not maintained when we analyzed the nonstructural region (i.e., genome start to capsid start). We found that SW278 and Ehime1107 clustered into GII for the nonstructural region–based grouping but clustered into GIV for the structural region–based grouping. All genogroups were supported by bootstrap values (10), except for the structural region–based grouping of GI, which had a slightly lower value of 897. Nevertheless, these results indicate that the nonstructural region of SW278 and Ehime1107, i.e., a GIV sequence, did not belong to a distinct genogroup, unlike their structural region, which belonged to a distinct genogroup (proposed as GIV). Comparisons of the complete genome sequences showed that SW278 and Ehime1107 shared >97% nucleotide identity and likely represented the same strain, although it was isolated from different countries; however, the lengths were different. Either SW278 or Ehime1107 had a 10-nucleotide insertion or deletion in the nontranslated region at the 3′ terminus. A number of closely matching partial sequences to SW278 and Ehime1107, which included both the polymerase and capsid gene, were available on the database, which indicates the circulation of similar strains in other countries.

We next used SimPlot (available from http://sray.med.som.jhmi.edu/SCRoftware/simplot/) with a window size of 100 and an increment of 20 bp (11) to further analyze these novel recombinant SW278 and Ehime1107 strains. We analyzed 7 complete genome SaV sequences. The Mc10 genome sequence was compared to C12, Bristol, Mc2, SK15, SW278, and Ehime1107. We observed a sudden drop in nucleotide similarity after the polymerase region for SW278 and Ehime1107 (Figure 2A). Nucleotide sequence analysis of the nonstructural region showed that SW278 and Ehime1107 shared between 74.0% to 77.6% nucleotide identity to the Mc2, C12, Mc10, and SK15 sequences, whereas analysis of the structural region showed that SW278 and Ehime1107 had only 54.0%–55.2% nucleotide identity to the Mc2, C12, Mc10, and SK15 sequences (Table); i.e., the nonstructural and structural regions of SW278 and Ehime1107 were »20% different. A similar result was observed with the nonstructural and structural regions of the already-established recombinant Mc10 and C12 strains, which had an 18.6% difference (3). When we analyzed the nonstructural and structural regions of Mc2 and SK15, we found only a 1.5% difference. Likewise, all other SaV strains generally maintained their nucleotide identities over the complete genome (Table). This result can be best explained as a recombination event at the polymerase and capsid junction for the SW278 and Ehime1107 strains, i.e., the nonstructural region originated from a GII strain, and the structural region originated from a strain belonging to another genogroup. The SaV GI, GIV, and GV genomes are predicted to encode an ORF3, whereas the SaV GII and GIII genomes have 2 main ORFs. We found that SW278 and Ehime1107 each had an ORF3, which is predicted to encode a yet-unknown protein of 161 amino acids. Notably, the structural region–based grouping showed that GI, GIV, and GV grouped in 1 major branch, while GII and GIII represented 2 other branches. These data provide further evidence of the intergenogroup recombination for SW278 and Ehime1107 strains.

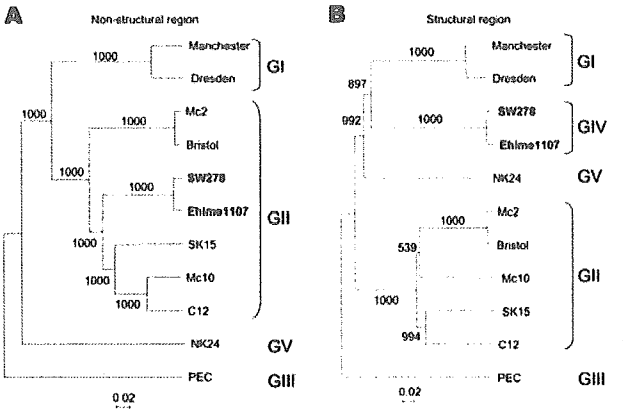

Figure 1. Phylogenetic analysis of (A) the nonstructural region (i.e., genome start to capsid start) and (B) the structural region (i.e., capsid start to genome end), showing the different genogroups. The numbers on each branch indicate the bootstrap values for the genotype. Bootstrap values ≥950 were considered significant for the grouping (10). The scale represents nucleotide substitutions per site. GenBank accession numbers are as follows: Mc10, AY237420; Manchester, X86560; Dresden, AY694184; SW278, DQ125333; Ehime1107, DQ058829; NK24, AY646856; C12, AY603425; Bristol, AJ249939; Mc2, AY237419; PEC, AF182760; and SK15, AY646855.

The SaV subgenomic RNA has not yet been identified, but for other caliciviruses the subgenomic RNA was identified (12–14). We recently provided evidence that the SaV viral protease was responsible for the cleavage of nonstructural and capsid proteins on ORF1 (15). Therefore, SaV replication may occur through at least 2 pathways: 1) the capsid protein was transcribed as a polyprotein on ORF1 and then cleaved and translated, or 2) the capsid protein was transcribed as subgenomic RNA and then translated. The suspected recombination site occurred at

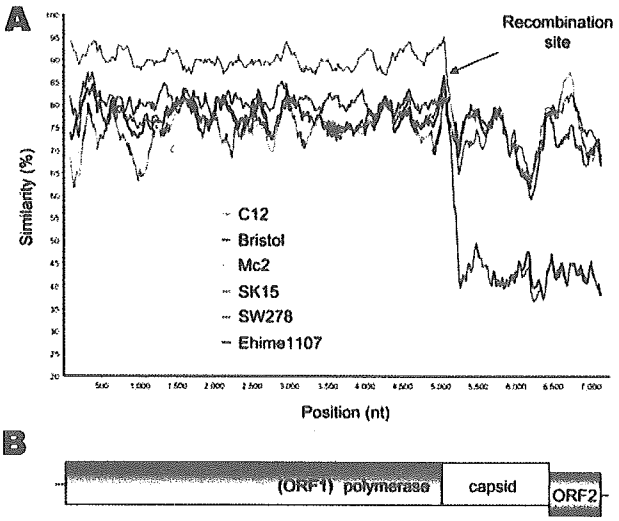

Figure 2. A) SimPlot analysis of 7 sapovirus (SaV) complete genome sequences. The Mc10 genome sequence was compared to C12, Bristol, Mc2, SK15, SW278, and Ehime1107 by using a window size of 100 bp with an increment of 20 bp. All gaps were removed. The recombination site is suspected to be located between the polymerase and capsid gene, as shown by the arrows. B) Genomic organization of the SaV GII and GIV strains.

the highly conserved polymerase and capsid junction for human SaV, as shown in Figure 3. Recombination is thought to occur when nucleic acids of the parental strains come into physical contact in infected cells, e.g., during copy choice recombination (4). These data suggest that recombinant SaV strains were formed either by full-length RNA template switching or full-length and subgenomic template switching.

Discussion

These results are noteworthy because this is the first report of intergenogroup recombination for any calicivirus. These findings provide evidence that zoonoses could occur within the *Sapovirus* genus because strains that infect pig species belong to GIII. Furthermore, since the parent nonstructural region of SW278 and Ehime1107 has not yet been identified, we could not rule out that the parents of SW278 and Ehime1107 came from a strain that infects animals. We have conducted a number of molecular epidemiologic studies using broad-range primers and found that GIV strains were infrequently compared to other genogroups (6,8,9,16,17). This finding suggests 1) the emergence and/or recombination of GIV strains from an animal reservoir, 2) a lower prevalence of GIV strains, though a number of similar sequences were identified in the United States, or 3) our primers were less sensitive in detecting variant GIV sequences. Nevertheless, further complete genome analysis of other SaV strains is needed to identify other recombinant strains and determine the extent of recombination in the *Sapovirus* genus. Although we cannot easily pinpoint where and when the recombination event took place, screening of animals with primers designed against human SaV strains may also help identity the potential parental strain(s) of these 2 novel recombinants.

Conclusions

To date, we have identified 4 different recombinant SaV strains, Mc10, C12, SW278, and Ehime1107. Collectively, these strains have 2 kinds of nonstructural sequences but 3 kinds of structural sequences (Figure 1). In addition, all nonstructural sequences belonged to GII. These data suggest that SaV could evade host immunity by readily changing their structural region (immunoreactive, i.e., capsid protein) and that GII strains (nonstructural–based grouping) are more capable of recombination than other genogroups. In 1999, Jiang et al. (18) identified the first naturally occurring human recombinant NoV, and several other strains were later described as recombinants (5,6,19–21). The site of genetic recombination for NoV was also between the polymerase and capsid genes. Human SaV and NoV strains cannot be cultivated, but the expression of the recombinant capsid

Table. Nucleotide identity (%) among sapovirus strains*

Nonstructural region	Structural region									
	Ehime1107	SW278	Mc2	Mc10	C12	SK15	Dresden	Manchester	NK24	PEC
Ehime1107		96.9	54.6	55.1	54.4	55.2	58.3	58.8	58.3	51.0
SW278	97.5		54.6	55.2	54.0	54.7	58.1	58.3	58.3	50.8
Mc2	73.8	74.0		72.7	73.0	71.8	54.5	54.0	53.7	51.2
Mc10	77.5	77.6	74.4		71.5	71.1	55.3	54.6	55.2	50.7
C12	77.3	77.3	74.4	90.1		75.0	55.4	55.7	55.5	51.8
SK15	77.5	77.5	73.3	81.0	80.3		56.2	56.1	55.5	50.4
Dresden	62.6	62.7	63.3	63.0	63.1	62.4		92.9	57.3	52.5
Manchester	63.5	63.3	63.2	63.7	64.0	62.8	90.5		57.4	52.1
NK24	55.4	55.6	55.8	55.7	55.0	55.2	56.3	56.8		53.3
PEC	52.5	52.5	53.0	52.3	52.4	52.3	51.7	51.5	52.1	

*Values on the lower left represent the nonstructural region, i.e., genome start to capsid start; values on the upper right represent the structural region, i.e., capsid start to genome end.

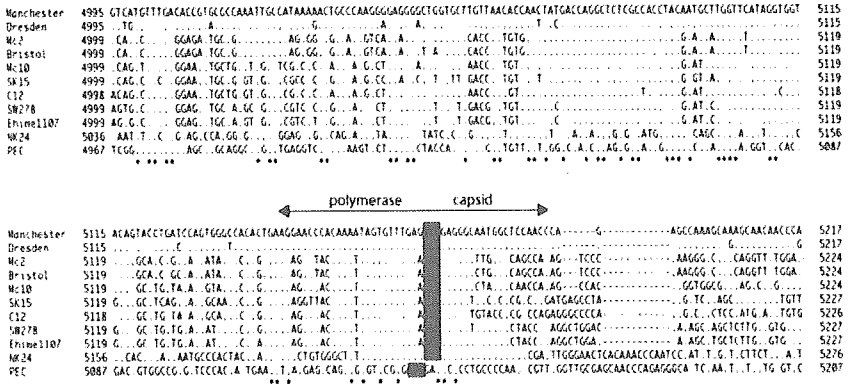

Figure 3. Nucleotide alignment of Manchester, Dresden, Mc2, Bristol, Mc10, SK15, C12, SW278, Ehime1107, NK24, and PEC sequences, showing the conserved polymerase and capsid junction. The asterisks represent conserved nucleotides. The shaded nucleotides represent the putative capsid start codons.

protein (rVP1) in a baculovirus expression system results in the self-assembly of viruslike particles (VLPs) that are morphologically similar to native SaV. In a recent study, we genetically and antigenically analyzed 2 recombinant NoV strains (strains 026 and 9912-02F) (17). When polymerase-based grouping was performed, these 2 strains clustered together, but when capsid-based grouping was performed, these 2 strains belonged in 2 distinct genotypes. When we compared the cross-reactivity of these VLPs with an antibody enzyme-linked immunosorbent assay (ELISA), the titers of 026 antiserum against 026 and 9912-02F VLPs were 1:2,058,000 and 1:512,000, respectively, a 4-fold difference, whereas the titers of 9912-02F antiserum against 9912-02F and 026 VLPs were 1:1,024,000 and 1:128,000, respectively, an 8-fold difference. These results demonstrated that 026 and 9912-02F likely represented distinct antigenic types, which correlated with the genetic analysis. The expression of SaV VLPs is also needed to determine the cross-reactivity among these recombinant strains, although our results have shown that GI and GV VLPs (capsid-based grouping) were antigenically distinct by an antibody and antigen ELISA (22), which suggests that these 2 recombinant strains are also antigenically distinct from GII strains. And finally, these results will have a major influence on the future phylogenetic classification of SaV strains. Therefore, the genetic classification of SaV strains needs to be addressed, and a consensus of prototype strains representing genogroups and genotypes should be established to avoid further grouping conflicts.

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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)

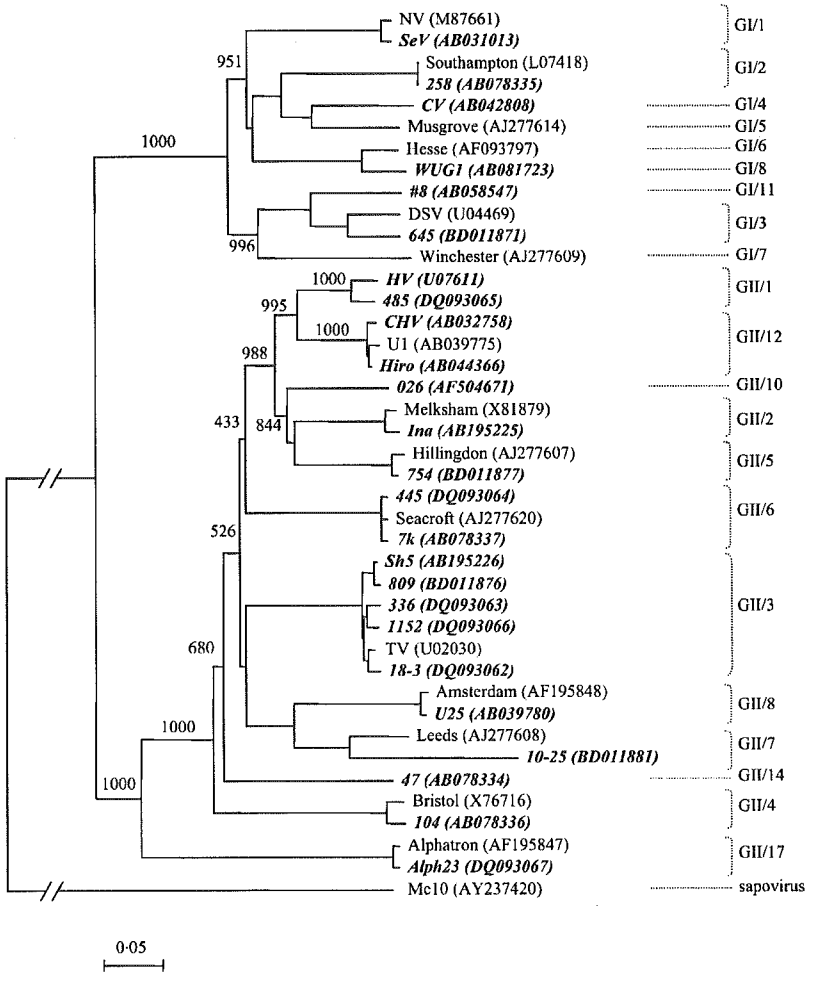


Fig. 1. Phylogenetic tree of NoV sequences examined in this study (shown in bold italic). NoV amino acid sequences were constructed using the entire VP1 sequence (the complete sequence for Mc24 was unavailable). Numbers on branches indicate bootstrap values for the clusters; values of 950 or higher were 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,

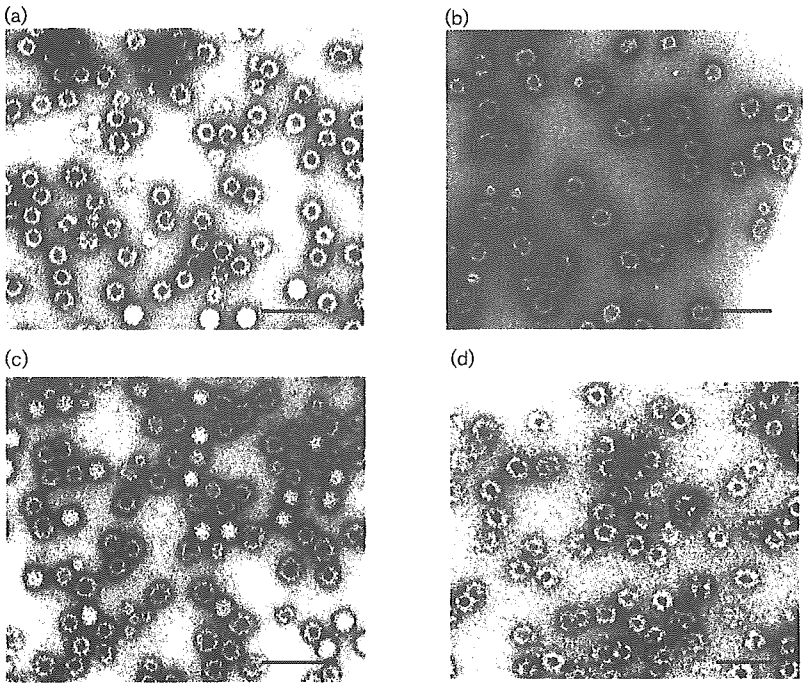
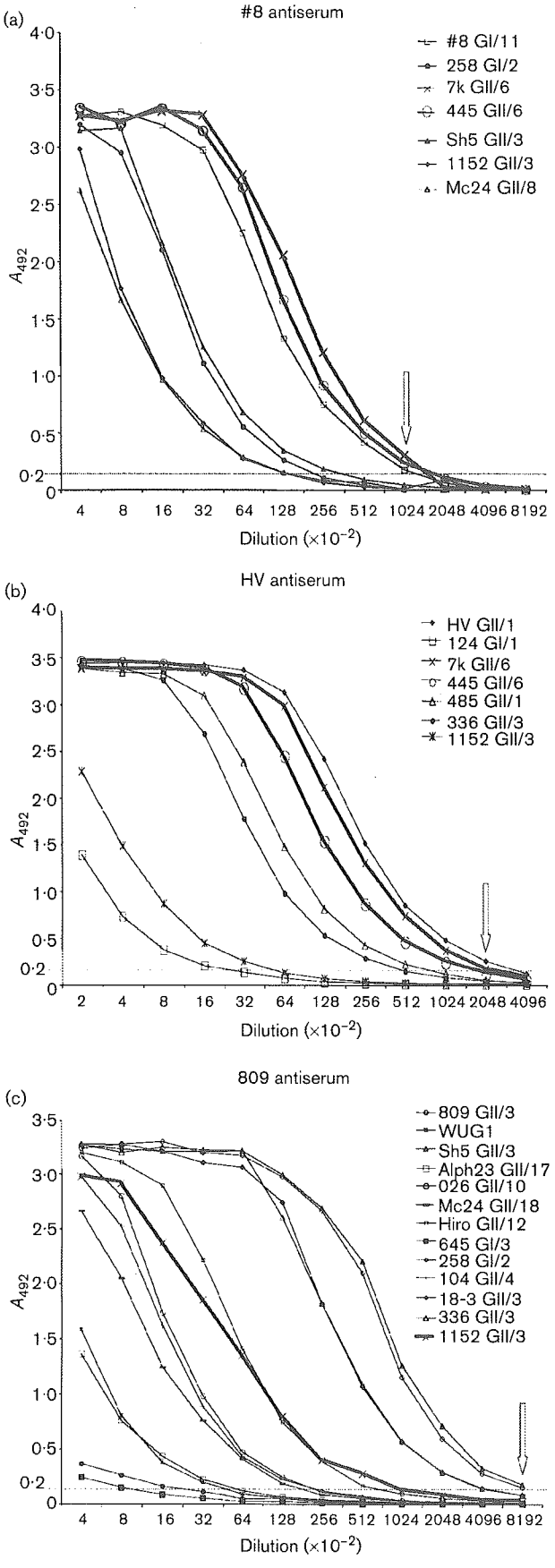


Fig. 2. EM images of CsCl-purified NoV VLPs negatively stained with 2% uranyl acetate (pH 4). (a) Strain 7k, (b) strain 485, (c) strain 445 and (d) strain 645. Bar, 100 nm.

Table 2. Antibody ELISA cross-reactivity among the 26 different VLPs and antisera

Titres are expressed as $\times 10^{-2}$.

Genogroup			GI						GII																				
	Genotype	VLPs	1	2	3	4	8	11	1	1	2	3	3	3	3	3	4	5	6	6	7	8	8	10	12	12	14	17
	Antiserum	Strains	SeV	258	645	CV	WUG1	#8	HV	485	Ina	809	Sh5	18-3	336	1152	104	754	7k	445	10-25	Mc24	U25	026	CHV	Hiro	47	Alph23
GI	1	SeV	**8192**	1042	1024	1024	1024	1024	256	128	256	256	256	256	256	128	128	256	256	256	128	128	256	256	256	256	256	256
	2	258	512	**8192**	512	1024	512	512	64	16	64	32	128	64	32	16	128	128	64	64	32	32	64	128	32	64	32	128
	3	645	256	256	**8192**	256	512	1024	32	8	32	64	128	128	64	64	64	64	32	64	128	128	128	128	16	32	16	64
	4	CV	128	512	256	**8192**	512	128	64	4	32	64	128	64	32	64	32	32	32	32	64	64	64	64	16	64	32	64
	8	WUG1	256	512	256	256	**8192**	256	32	32	16	32	64	64	64	16	32	32	32	32	64	8	32	32	32	16	32	64
	11	#8	128	128	128	256	256	**1024**	256	128	256	256	256	256	256	128	256	256	1024	1024	256	128	128	256	128	256	128	256
GII	1	HV	16	16	8	16	32	256	**2048**	512	128	128	256	512	512	32	256	256	2048	1024	128	128	256	512	512	512	256	256
	1	485	128	128	16	128	128	128	1024	**4096**	128	256	256	256	256	64	128	256	128	128	128	64	256	256	512	256	256	128
	2	Ina	<4	32	16	16	64	32	256	256	**4096**	128	128	256	128	64	512	512	256	128	128	256	128	512	256	512	128	128
	3	809	<2	4	4	8	32	4	512	128	128	**8192**	8192	4096	4096	1024	64	256	64	128	256	128	256	128	256	512	64	32
	3	Sh5	8	16	16	16	16	16	128	64	64	2048	**2048**	1024	1024	256	128	128	64	64	128	64	128	128	128	128	64	64
	3	18-3	16	16	16	16	32	32	64	64	64	256	512	**1024**	512	128	64	64	64	64	128	64	128	128	128	128	64	64
	3	336	32	16	32	32	32	64	128	128	128	1024	1024	1024	**2048**	512	128	128	128	128	256	128	256	128	256	256	128	128
	3	1152	<4	<4	<4	<4	8	<4	16	16	32	512	1024	1024	1024	**1024**	32	64	32	32	32	32	32	32	16	32	8	16
	4	104	32	64	128	64	256	128	512	512	512	512	512	1024	1024	128	**8192**	1024	256	512	512	256	512	512	512	512	512	256
	5	754	128	128	64	128	256	512	1024	1024	1024	512	1024	1024	1024	256	512	**8192**	512	1024	512	256	512	1024	1024	1024	512	512
	6	7k	64	128	64	128	128	1024	512	128	128	128	256	256	128	32	256	256	**4096**	2048	128	64	256	256	256	256	128	256
	6	445	16	16	8	16	32	128	64	32	64	128	128	128	128	64	64	64	2048	**4096**	64	64	64	128	64	64	64	128
	7	10-25	32	64	128	128	64	128	512	512	256	1024	512	1024	512	128	512	512	512	512	**16384**	512	1024	512	512	512	512	256
	8	Mc24	8	16	4	8	16	NT	64	32	NT	16	NT	32	32	8	32	32	16	16	128	**2048**	2048	32	32	32	16	4
	8	U25	16	32	32	32	32	32	256	256	128	128	256	256	256	64	256	256	128	128	512	4096	**4096**	256	256	256	128	128
	10	026	32	32	32	64	128	16	512	512	256	128	128	256	256	128	256	512	256	256	512	256	256	**2048**	512	512	256	128
	12	CHV	32	64	64	64	128	128	1024	1024	512	256	256	512	64	512	256	512	256	256	256	128	512	1024	**4096**	2048	512	256
	12	Hiro	<2	16	4	2	8	4	256	128	64	64	64	64	64	16	64	128	32	64	64	32	64	128	1024	**1024**	64	32
	14	47	128	128	64	256	256	256	512	256	512	512	512	512	512	128	512	512	256	256	256	128	512	512	512	512	**4096**	256
	17	Alph23	64	64	32	32	8	32	64	32	128	32	128	64	32	256	64	64	64	64	256	64	128	256	64	256	32	**2048**



which was equal to the homologous VLP titre. We also found that GII/1 HV antiserum cross-reacted strongly (i.e. equal to the homologous VLP titre) against GII/6 7k VLPs (titre 204 800) and moderately strongly (i.e. twofold lower than the homologous VLP titre) against GII/6 445 VLPs (titre 102 400) (Fig. 3b and Table 2). We observed several antisera that cross-reacted moderately against different genotypes (i.e. fourfold lower than the homologous VLP titres). For example, GI/11 #8 antiserum cross-reacted moderately with GI/4, GI/8, GII/1, GII/2, GII/3, GII/4, GII/5, GII/7, GII/10, GII/12 and GII/17 VLPs (Fig. 3a and Table 2). GII/1 HV antiserum also cross-reacted moderately with several different genotypes, including GII/1 (strain 485), GII/3, GII/10 and GII/12 (Fig. 3b and Table 2). GII/1 485 antiserum cross-reacted moderately only with GII/1 HV VLPs; GII/6 7k antiserum cross-reacted moderately with GI/11 VLPs; GII/10 026 antiserum cross-reacted moderately with several different genotypes, including GII/1, GII/5, GII/7 and GII/12; and GII/12 CHV antiserum cross-reacted moderately with GII/1 and GII/10 VLPs (Table 2).

Genotype-specific reactivities

We observed weak cross-reactivities among different genotypes (i.e. greater than eightfold dilutions). We found that GI/1, GI/2, GI/3, GI/4 and GI/8 antisera cross-reacted weakly with other genotypes (Table 2). We also observed similar weak cross-reactivities with GII/1 (strain 485), GII/2, GII/3 (all five strains), GII/4, GII/5, GII/6 (strain 445), GII/7, GII/8 (both strains), GII/14 and GII/17 antisera. For several GII genotypes, only one type of antiserum was produced, but for five GII genotypes, we produced two or more different antisera against VLPs belonging to the same genotype (Table 2). Some interesting results were observed. For example, the antigenicities of HV and 485 were considerably different, despite the fact that both strains belong to GII/1 and share approximately 94 % amino acid identity. As shown in Fig. 3(b), HV antiserum cross-reacted strongly with GII/6 VLPs, but 485 antiserum showed little cross-reactivity with these GII/6 VLPs (Table 2). This unusual cross-reactivity pattern was also observed with other antisera. For example, for GII/6, we found that 7k antiserum cross-reacted moderately with GI/11 #8 VLPs, whereas 445 antiserum cross-reacted weakly (i.e. 32-fold lower than the homologous VLP titre; Table 2). More uniquely, we found that GII/3 1152 antiserum, which was genotype-specific, had unusual antigenicity. We found that

Fig. 3. Antibody ELISAs for NoV VLPs. Wells were coated with 100 µl purified VLPs. After washing, hyperimmune rabbit antiserum raised against the VLPs was used to detect antigens. Antisera were diluted twofold in PBS-T-SM from a starting dilution as indicated (dilutions × 10^{-2}). The arrows indicate the endpoint. (a) GI/11 #8 antiserum cross-reacts strongly with GII/6 7k and 445 VLPs. (b) GII/1 HV antiserum cross-reacts strongly with GII/6 7k and moderately strongly with 445 VLPs. (c) GII/3 809 antiserum cross-reacts weakly with GII/3 1152 VLPs.

three different GII/3 antisera (strains 809, Sh5 and 18-3) cross-reacted weakly with 1152 VLPs (i.e. eightfold lower than the homologous VLP titre; Table 2 and Fig. 3c). This unusual cross-reactivity result was not evident with the other genotypes in which we produced two different antisera (i.e. GII/1, GII/6, GII/8 and GII/12; see Table 2).

Amino acid alignment and secondary structure prediction

An alignment of 25 VP1 amino acid sequences used in this study (Mc24 complete capsid was unavailable) revealed that the N-terminal region (aa 1–49), shell domain (aa 50–225) and P1-1 domain (aa 226–278) had more conserved short continuous residues than the P2 domain (aa 279–405), P1-2 domain (aa 406–520) and C-terminal region (Fig. 4). These continuous residues may be the reason for the cross-reactivity among different genotypes, in particular, the strong cross-reactivity of #8 antiserum against GII/6 VLPs (Fig. 3a). However, this does not explain why GII/3 1152 VLPs cross-reacted weakly with GII/3 809, Sh5 and 18-3 antisera (i.e. eightfold lower than the homologous VLP titre) and moderately against GII/3 336 antiserum (i.e. fourfold lower than the homologous VLP titre). An amino acid

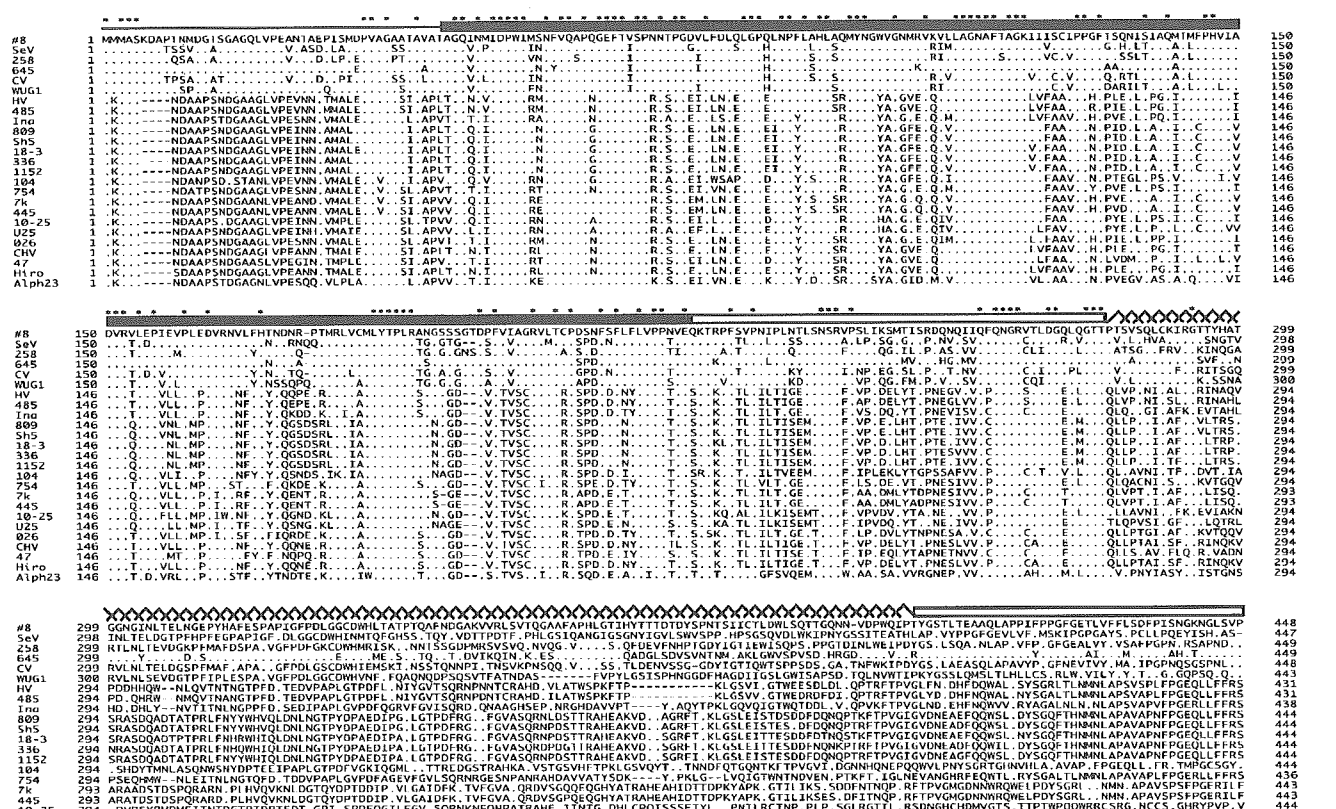


Fig. 4. Amino acid alignment of VP1 sequences of the NoV sequences examined in this study. The following regions are indicated above the sequences (in order): N-terminal region (line); shell domain (filled box), P1-1 domain (open box); P2 domain (XXX); P1-2 domain (open box) and C-terminal region (line) (Chen *et al.*, 2004). Asterisks indicate conserved amino acids.

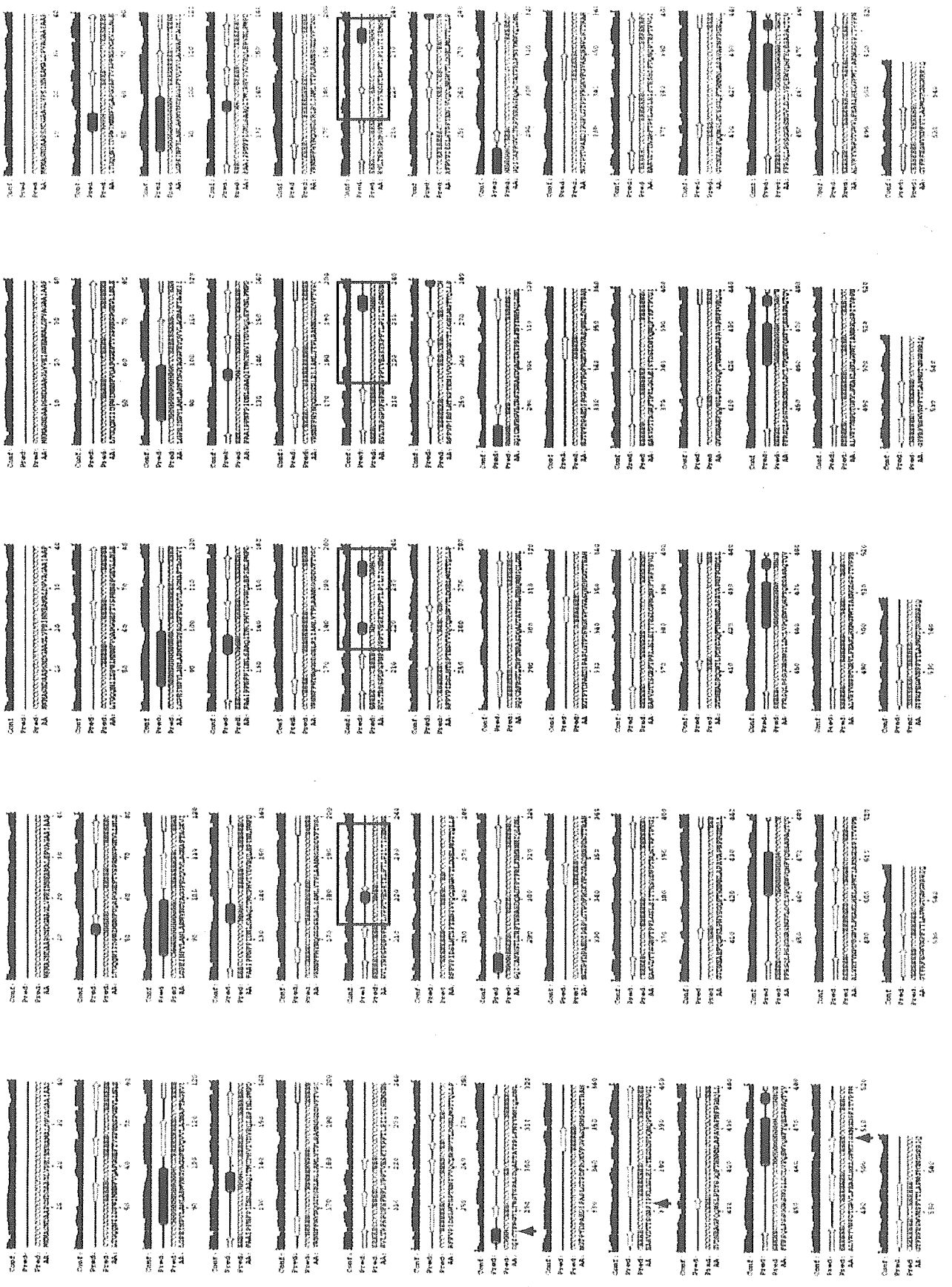

1152
18-3
336
809
Sb5

Table 3. Summary of cross-reactivities among VLPs

Each letter represents one strain. For example, GII/1 antiserum cross-reacted with two GII/6 strains (A and B), where A, strongly (i.e. identical to the homologous VLP titre), B, moderately strongly (i.e. twofold lower than the homologous VLP titre), and C, moderately (i.e. fourfold lower than the homologous VLP titre). For simplicity, we have excluded the homologous reactivities.

			VLPs												
	Genogroup		GI			GII									
		Genotype	4	8	11	1	2	3	4	5	6	7	10	12	17
Antiserum	GI	11 (strain #8)	C	C		C	C	CCCC	C	C	AA	C	C	C	C
	GII	1 (strain HV)						CC			AB		C	CC	
		6 (strain 7k)			C										
		10 (strain 026)				CC				C		C		CC	
		12 (strain CHV)				CC							C		

alignment of these five GII/3 VP1 sequences showed no unusual insertions, deletions or recombination sites; in fact, the shell domain was highly conserved among the GII/3 sequences (data not shown). However, the 1152 VP1 sequence had three unique amino acid residues (Thr-285, Ile-372 and Ser-508) when compared with the other four GII/3 VP1 sequences. The first two residues were located in the outermost region of the P2 domain, whilst the third residue was located within the P1 domain (data not shown). We used the PSIPRED secondary structure prediction software (McGuffin *et al.*, 2000) to compare the five GII/3 VP1 structures. We found that the predicted VP1 structures for 809, Sh5, 18-3 and 336 had a helix between residues 219 and 237, whereas this helix structure was absent for 1152 (Fig 5). These data suggested that the helix structure may play an important role in influencing the cross-reactivity among the GII/3 VLPs and antisera.

DISCUSSION

In this study, we analysed NoV capsid-based grouping and cross-reactivity among 26 different VLPs belonging to six GI and 12 GII genotypes. Using an antibody ELISA, we found that the antisera reacted strongly against the homologous VLPs with titres ranging from 102 400 to 1 638 400. As summarized in Table 3, we also observed strong, moderately strong and moderate cross-reactivities among different genotypes (i.e. equal to the homologous VLP titre and to twofold and fourfold dilutions, respectively). For example, GI/11 antiserum had a broad range of cross-reactivities, detecting two GI genotypes (GI/4 and GI/8) and 10 GII genotypes (GII/1–7, GII/10, GII/12 and GII/17); GII/1 antiserum (strain HV) had a broad range of cross-reactivities, detecting four GII genotypes (GII/3, GII/6, GII/10 and GII/12); GII/10 antisera also had a broad range of cross-reactivities, detecting four GII genotypes (GII/1, GII/5, GII/7 and GII/12); GII/6 antiserum detected GI/11 VLPs; and GII/12 antiserum (strain CHV) detected GII/1 and GII/10 VLPs.

Although antigen ELISAs are generally broadly reactive (Jiang *et al.*, 2000), this is the first report of a GI (strain #8) polyclonal antiserum cross-reacting strongly with other GII genotypes and the first report of a GII (strain HV) polyclonal antiserum cross-reacting strongly with other GII genotypes (Jiang *et al.*, 2002; Kamata *et al.*, 2005; Kitamoto *et al.*, 2002). These broad-range cross-reactivities may be due to unfolded VLPs on the microtitre plates at the high pH used (carbonate/bicarbonate buffer, pH 9·6) (White *et al.*, 1997). However, we have not found such broad-range cross-reactivities in any of our other studies (Kamata *et al.*, 2005). Conserved continuous residues in the shell and/or P1-1 domains may be the reason for these cross-reactivities against different genotypes (Fig. 4 and Table 2). However, we found that several antisera were genotype-specific, indicating that VLPs have unique epitopes.

Interestingly, we found that four types of GII/3 antisera (strains 809, Sh5, 18-3 and 336) cross-reacted moderately to weakly against GII/3 1152 VLPs (i.e. up to eightfold lower than the homologous VLP titre; Table 2). Amino acid alignments of these five GII/3 sequences revealed that 1152

Fig. 5. Schematic representations of the complete predicted secondary structures of VP1 of NoV (GII/3) strains 1152, 18-3, 336, 809 and Sh5. The level of confidence of prediction (Conf) is shown on the first line, where a tall box represents a high confidence of prediction and a short box represents a low confidence of prediction. The predicted secondary structure (Pred) is shown on the second line, where a helix is represented by a cylinder, a β-strand by an arrow and a coil by a line. The third line also shows the predicted secondary structure (Pred), where H represents a helix, E a β-strand and C a coil. The amino acid sequence (AA) is shown on the bottom line. The boxed regions in 18-3, 336, 809 and Sh5 VP1 indicate a helix structure that is absent in 1152 VP1. The amino acid residues that are unique to the 1152 sequence when compared with the other four GII/3 sequences are indicated by arrows.

had three unique amino acid residues compared with the other four GII/3 sequences (Thr-285, Ile-372 and Ser-508), two of which were located within the P2 domain (Thr-285 and Ile-372). Amino acid secondary structure predictions made using the PSIPRED secondary structural prediction software revealed that the VP1 secondary structures for 809, Sh5, 18-3 and 336 had a helix structure between residues 219 and 237; this helix structure was absent for 1152 (Fig. 5). This helix structure may, in part, influence the cross-reactivity among the GII/3 VLPs (i.e. without the helix structure); GII/3 1152 VLPs cross-reacted weakly with the other four GII/3 antisera. This suggestion may also explain NoV virulence in which some strains appear to infect a certain population over an extended period of time (Dingle, 2004; Noel *et al.*, 1999). In a recent report, single amino acid changes were suggested to represent a possible way for the virus to evade the host immunity (Dingle, 2004). In addition, one report suggested that a change in VP1 secondary structure (i.e. the disappearance of a helix structure) was responsible for a chronic NoV infection in an immunocompromised patient for over 2 years (Nilsson *et al.*, 2003).

Almost half of our constructs (strains SeV, 645, CV, HV, Ina, 809, Sh5, 18-3, 1152, 104, 754, CHV and Alph23) did not include the ORF3 sequence, which encodes a minor capsid protein (VP2) thought to increase the stability of NoV VLPs and may function in RNA genome packaging (Bertolotti-Ciarlet *et al.*, 2003). For rabbit haemorrhagic disease virus, VP2 is essential for the production of infectious virus (Sosnovtsev & Green, 2000). Nevertheless, we found that all constructs with or without ORF3 sequences expressed VLPs that were morphologically similar to native NoV (Fig. 2). Further studies are needed to determine whether VP2 has some influence on antigenicity.

In conclusion, this cross-reactivity study represents the most extensive undertaken for any genera in the family *Caliciviridae*. Since human NoV strains cannot be propagated in cell culture systems and human serological studies have found that VLPs and native virions share similar antigenic properties, VLPs have been used to understand antigenic relationships in more detail. Further studies, such as high-resolution structural analysis of other NoV genotypes and antigenic mapping, are needed in order to explain the complex NoV antigenicity, as previously suggested (Chen *et al.*, 2004). Finally, the results and reagents from this study can be used to design detection systems capable of detecting a broad-range of genotypes in clinical specimens; in particular, GI/11 antisera may be capable of detecting at least 32 % (12/37) of the recently described NoV genotypes (Kageyama *et al.*, 2004).

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Identification of Genogroup I and Genogroup II Broadly Reactive Epitopes on the Norovirus Capsid

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Norwalk virus, a member of the family *Caliciviridae*, is an important cause of acute epidemic nonbacterial gastroenteritis. Norwalk and related viruses are classified in a separate genus of *Caliciviridae* called *Norovirus*, which is comprised of at least three genogroups based on sequence differences. Many of the currently available immunologic reagents used to study these viruses are type specific, which limits the identification of antigenically distinct viruses in detection assays. Identification of type-specific and cross-reactive epitopes is essential for designing broadly cross-reactive diagnostic assays and dissecting the immune response to calicivirus infection. To address this, we have mapped the epitopes on the norovirus capsid protein for both a genogroup I-cross-reactive monoclonal antibody and a genogroup II-cross-reactive monoclonal antibody by use of norovirus deletion and point mutants. The epitopes for both monoclonal antibodies mapped to the C-terminal P1 subdomain of the capsid protein. Although the genogroup I-cross-reactive monoclonal antibody was previously believed to recognize a linear epitope, our results indicate that a conformational component of the epitope explains the monoclonal antibody's genogroup specificity. Identification of the epitopes for these monoclonal antibodies is of significance, as they are components in a commercially available norovirus-diagnostic enzyme-linked immunosorbent assay.

Norwalk virus (NV) is the prototype member of the genus *Norovirus* in the virus family *Caliciviridae*. These viruses are responsible for 98% of all nonbacterial acute epidemic outbreaks of gastroenteritis in the United States, resulting in an estimated 23 million cases per year (4). Although it has been 30 years since NV was first identified, the study of this virus is still hampered by the lack of a cell-culture system or an animal model. However, expression of the 3′ end of the genome in a baculovirus expression system results in the production of recombinant NV virus-like particles (rNV VLPs) that are morphologically and antigenically similar to native NV virions (7, 15, 28). The availability of these rNV VLPs (as well as those of other noroviruses) has also enabled the generation of reagents, monoclonal antibodies in particular, for the study of the serological and antigenic properties of these viruses.

The Norwalk virus has icosahedral symmetry and is composed of 180 molecules of a single major capsid protein, VP1, organized into 90 dimers (29). The virus has a surface structure characteristic of animal and human caliciviruses, in which arch-like structures protrude from the surfaces surrounding cuplike depressions at the three- and fivefold axes of symmetry.

The capsid protein (530 amino acids [aa]) itself folds into two domains. The amino-terminal shell (S) domain is highly conserved among animal caliciviruses (3, 6, 28). The sequence of the C-terminal protruding (P) domain, which forms protruding arches on the capsid, is more diverse, with the most variation seen in the P2 subdomain at the outer surface of the virion (3, 10).

Norwalk and related viruses are classified as noroviruses in a separate genus of the family *Caliciviridae*, which is comprised of at least three genogroups based on sequence similarities in the polymerase and capsid regions of the genome (36). Furthermore, these viruses have been classified into multiple antigenic groups based on results from immune electron microscopy and cross-protection studies done in volunteers (19–23), but it is unclear how these antigenic and genetic characterizations relate to each other. Information about the location of the type-specific and cross-reactive epitopes on the virus capsid is limited. Specific identification of these epitopes is essential for designing broadly reactive diagnostic assays and for helping to dissect the immune response to calicivirus infection and may be useful in identifying potential targets for antivirals.

We previously generated a panel of monoclonal antibodies (MAbs) to the rNV VLPs (9, 10, 16). Several of these MAbs are cross-reactive between viruses within distinct genogroups (9, 10, 16). Therefore, mapping the residues these MAbs recognize on the NV capsid will provide information on the determinants of cross-reactivity for caliciviruses. Because generation of neutralization escape mutants of the virus is unavailable for the noroviruses, we approached the problem using biochemical methods such as those involving deletion mutants and site-directed mutagenesis. We describe here the identification of the epitopes for the genogroup I (GI)-cross-reactive MAbs NV3901 and NV3912 and the genogroup II (GII)-cross-reactive MAb NS14. MAbs NV3901 and NV3912 map to the same epitope by competition enzyme-linked immunosorbent assay (ELISA) and recognize a

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TABLE 1. NV deletion mutant primers

Primer	Sequence (5′ to 3′)	Position[a] (nt)	Polarity
NV *Bgl II* 225	TCT GTT AGA TCT GAG CAG AAA ACC AGG CCC	6030–6052	sense
NV *Bgl II* 278	TCT GTT AGA TCT GGC ACC ACC CCA GTT TCA	6189–6211	sense
NV *Bgl II* 406	CGG AGA TCT TTA GGC TAG ATG TGT TGC CTC	6553–6575	antisense
NV *Bgl II* 530	CAA GGA GAT CTT TAT CGG CGC AGA CCA AGC	6927–6950	antisense
NV *Bgl II* 514	CAA GGA GAT CTT TAC TTT AAT TGA TAA AAT	6880–6899	antisense
NV *Bgl II* 454	CGC GTT AGA TCT GGT GAG GCT GCC CTG CTC	6717–6734	sense
NV *Bgl II* 466	CGC GTT AGA TCT ACC GGT CGG AAT CTT GGG	6753–6770	sense
NV *Mfe I* 520	CGG CAA TTG TTA GCT GGC AGT TCC CAC AGG C	6899–6918	antisense

[a] NV accession number M87661 in GenBank.

common epitope shared by GI viruses, and MAb NV3901 is capable of detecting a high proportion of GI viruses in fecal samples (9). MAb NS14 reacts with multiple GII VLPs in both ELISAs and Western blots and reacts weakly with GI VLPs in ELISAs (16). Identification of these epitopes is of significance because MAbs NV3912 and NS14 are currently being used as capture antibodies in a commercially available NLV diagnostic ELISA kit, SRSV (II) AD (Denka Seiken, Tokyo, Japan) (1).

MATERIALS AND METHODS

Cloning of NV and HOV deletion mutants into GST expression vector. The glutathione *S*-transferase (GST) expression vector used to express the deletion mutants of VP1 was pGEX-2TK (Amersham Pharmacia Biotech, Piscataway, NJ). The NV primers used for this study are shown in Table 1. Primers for a genogroup II.4 virus called the Houston virus (HOV) used for this study are shown in Table 2. The HOV virus is related to Lordsdale virus (GenBank accession number X86557) and has 94% amino acid similarity and 91% amino acid identity therewith in the VP1 region. Each primer contains an engineered restriction site indicated in the name of the primer to facilitate cloning. The numbering of the sense primers indicates the N-terminal (first) norovirus residue contained within a construct generated with a particular primer. The numbering of the antisense primers indicates the C-terminal (last) norovirus residue contained within a construct generated with a particular primer.

PCR-amplified fragments of the NV or HOV ORF2 were generated using the primer pairs indicated by the names of the constructs. The template used for the PCR was the plasmid pG4145 (15) containing the complete NV ORF2. PCR fragments were either directly cloned into the pGEX-2TK vector or first cloned into the pCR4 Blunt-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA) and then subcloned into pGEX-2TK.

Expression and characterization of fusion proteins. Purified DNA for each clone was prepared and used to transform BL21 cells (Novagen, Madison, WI). Positive transformants were identified by PCR. To express the GST-NV fusion proteins, overnight cultures of BL21 cells transformed with each plasmid were diluted to a ratio of 1:10 in fresh LB broth supplemented with 100 μg/ml ampicillin. Cells were grown at 37°C until a density was reached where the A_{600} was 0.6 to 0.7. Expression was induced by addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen Life Technologies), and cultures were grown for an additional 3 h. Cells were pelleted by centrifugation for 15 min at 13,800 × *g* at 4°C. Supernatant was removed, and the cell pellet was suspended in 1/10 volume lysis buffer (50 mM Tris, pH 8, 120 mM NaCl, 50 mM EDTA, 3 mg/ml lysozyme) and incubated on ice for 15 min. Following incubation, Triton X-100 and 2-mercaptoethanol were added to concentrations of 1% and 10 mM, respectively, as were the following protease inhibitors: leupeptin, pepstatin, and phenylmethylsulfonyl fluoride. The suspension was subjected to two freeze-thaw cycles, and the insoluble fraction was removed by centrifugation for 15 min at 19,800 × *g* at 4°C. The supernatant was reserved, and the insoluble pellet was suspended in 1/10 volume solubilization buffer containing 1.5% (wt/vol) sarkosyl, 25 mM triethanolamine, and 1 mM EDTA (pH 8) and incubated on ice for 10 min. Following incubation, the sample was separated by centrifugation at 19,800 × *g* at 4°C. The supernatant was pooled with the reserved supernatant from the previous centrifugation adjusted to 1% Triton X-100 and 1 mM $CaCl_2$, aliquoted into 1-ml fractions, and stored at −20°C.

Western blot analysis of fusion proteins. Analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli with modifications (18). Polyacrylamide 12% resolving gels were used with a 4% acrylamide stacking gel. Prewashed glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) were added to an aliquot of supernatant and incubated with agitation for 1.5 h at 4°C. Beads were pelleted by centrifugation for 5 min at 500 × *g* at 4°C. The supernatant was discarded, and the beads were washed with 10 bed volumes of ice-cold phosphate-buffered saline (PBS), and then with PBS with 500 mM NaCl, and then again with PBS. Following the final wash, the beads were suspended in sample buffer containing 1% SDS, 10% 2-mercaptoethanol, 0.05 M Tris-HCl (pH 6.8), 10% glycerol, and 0.0025% phenol red and boiled for 5 min.

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech) as described previously (37). The proteins were detected using a polyclonal goat anti-GST antibody (Amersham Pharmacia) at a dilution of 1:8,000, a mouse hyperimmune anti-rNV VLP serum at a dilution of 1:5,000, or a rabbit hyperimmune anti-rHOV VLP serum at a dilution of 1:5,000 in 0.5% Blotto (Carnation nonfat dry milk in 0.01 M PBS). MAb NV3901, MAb NV3912 (10), and MAb NS14 (16) ascites were used for detection at dilutions of 1:1,000. All secondary antibodies used were conjugated to horseradish peroxidase (Sigma, St. Louis, MO). Membranes were developed by chemiluminescence using Western lightning detection reagent (Perkin-Elmer Life Sciences, Inc., Boston, MA) following the manufacturer's protocol.

Site-directed mutagenesis of GST-NV 454-520. Specific residues in the GST-NV 454-520 construct were altered using the Quickchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Briefly, the indicated primer pairs (Table 3) containing the desired mutation were annealed to the complementary regions of the parental plasmid

TABLE 2. HOV deletion mutant primers

Primer	Sequence (5′ to 3′)	Polarity
HOV *BamHI* 221	GGT GGA TCC GAG TCA AGA ACC AAA CCA TTC ACC	sense
HOV *BamHI* 417	CGC GGA TCC CAC CTA GCC CCT GCC	sense
HOV *BamHI* 453	CGC GGA TCC CTC CCC CAG GAA TGG	sense
HOV *BamHI* 473	CGC GGA TCC GCT CTG CTG AGA TTT GTG	sense
HOV *BamHI* 488	CGC GGA TCC GAG TGC AAA CTT CAT AAA TC	sense
HOV *EcoRI* 488	GGG AAT TCT TAG CAC TCA AAC AGG ACC CTA CC	antisense
HOV *EcoRI* 494	GCC GAA TTC TTA TGA TTT ATG AAG TTT GC	antisense
HOV *EcoRI* 540	CAA GGG AAT TCT TAT AAC GCA CGT CTA CGC CC	antisense

TABLE 3. Site-directed mutagenesis primers

Primer or primer pair	Sequence[a] (5′ to 3′)	Position[b] (nt)	Mutation
NV G6855T, T6856A	CAGCTGCCGATCAATGGG**TA**CTTTGTCTTTGTTTCATG	6837–6874	V500Y
NV C6855A, A6856T	CATGAAACAAAGACAAAG**TA**CCCATTGATCGGCAGCTG	6837–6874	V500Y
NV A6897G, A6898C	GTGTCCAGATTTTATCAATTA**GC**GCCTGTGGGAACTGCCAGCTC	6876–6919	K514A
NV T6897C, T6898G	GAGCTGGCAGTTCCCACAGG**CG**CTAATTGATAAAATCTGGACAC	6876–6919	K514A
NV G6916A	GCCTGTGGGAACTGCCA**A**CTCGGCAAGAGGTAGGC	6899–6933	S520N
NV C6916T	GCCTACCTCTTGCCGAGT**T**GGCAGTTCCCACAGGC	6899–6933	S520N
NV A6989G	CCAGATTTTATCAATTAA**G**GCCTGTGGGAACTGCCAG	6880–6916	K514R
NV T6898C	CTGGCAGTTCCCACAGGCCTTAATTGATAAAATCTGG	6880–6916	K514R
NV A6773C	GTCGGAATCTTGGGGA**C**TTCAAAGCATACCCTG	6757–6789	E472D
NV T6773G	CAGGGTTAGCTTTGAA**G**TCCCCAAGATTCCGAC	6757–6789	E472D
NV A6772C	CGGTCGGAATCTTGGGGCATTCAAAGCATACCCTG	6755–6789	E472A
NV T6772G	CAGGGTATGCTTTGAATGCCCCAAGATTCCGACCG	6755–6789	E472A

[a] Mutant nucleotides shown in bold.
[b] NV accession number M87661 in GenBank.

template and extended using *Pfu* polymerase to generate a mutated plasmid containing staggered nicks. The methylated parental DNA was digested by treatment with DpnI, and the newly synthesized nicked vector DNA was used to transform competent bacterial cells. Mutant clones were confirmed by sequencing.

Peptide competition ELISA. Synthetic peptides (Sigma-Genosys, The Woodlands, TX) were used as competitors for MAb binding to rHOV VLPs in an antigen detection ELISA. The sequence of the HOV peptide is ALLRFVNPD TGRVLFECKLHKS. MAb NS14 was purified using a protein G column (Pierce, Rockford, IL) according to the manufacturer's instructions. Optimized concentrations of coating rVLP and detector MAb were determined by checkerboard serial dilution. rHOV VLPs prepared in 0.05 M carbonate bicarbonate buffer, pH 9.6, were used to coat flat-bottomed polyvinylchloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) overnight at 4°C. In separate tubes, constant concentrations of MAb NS14 (to give an optical density at 450 nm of between 0.5 and 0.6) were added to increasing concentrations of competitor peptide (1, 5, 10, and 15 μM) in 0.5% Blotto and incubated overnight at 4°C. A control MAb in 0.5% Blotto without peptide was included in each plate. The VLP-coated microtiter plates were washed three times with PBS containing 0.05% Tween 20 and blocked with 5% Blotto for 1 h at 37°C.

Following six washes with PBS-Tween, each of the MAb-peptide mixtures was added to duplicate wells, and the plates were incubated for 2 h at 37°C. After washing six times, a 1:5,000 dilution of goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Sigma) in 0.5% Blotto was added to each well, and the plates were incubated for 1 h at 37°C. To develop the ELISA, 100 μl of 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added to each well, and the color reaction was stopped by the addition of 100 μl of 1 M phosphoric acid. The optical density at 450 nm was read, and the average values for duplicate wells were calculated.

RESULTS

Monoclonal antibodies NV3901 and NV3912 recognize GST-NV fusion proteins corresponding to the C-terminal region of the P1 subdomain of VP1. Three initial GST-NV VP1 capsid fusion proteins were constructed based on structural information available for NV. These constructs corresponded to the full-length protruding domain of the capsid protein (aa 225 to 530), the P2 subdomain (aa 278 to 406), and the C-terminal 252 amino acids comprising the P2 and the far C-terminal portion of the P1 subdomains (aa 278 to 530) (Fig. 1C). These proteins were expressed as GST fusions in bacteria and purified using glutathione-Sepharose beads. Purified proteins were analyzed by Western blotting for reactivity with anti-GST- and anti-NV-specific antisera. Each of the fusion proteins was detected using the anti-GST serum, and NV-specific proteins but not GST alone were detected in lanes corresponding to the

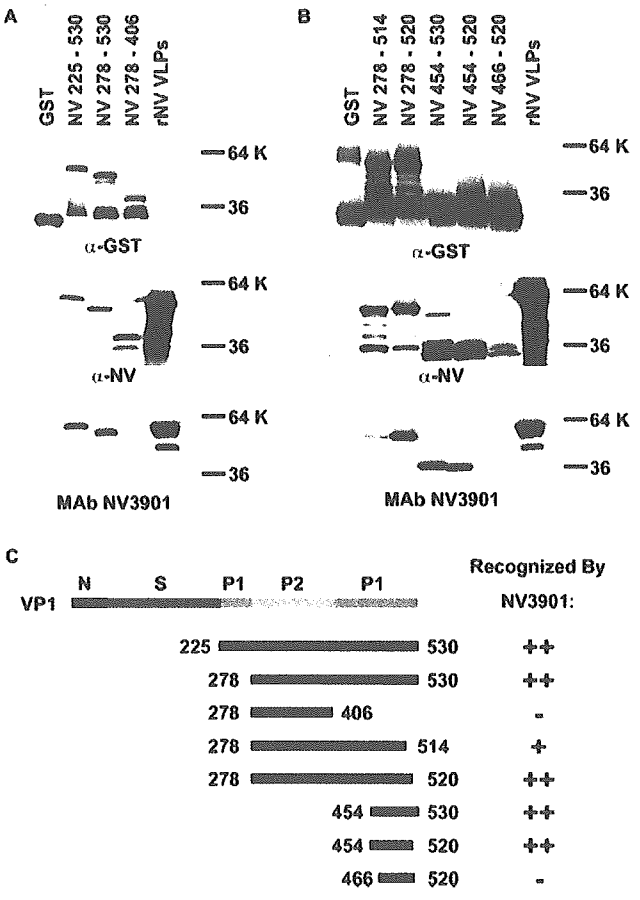

FIG. 1. Analysis of MAb NV3901 binding to GST-NV fusion proteins. Purified NV capsid protein domains (A) or deletion mutants (B) containing the indicated residues were analyzed by Western blotting with anti-GST antiserum (top panels), polyclonal mouse anti-NV VLP antiserum (middle panels), or MAb NV3901 (bottom panels). GST, purified GST protein; rNV VLPs, purified Norwalk virus VLPs; K, kDa. (C) Schematic representation of the locations of the constructs relative to the full-length NV VP1 protein and summary of recognition by MAb NV3901. ++, strongly recognized; +, weakly recognized; −, not recognized.