

Murine Noroviruses Comprising a Single Genogroup Exhibit Biological Diversity despite Limited Sequence Divergence^{∇†}

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Viruses within the genus *Norovirus* of the family *Caliciviridae* are the major cause of acute, nonbacterial gastroenteritis worldwide. Human noroviruses are genetically diverse, with up to 57% divergence in capsid protein sequences, and comprise three genogroups. The significance of such genetic diversity is not yet understood. The discovery of murine norovirus (MNV) and its ability to productively infect cultured murine macrophages and dendritic cells has provided an opportunity to determine the functional consequences of norovirus diversity in vitro and in vivo. Therefore, we compared the full-length genomes of 21 new MNV isolates with five previously sequenced MNV genomes and demonstrated a conserved genomic organization consisting of four open reading frames (ORFs) and a previously unknown region of nucleotide conservation in ORF2. A phylogenetic analysis of all 26 MNV genomes revealed 15 distinct MNV strains, with up to 13% divergence at the nucleotide level, that comprise a single genotype and genogroup. Evidence for recombination within ORF2 in several MNV genomes was detected by multiple methods. Serological analyses comparing neutralizing antibody responses between highly divergent strains suggested that the MNV genogroup comprises a single serotype. Within this single genogroup, MNV strains exhibited considerable biological diversity in their ability to grow in culture and to infect and/or persist in wild-type mice. The isolation and characterization of multiple MNV strains illustrate how genetic analysis may underestimate the biological diversity of noroviruses and provide a molecular map for future studies of MNV biology.

Human noroviruses are the major etiologic agent of nonbacterial, epidemic gastroenteritis worldwide, causing an estimated 23 million infections per year in the United States alone (53). Noroviruses cause most outbreaks of gastroenteritis, as well as a significant proportion of sporadic cases of gastroenteritis in children and adults (7, 9, 16, 20, 37, 54). Symptoms of norovirus infection, which include vomiting, diarrhea, low-grade fever, malaise, and abdominal cramping or pain, usually resolve within 48 h, although noroviruses can be shed for 3 weeks after infection in adults and at least 6 weeks after infection in children of less than 6 weeks of age (13, 21, 57, 68). Norovirus infection is usually mild and self-limiting; however, severe disease and long-term virus shedding (lasting from 4 months to >2 years) have been documented previously in patients with underlying chronic conditions or immunosuppression (17, 41, 52, 58).

Human noroviruses are members of the genus *Norovirus* in the family *Caliciviridae*. Noroviruses are nonenveloped, icosahedral viruses with a positive-sense, single-stranded RNA genome of ~7.5 kb that contains three open reading frames (ORFs) (23). ORF1 encodes a polyprotein that is further

cleaved into at least six nonstructural (NS) proteins by the viral 3C-like protease (6, 44, 45, 71). ORF2 encodes the major capsid protein, or viral protein 1 (VP1), which consists of a shell domain (S domain) and a protruding domain (P domain) (35, 65). The S domain is more highly conserved than the P domain. The P domain is subdivided into the P1 and P2 subdomains, with the hypervariable P2 subdomain containing both immune and cellular recognition sites (26, 29, 46, 73). ORF3 encodes the small basic protein, or VP2, which is found in the virion and has a role in virion stability (5, 19).

The lack of a permissive cell culture system and animal model has prevented the serotyping of human noroviruses. Consequently, genetic analysis has been used to classify these viruses. Five genetic groups, or genogroups, of noroviruses have been identified using phylogenetic analysis of VP1 sequences (82). Genogroups I, II, and IV contain human noroviruses, while genogroups III and V contain bovine noroviruses and murine noroviruses (MNVs), respectively. Genogroup II also contains porcine noroviruses. Genogroups I, II, and IV can be further subdivided into 29 distinct phylogenetic clusters, or genotypes (82). Human noroviruses are genetically diverse; over 100 strains have been sequenced to date. Full-length human norovirus genomes diverge by as much as 45% at the nucleotide level, while VP1 sequences diverge by as much as 57% (40, 82).

In 1995 and 2002, genogroup II/4 noroviruses emerged and subsequently spread globally to become the dominant norovirus strain (12, 15, 47, 59, 77). The mechanisms that drive the emergence of norovirus strains are not understood. The identification of the first MNV strain, MNV1, and its routine lab-

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oratory propagation in the murine macrophage cell line RAW 264.7 (RAW) provided the first cell culture system and animal model in which to investigate the molecular mechanisms that promote norovirus evolution (39, 78). MNVs are enteric pathogens that share many molecular and biological properties with human noroviruses (79). Clinical symptoms during MNV infection in immunocompetent mice have not been reported, although MNV infection in mice deficient in components of innate immunity can cause diarrhea and lethality (31, 39, 56). MNVs are widespread in research mice today; MNV1-reactive antibodies were found in 22.1% of serum samples submitted for testing from research colonies in the United States and Canada, and at least 18 additional MNVs have been isolated from research colonies in the United States and Germany (GenBank accession numbers DQ269192 to DQ269205 and DQ911368) (31, 32). However, until now only five full-length sequences have been available.

The goal of this work was to determine the extent of MNV diversity by isolating and genetically characterizing multiple full-length MNVs. In addition, we wanted to determine whether phylogenetic analysis would predict the biological properties of MNV strains. In this study, the analysis of 26 full-length MNV genomes, 21 of which were sequenced for the first time, revealed 15 distinct MNV strains with a divergence of at most 13% at the nucleotide level. These strains comprise a single genotype and genogroup. Consistent with the limited sequence divergence reported here, serological cross-reactivity between highly divergent MNV strains was demonstrated. These strains have been named according to the convention suggested by Green et al. (22). Even closely related MNV strains differed in their abilities to form plaque on RAW cell monolayers and to infect and/or persist in wild-type mice.

MATERIALS AND METHODS

Cell lines and mice. RAW cells were maintained as previously described (78). Wild-type C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). STAT1^{-/-} mice (55) were bred and housed at Washington University, St. Louis, MO, under specific-pathogen-free conditions in accordance with federal and university guidelines. All mice used in this study were initially seronegative for MNV as determined by an enzyme-linked immunosorbent assay (ELISA) using MNV1 virions (78). Eight-week-old, sex-matched mice were given 25 μ l of virus inoculum orally. At various times after infection, tissue samples were aseptically removed and stored at -80°C . Tissue samples were homogenized in 1 ml of growth medium with 1.0-mm-diameter zirconia-silica beads by using a MiniBeadbeater (Biospec Products, Bartlesville, OK) or a MagNALyser (Roche Applied Science, Hague Road, IN) and clarified by centrifugation before analysis.

Quantification of MNV titers and genome copies. Plaque assays and plaque neutralization assays were performed as previously described (78). As not all MNV strains formed plaques on RAW cell monolayers, a 50% tissue culture infective dose (TCID₅₀) assay was developed. RAW cells were seeded into 96-well plates at a density of 3×10^4 viable cells per well. Serial dilutions of virus were prepared in growth medium and added to the 96-well plate. Plates were incubated at 37°C and 5% CO₂ for 1 week. Cells were then fixed using crystal violet, and the cytopathic effect (CPE) was evaluated visually. The TCID₅₀/ml was calculated using the Reed-Muench formula (66).

To measure the number of MNV genome copies, tissue samples or feces were submitted to the Research Animal Diagnostic Services (Charles River Laboratories, Wilmington, MA) for quantitative PCR (qPCR). RNA was extracted from samples by using either the RNeasy mini kit (QIAGEN, Valencia, CA) or the MagAttract RNA tissue mini M48 kit (QIAGEN) on a KingFisher robotic extraction station (Thermo Labsystems, Franklin, MA). RNA was reverse transcribed to yield cDNA in a 30- μ l reaction mixture containing 5.5 mM MgCl₂, 2.0 mM (each) deoxynucleoside triphosphates, 2.5 μ M random hexamers, 1 \times PCR buffer 2 (Applied Biosystems, Foster City, CA), 20 U of RNase inhibitor (Ap-

plied Biosystems), 37.5 U of MultiScribe reverse transcriptase (Applied Biosystems), and 5 μ l of RNA template. The reaction mixture was incubated at 25°C for 10 min and 48°C for 30 min and then heat inactivated at 95°C for 5 min. The primers and probe used by the Research Animal Diagnostic Services for PCR amplification targeted the ORF1-ORF2 junction, which was completely conserved among all viruses used in this investigation. PCR-amplified product from CR1 was cloned into a plasmid for use as a positive control. A series of 10-fold dilutions (10^1 to 10^6) of the plasmid construct was used to create a standard curve ($R^2 \geq 0.993$). PCR amplification was performed with the 7300 real-time PCR system, and the number of genome copies per reaction was determined using the 7300 system SDS software (Applied Biosystems). The number of copies per sample was estimated based on the amount of the sample used for RNA isolation.

Isolation of new MNVs. Fecal material from adult mice was placed into 1 ml of sterile phosphate-buffered saline and homogenized with 1.0-mm zirconia-silica beads, using a MiniBeadbeater (Biospec Products). The fecal homogenate was clarified by centrifugation, passed through a 0.22- μ m-pore-size filter, and inoculated onto RAW cells. After 4 days, the RAW cells were frozen at -80°C . The thawed virus was clarified by centrifugation and used to generate viral stocks.

Consensus sequence analysis of viral RNA. Total RNA was extracted from MNV-infected RAW cells with TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and an oligo(dT) primer. Genome-specific sequences were amplified with Expand Long (Roche Applied Science) to produce overlapping PCR products. DNA products were sequenced directly with reagents from the Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) on an ABI 3730XL DNA analyzer, using MNV1-specific primers. When these primers failed due to nucleotide mismatching, additional sequence-specific primers were designed and used for sequencing. The termini of the MNV genomes were obtained using the 5' and 3' rapid amplification of cDNA ends system (Invitrogen) and sequence-specific primers. Oligonucleotide primer sequences are available upon request.

Sequence alignments. ORF1, ORF2, or ORF3 of each norovirus was translated using custom scripts from Python version 2.4, and the protein products were aligned using ClustalW (10) with BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). These protein alignments were used for subsequent phylogenetic analyses and also as a basis for the nucleotide sequence alignments. Specifically, Python scripts were used to replace the amino acids in the protein alignment with the nucleotides from the original untranslated sequence, codon by codon, in order to conserve the spatial architecture generated using the more reliable multiple-protein-sequence alignment. The nucleotide sequences of the 5' and 3' untranslated regions (UTRs) were aligned separately using ClustalW and checked manually for accuracy.

Phylogenetic analyses. Bayesian phylogenetic methods were used with MrBayes (version 3.1.2) (33) to determine the evolutionary relationships among noroviruses and to detect potential instances of recombination. ORF1, ORF2, and ORF3 nucleotide and protein alignments and the alignment of the nucleotide sequences of the 3' UTRs were analyzed separately and combined into a single data set for analysis. For the nucleotide sequences, we created a total of 11 data partitions, including first, second, and third codon positions for all three ORFs (9 partitions) and 2 additional partitions for the 5' and 3' UTRs. The MODELTEST program (64) was used to determine the most appropriate nucleotide evolution model for these sequences, which turned out to be the general time-reversible model with a proportion of invariable sites and gamma-distributed rate variation (81), which allows for site-rate heterogeneity. To select the most appropriate model of protein sequence evolution, each of the protein sequence data sets was tested with MrBayes by running 10,000 Markov chain Monte Carlo (MCMC) generations under all nine models of protein sequence evolution to find the amino acid substitution model that best fit the data, which was then used for the full analysis. The WAG model (76) proved to be the best for all of the protein alignments. In the final analysis of all separate and combined protein and nucleotide alignments, we ran four independent Markov chains for one million generations with a burn-in of 5,000 generations. Posterior probabilities of all the trees were calculated by using the majority-rule consensus of all the trees found during the MCMC runs, minus the burn-in trees.

Preparation of polyclonal anti-MNV sera. Five CD-1 mice were orally inoculated with 300 TCID₅₀s of WU11, CR1, CR3, CR6, or CR7 or MNV1.CW3, passage 4, and given an identical dose of the same virus 2 weeks later. Serum samples were collected 4 weeks after the administration of the booster dose, pooled, and heat inactivated before use. An ELISA to detect the binding of polyclonal antisera to purified MNV1 virions was performed as previously described (78).

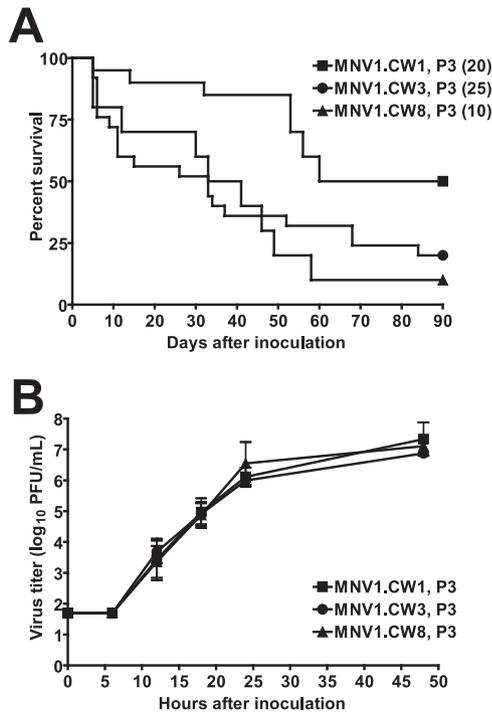


FIG. 1. The virulence of plaque-purified MNV1 clones differed in vivo. (A) MNV1.CW3, passage 3 (P3), and MNV1.CW8, passage 3, were virulent in $STAT1^{-/-}$ mice. $STAT1^{-/-}$ mice were orally inoculated with 3×10^4 PFU of virus. The numbers of mice analyzed are indicated in parentheses. Differences in survival rates corresponding to MNV1.CW1, passage 3, and MNV1.CW3, passage 3 ($P = 0.0109$), and to MNV1.CW1, passage 3, and MNV1.CW8, passage 3 ($P = 0.0017$), were analyzed by the log rank test. (B) Plaque-purified MNV1 clones showed similar growth rates in vitro. RAW cells were inoculated at a multiplicity of infection of 0.05. Error bars represent the standard error of the mean of results from two independent experiments. No differences between the growth curves were detected by analysis of variance ($P = 0.9983$).

Nucleotide sequence accession numbers. The MNV sequences determined in this study have been deposited in GenBank under accession numbers EU004654 to EU004683.

RESULTS

The virulence of plaque-purified MNV1 clones differed in $STAT1$ -deficient mice. The first attempt to plaque purify and passage the MNV1 isolate (GenBank accession number AY228235) in tissue culture generated the clone MNV1.CW1, passage 3, which is attenuated in vivo (78). To determine whether clonal selection and amplification in vitro always lead to the attenuation of MNV in vivo, the MNV1 isolate was triply plaque purified and passaged three times in RAW cells to generate 10 additional clones (MNV1.CW2 through MNV1.CW11). Two of these clones, MNV1.CW3, passage 3, and MNV1.CW8, passage 3, were significantly more virulent in orally inoculated $STAT1^{-/-}$ mice than MNV1.CW1, passage 3 (Fig. 1A). However, all three of these clones had similar growth rates in vitro (Fig. 1B).

In order to determine whether specific amino acid changes were associated with MNV1 virulence in vivo, the consensus sequence of each new passage 3 clone was determined and

compared to the sequences of MNV1 and MNV1.CW1, passage 1 (Table 1). Only two nonsynonymous mutations distinguished the genomes of virulent and attenuated MNVs (Table 1), suggesting that a few mutations can alter the virulence of a norovirus in vivo.

Isolation of new MNVs. The observed in vivo biological differences between MNV1 clones that were less than 0.01% divergent at the nucleotide level raised the possibility of obtaining new isolates of MNV possessing biological properties that differed from those of MNV1. Since the previous isolation of an enteric virus from the brain of an immunocompromised mouse (39) may have altered the biological properties of MNV1, 21 additional MNVs were isolated from the feces of either wild-type or genetically modified mice (Table 2). To increase the diversity of the MNVs isolated, samples were obtained from two research colonies, those at Washington University, St. Louis, MO, and Charles River Laboratories, Wilmington, MA, between March and October 2005. Twenty-one viruses causing a detectable CPE in RAW cells were passaged twice in RAW cells to generate a working stock. This approach was taken to minimize possible tissue culture adaptations from plaque purification and extensive passage in RAW cells and yet generate enough virus for analysis. Of note, minimal CPEs were observed in RAW cells during the amplification of the genomes of many of the new viruses.

The isolation of multiple MNVs as reported here and in other studies presents a nomenclature challenge. Therefore, in this report, we have adopted the nomenclature presently used for caliciviruses (22). Isolates are designated with a cryptogram that is organized as follows: host of origin/genus abbreviation/genogroup abbreviation/virus name/year of occurrence/country of origin. In order to emphasize the established convention for naming noroviruses in which the virus name reflects the location of origin (illustrated in Zheng et al. [82]), three previous isolates, MNV2 to MNV4 (31), are herein called UM2 to UM4 to reflect their isolation at the University of Missouri.

Sequence analysis of MNVs uncovered regions of conservation within the genomes. The consensus sequence of each new MNV genome was determined and compared to the full-length genome sequences available for MNV1, UM2 to UM4, and Berlin (Table 2). All of these MNVs have the three ORFs and the polyadenylated tail that are characteristic of noroviruses (23). However, WU11 has a codon inserted in the region coding for NS1-2 (also called the N-terminal protein) (71), while CR10, CR11, and CR13 have a codon inserted in the region coding for NS4 (also called the 3A-like protein) (79) (Fig. 2A). CR18 has a codon deleted in ORF2 and a single nucleotide

TABLE 1. Nonsynonymous mutations that distinguish virulent and attenuated MNVs

Virus	GenBank accession no.	Phenotype in $STAT1^{-/-}$ mice (reference)	Nucleotide at position ^a :	
			2151	5941
MNV1	AY228235	Virulent (39)	G (V)	A (K)
MNV1.CW1, passage 1		Virulent (78)	G (V)	A (K)
MNV1.CW1, passage 3	DQ285629	Attenuated (78)	A (I)	G (E)
MNV1.CW3, passage 3	EF014462	Virulent	G (V)	A (K)
MNV1.CW8, passage 3	EU004659	Virulent	G (V)	A (K)

^a Amino acids encoded by these nucleotides are shown in parentheses.

TABLE 2. Sources of MNV isolates

Virus	GenBank accession no.	Date of isolation	Location of research colony	Country of origin	Mouse strain	Genetic deficiency(ies) or description ^d	Symptom
MNV1	AY228235	2002	Washington University	U.S.	129Pas	IFN- $\alpha\beta$ R ^{-/-} IFN- γ R ^{-/-}	Sporadic lethality (39)
WU11	EU004663	March 2005	Washington University	U.S.	C57BL/6	OTI/Rag1 ^{-/-} IFN- γ R ^{-/-}	Diarrhea
WU12	EU004664	April 2005	Washington University	U.S.	BALB/c	β_2 M ^{-/-}	Diarrhea
WU20 ^a	EU004665	July 2005	Washington University	U.S.	C57BL/6	Rag1 ^{-/-} IFN- γ R ^{-/-}	None
WU21 ^a	EU004666	July 2005	Washington University	U.S.	C57BL/6	Rag1 ^{-/-} IFN- γ R ^{-/-}	None
WU22 ^a	EU004667	July 2005	Washington University	U.S.	C57BL/6	Rag1 ^{-/-} IFN- γ R ^{-/-}	None
WU23 ^b	EU004668	July 2005	Washington University	U.S.	C57BL/6	OTI/Rag1 ^{-/-} IFN- γ R ^{-/-}	Diarrhea
WU24 ^b	EU004669	July 2005	Washington University	U.S.	C57BL/6	OTI/Rag1 ^{-/-} IFN- γ R ^{-/-}	None
WU25	EU004670	July 2005	Washington University	U.S.	C57BL/6	OTI/Rag1 ^{-/-} IFN- γ R ^{-/-}	Diarrhea
WU26	EU004671	August 2005	Washington University	U.S.	BALB/c	β_2 M ^{-/-}	None
CR1	EU004672	October 2005	Charles River Laboratories	U.S.	CD-1		None
CR3	EU004673	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR4	EU004674	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR5	EU004675	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR6	EU004676	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR7	EU004677	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR10	EU004678	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR11	EU004679	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR13	EU004680	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR15	EU004681	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR17	EU004682	October 2005	Charles River Laboratories	U.S.	C57BL/6	GM	None
CR18	EU004683	October 2005	Charles River Laboratories	Germany	DBA/1		None
UM2	DQ223041	2005	NR ^c	U.S.		Samples pooled from WT and GM mice (31)	NR
UM3	DQ223042	2005	NR	U.S.		Samples pooled from WT and GM mice (31)	NR
UM4	DQ223043	2005	NR	U.S.		Samples pooled from WT and GM mice (31)	NR
Berlin	DQ911368	April 2006	Robert Koch Institute	Germany	NR	IFN- γ R ^{-/-}	NR

^a WU20, WU21, and WU22 were isolated from littermates.

^b WU23 and WU24 were isolated from littermates.

^c NR, not reported.

^d IFN- $\alpha\beta$ R, alpha/beta interferon receptor; IFN- γ R, gamma interferon receptor; OTI and OTII, ovalbumin-specific T-cell receptor transgenic; Rag1, recombination activation gene; β_2 M, β_2 -microglobulin; GM, genetically modified; WT, wild-type.

insertion in ORF3. The insertion in ORF3 introduces a frame-shift in CR18 that is predicted to generate a truncated ORF3 product (Fig. 2A). Interestingly, all sequenced MNV genomes have a fourth ORF (named ORF4) that overlaps ORF2 but in a different reading frame (+1) (Fig. 2A and B). Although ORF4 has not been found in human, bovine, or porcine noroviruses, a similar ORF which overlaps the VP1 coding region is found in the genomes of genogroup I sapoviruses, members of another genus of the *Caliciviridae* (11, 70).

A comparison of all sequenced full-length MNV genomes revealed only three regions in which more than 30 nucleotides were completely conserved. Thirty-two nucleotides at the 5' ends of the genomes were conserved, while 64 nucleotides at the ORF1-ORF2 junctions were conserved (Fig. 2B). A role for each of these regions in norovirus replication has been proposed previously (4, 11, 24, 25, 63). The third region of conservation lay within ORF2 and ORF4. Forty-seven nucleotides between positions 5401 and 5447 were completely conserved in all sequenced MNV genomes. In addition, a comparison of all sequenced MNV genomes showed that the 3' UTRs of MNVs contained both conserved and variable regions and ranged from 73 to 82 nucleotides in length (Fig. 2C).

A comparison of the predicted protein products of all sequenced MNV genomes revealed that most of the polyprotein cleavage sites were conserved at the P1 position, with only a

conservative N706S substitution at the P1' position in CR3, CR6, CR7, and UM2 and a conservative G871S substitution at the P1' position in CR3, CR4, CR10, CR11, CR13, and UM3. These observations lend further support to the cleavage map of the MNV NS polyprotein that was determined previously (71). In that study, the comigration of an expressed NS1-2 protein and a protein from in vitro translation of the MNV genome indicated that the translation of the ORF1 polyprotein begins at the first or second start codon. A comparison of the predicted NS1-2 protein products of all sequenced MNV genomes supported this conclusion, as the first and second translational start codons were completely conserved (Fig. 2B). In addition, the third translational start codon was not conserved in all sequenced MNV genomes; WU11, WU20 to WU25, CR4, CR7, CR10, CR11, CR13, and UM4 each had an M48L substitution.

A mutational analysis was used previously to identify two caspase 3 cleavage sites in the NS1-2 protein, ¹¹⁸DXXD¹²¹ and ¹²⁸DXXD¹³¹ (71). However, neither of these motifs is completely conserved in all sequenced MNV genomes. A D121G substitution is found in CR15, while a D131E substitution is found in CR11 and CR13. Interestingly, Sosnovtsev et al. found that the putative caspase 3 cleavage site ¹⁰⁰DXXD¹⁰³ is not recognized by caspase 3 in an in vitro cleavage assay (71). The complete conservation of this motif in all sequenced MNV

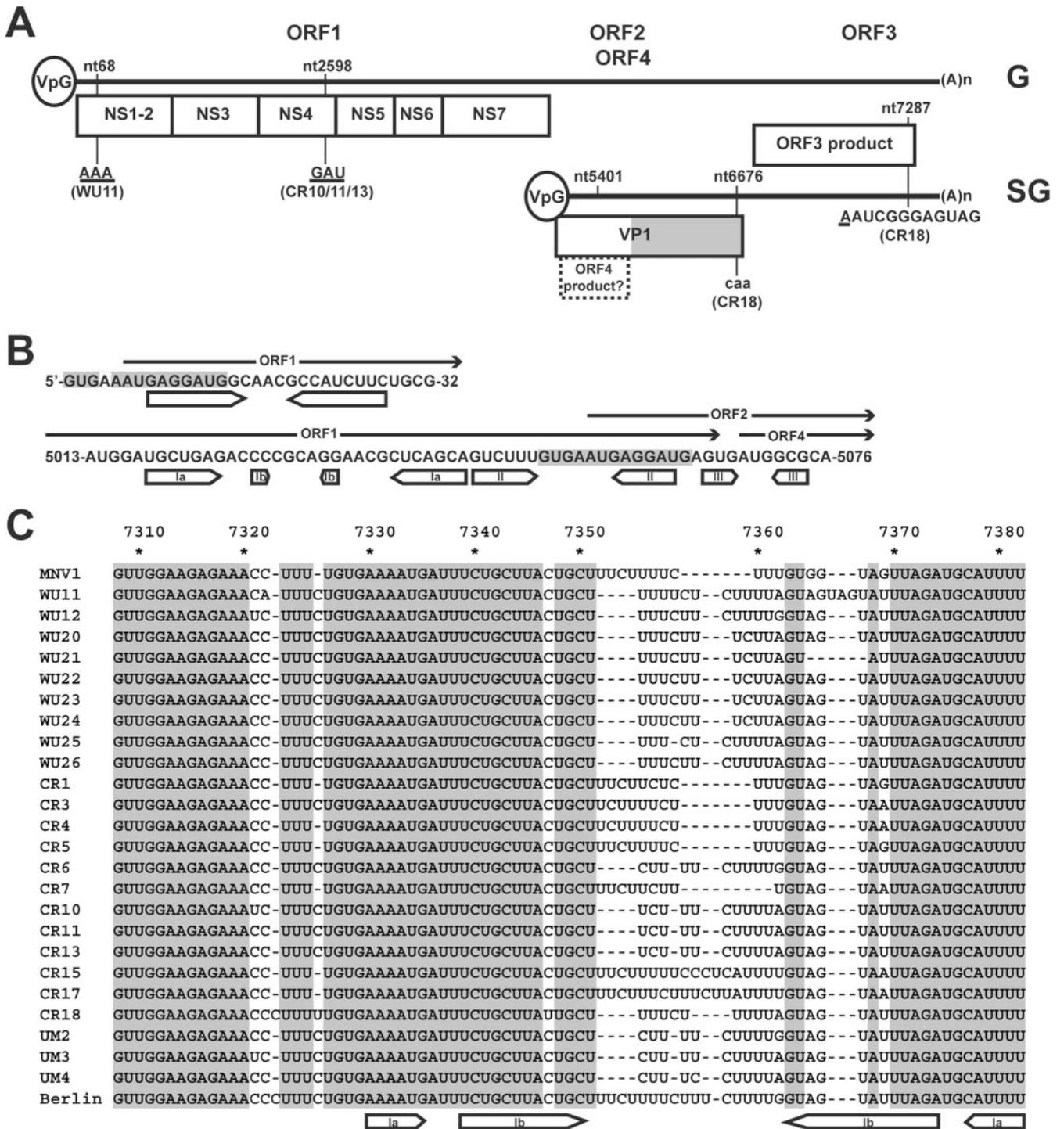


FIG. 2. Alignment of multiple full-length MNV genomes. (A) The genome organization was conserved among all sequenced MNV genomes. The presence of a viral protein (VpG) linked to the genomic (G) and subgenomic (SG) RNA is predicted, but not proven, for MNV. The nucleotide positions of insertions and deletions identified in specific MNVs are indicated, as is the beginning of a 47-nucleotide (nt) region of nucleotide conservation in ORF2. Nucleotide numbering is based on the MNV1 sequence (GenBank accession number AY228235). Insertions are underlined, while deletions are indicated in lowercase. A truncated ORF3 coding region caused by the insertion in CR18 is also shown. The P domain of VP1 is shaded in gray. (B) The 5' ends and ORF1-ORF2 junctions of all sequenced MNV genomes were conserved. Thirteen nucleotides conserved at the 5' ends of the genomic and subgenomic RNAs are shaded in gray. Inverted repeats that form the stems of hairpins predicted using mfold (version 3.2) (51, 83) are represented by open arrows. (C) The 3' UTRs of all sequenced MNV genomes had conserved and variable regions. Conserved nucleotides are shaded in gray. Inverted repeats that form the stems of hairpins predicted using mfold (version 3.2) (51, 83) are represented by open arrows.

TABLE 3. Sequence divergences between MNVs

Genome region or corresponding protein product	Range of uncorrected pairwise sequence divergences ^a :	
	Nucleotides ^b	Amino acids
Whole genome	0–0.13	
ORF1	0–0.14	
NS1-2 (N-terminal protein)	0–0.15	0–0.08
NS3 (NTPase)	0–0.17	0–0.03
NS4 (3A-like protein)	0–0.19	0–0.09
NS5 (VpG)	0–0.15	0–0.11
NS6 (Pro)	0–0.16	0–0.05
NS7 (Pol)	0–0.13	0–0.05
ORF2	0–0.13	0–0.07
S domain	0–0.05	0–0.02
P domain	0–0.19	0–0.11
ORF3	0–0.13	0–0.11

^a Uncorrected sequence divergences were calculated using PAUP* (72).

^b Similar sequence divergences were calculated using maximum-likelihood analysis (72).

genomes suggests that the cleavage of this motif by a protein other than caspase 3 may have a biological role in the life cycle of MNV.

The identification of a previously unrecognized ORF, as well as a novel region of nucleotide conservation within ORF2 and a conserved DXXD motif in the NS1-2 protein, illustrates how a comparison of multiple full-length norovirus genomes can reveal unsuspected features of the genomes. We speculate that this limited evolutionary divergence reflects as-yet-unidentified functional constraints.

Phylogenetic analysis of MNVs revealed 15 strains that comprise a single genotype and genogroup. To understand the evolution of the MNV genome in more detail, the sequence divergences for the whole genome, each ORF, each coding region, and each protein product from all sequenced full-length MNV genomes were calculated. All sequenced MNV genomes were 87% or more similar at the nucleotide level, with up to 19% divergence observed in the regions coding for NS4 and the P domain (Table 3). These MNV genomes were even more similar at the amino acid level, with no more than 11% divergence in any protein product (Table 3).

To examine the relationship of MNVs to other noroviruses, a consensus Bayesian tree was constructed using an alignment that compared VP1 sequences of MNVs with that of the prototype strain of each of the other genogroups: Norwalk (M87661), Hawaii (U07611), Jena (AJ011099), and Alpatron (AF195847). MNVs comprise a single, well-defined genogroup distinct from other genogroups (Fig. 3A). The division of the tree into five genogroups was supported by high posterior probabilities. The relationship among MNVs was not as well supported (data not shown). Similar tree topologies were generated using maximum-likelihood and neighbor joining analyses (72) (data not shown). These data confirmed previous observations about the phylogenetic relationship of MNVs to other noroviruses based on studies using a single or a limited number of MNV strains (30, 39, 79, 82). The examination of a histogram representing uncorrected pairwise divergences between VP1 sequences of MNVs and those of the prototype strains of other genogroups revealed a single peak for MNVs

(Fig. 3B), demonstrating that, unlike human noroviruses, all sequenced full-length MNV genomes comprise a single genotype, which we have named genotype I.

To resolve the phylogenetic relationships among MNVs, consensus Bayesian trees were constructed using alignments of the nucleotide sequences of the entire genome, ORF1, each coding region within ORF1, ORF2, the region coding for the S and P domains, and ORF3 for all sequenced full-length MNV genomes. The phylogenetic trees comparing the whole genome, ORF1, and ORF2 sequences revealed 15 well-supported phylogenetic clades, or strains (Fig. 3C and 4A). Similar tree topologies were generated using maximum-likelihood and neighbor joining analyses (72) (data not shown). The examination of a histogram representing uncorrected pairwise sequence divergences for the whole genome, ORF1, and ORF2 supported the division of MNVs into 15 strains, as the intra- and interstrain pairwise distances did not overlap (data not shown). The phylogenetic trees based on ORF3 and many of the individual coding regions were not supported by high posterior probabilities, probably due to the lack of divergence and/or the reduced lengths of these coding regions (see Fig. S1 in the supplemental material; also Table 3). Conversely, the phylogenetic trees based on the regions coding for NS3 (also called NTPase) (71), NS4, and the P domain were well supported, most likely due to the increased divergence in these coding regions compared to that in the other regions we examined (see Fig. S1 in the supplemental material; also Table 3). A sequence analysis of one of these three coding regions may be enough to initially identify new MNV isolates. However, only full-length genome sequences should be used to classify new MNV strains, as the trees based on the regions coding for NS3, NS4, and the P domain did not illustrate the same phylogenetic relationships (see Fig. S1 in the supplemental material).

Recombinant regions in several MNVs were detected. A comparison of the consensus Bayesian trees generated using alignments of the whole genome, ORF1, and ORF2 nucleotide sequences revealed considerable areas of disagreement among the trees (Fig. 4A). For example, the tree based on ORF1 sequences placed CR15 far away from MNV1, while the tree based on ORF2 sequences placed CR15 in close proximity to MNV1. To determine whether recombination may have occurred in MNV genomes, an analysis using six automated methods available in the Recombination Detection Program 2 was performed (50). Recombinant regions in several MNV genomes were predicted by three methods (Table 4), although recombinant regions in many MNV genomes were predicted by one or two methods (data not shown). Recombination in the genome of CR1, CR18, or Berlin was not predicted by any method. Of note, most of the predicted recombinant regions were located within ORF2 and ORF3. Interestingly, two recombinant regions in the genomes of WU11 and CR7 were predicted, possibly due to sequential recombination events in the ancestors of these two viruses. To examine the predicted recombinant regions in more detail, the nucleotide similarity between the putative recombinant genomes listed in Table 4 and those of the nonrecombinant viruses CR1 and CR18 was plotted using Simplot2 (http://sray.med.som.jhmi.edu/RaySoft/simplot_old/Version2/SimPlot_Doc_v24.html). A region in ORF2 coding for the P domain that showed increased similar-

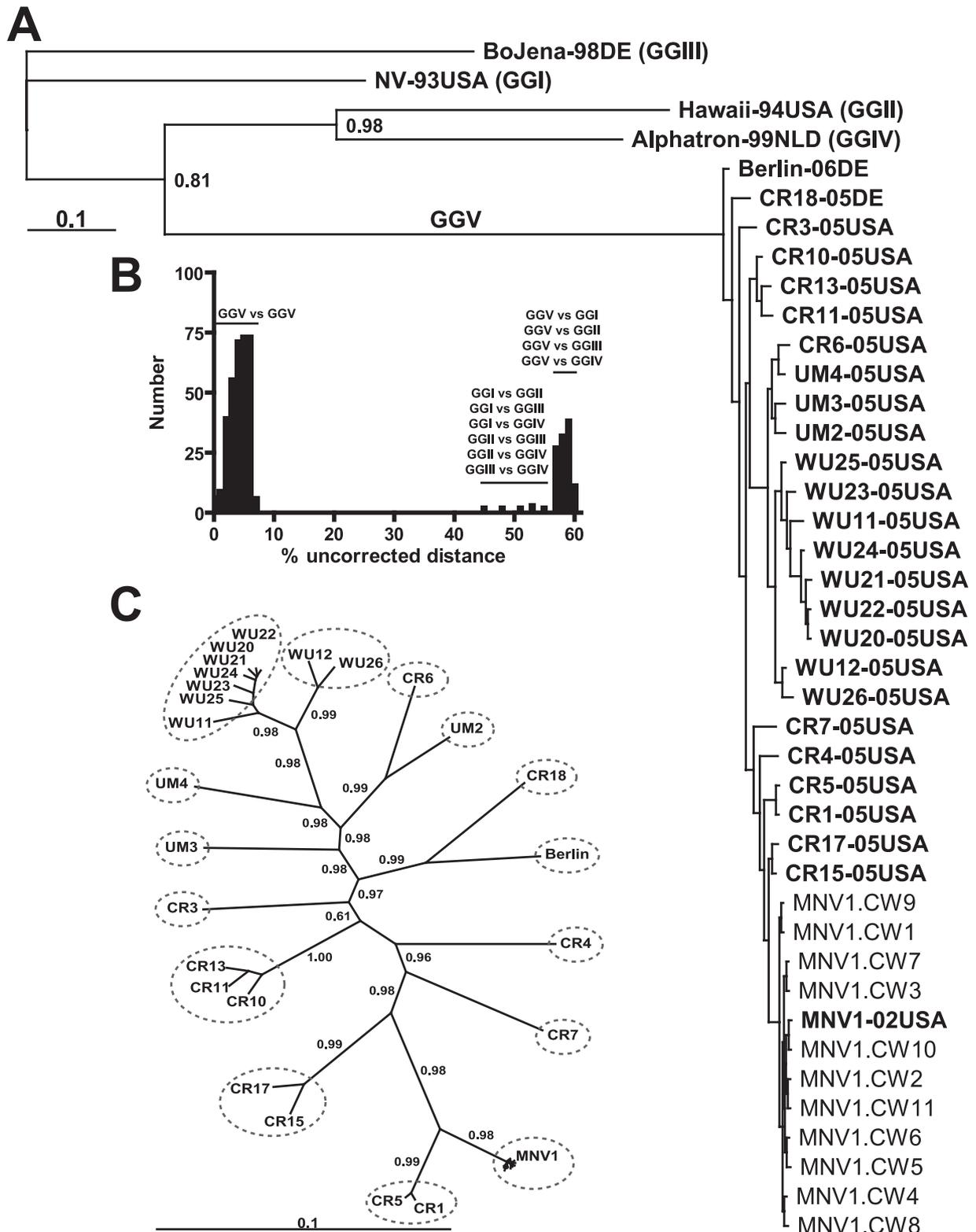


FIG. 3. Phylogenetic analysis of MNVs. (A) All sequenced MNV genomes comprise a single genogroup (GG). A consensus Bayesian tree based on VP1 sequences from MNVs and from prototype strains of other norovirus genogroups is shown. Posterior probabilities are presented next to the branches (1.00 is equivalent to 100% of the trees found in 1,000,000 MCMC generations contained in that particular grouping). Estimated sequence divergence, 0.1, or 10%. Sequence designations were assigned as follows: name-year, country code. Country codes: DE, Germany; NLD, The Netherlands. (B) All sequenced MNV genomes comprise a single genotype. The distribution of pairwise sequence divergences for comparisons of VP1 sequences from MNVs and from prototype strains of other norovirus genogroups is shown. Uncorrected pairwise sequence divergences were calculated using PAUP* (72). (C) All the sequenced MNV genomes comprise 15 distinct strains. A consensus Bayesian tree based on full-length MNV genomes is shown. Genetically distinct MNV strains are circled. For clarity, the names of plaque-purified MNV1 clones have been removed from the tree and are represented by branches.

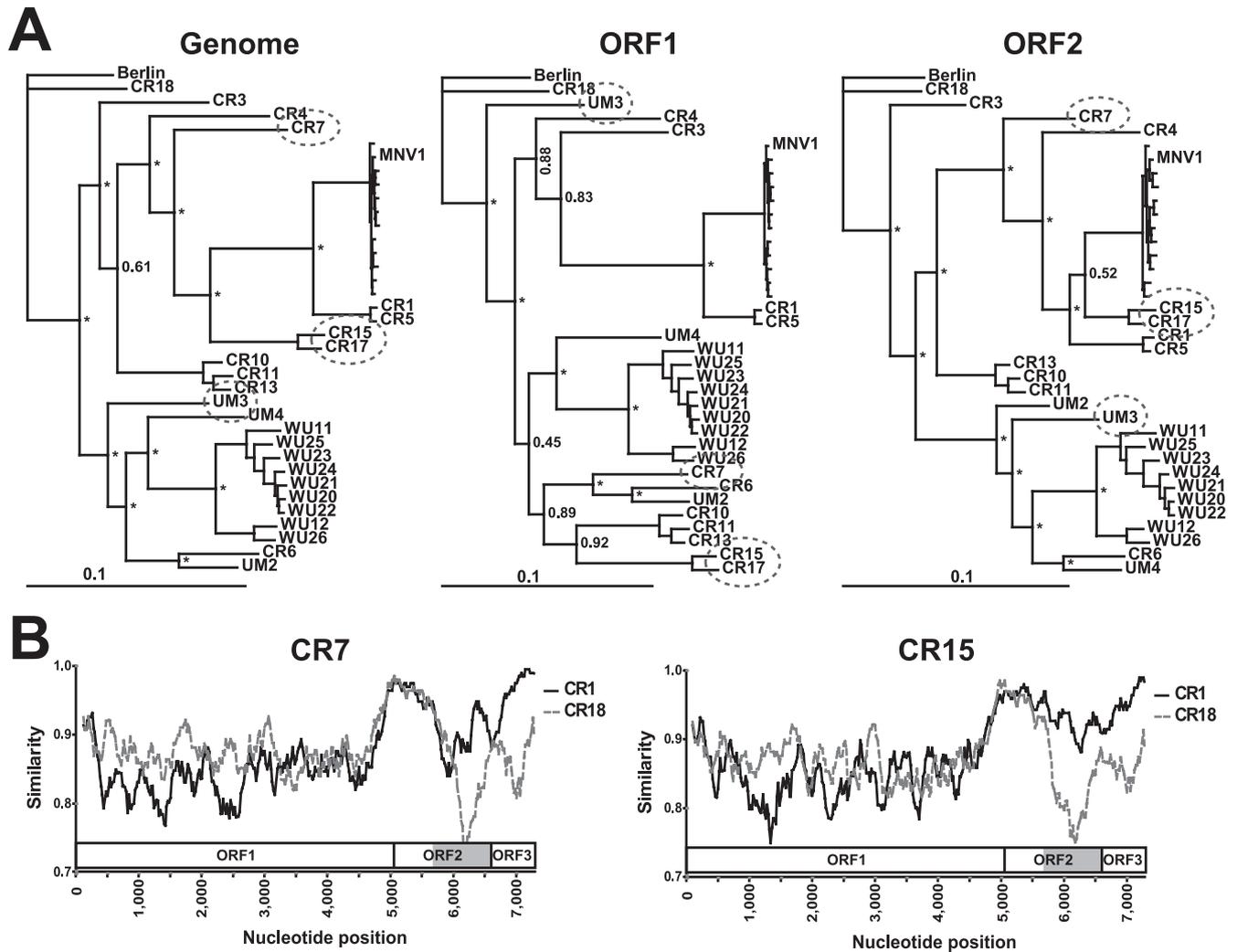


FIG. 4. Detection of recombinant MNVs. (A) Consensus Bayesian trees constructed using the whole genome, ORF1, and ORF2 nucleotide sequences are shown. MNVs with discordant positions are circled. A posterior probability of 0.95 or greater is represented by an asterisk. (B) The nucleotide similarity between the putative recombinant viruses CR7 and CR15 and the two nonrecombinant viruses CR1 and CR18 is shown. Nucleotide similarity was plotted using Simplot2 (http://sray.med.som.jhmi.edu/RaySoft/simplot_old/Version2/SimPlot_Doc_v24.html). A window size of 200 nucleotides with an increment of 20 was used. For similarity, 1.0 equals 100%. The P domain coding region of ORF2 is indicated by gray shading.

TABLE 4. Recombinant MNV genomes^a

Virus(es)	Recombinant region	Nucleotide position ^b
WU11	ORF1-ORF2	4661-5131
	ORF2-ORF3	6351-6692
WU12	ORF2-ORF3	6351-6692
WU20 to WU24	ORF2-ORF3	6159-7244
CR4	ORF2-ORF3	5831-6848
CR6	ORF2	6159-6448
CR7	ORF2-ORF3	5832-6849
	ORF3	7004-7286
CR15	ORF2-ORF3	5667-7357
CR17	ORF2-ORF3	5703-7010
UM3	ORF2	6159-6448
UM4	ORF2	6158-6563

^a Predicted by the Recombination Detection Program (49), GeneConv (60), and Sister-scanning (18) methods.

^b Nucleotide numbering is based on the sequence of MNV1 (GenBank accession no. AY228235).

ity to the corresponding region of CR1 and decreased similarity to the corresponding region of CR18 was detected only in CR4, CR7, CR15, and CR17 (Fig. 4B; also data not shown). Taken together, these data suggest that recombination has occurred in MNV genomes, although convergent evolution is also a possibility. Additional studies using coinfection with genetically distinct MNV strains will be needed to confirm that recombination can occur in MNV genomes.

Many new MNV strains exhibited altered plaque morphology. As noted above, many of the new MNVs caused minimal CPE during amplification in RAW cells. To examine the in vitro growth of the new MNVs in more detail, the ability of each MNV to form plaques on RAW cell monolayers was assessed. Several of the new MNVs, WU11 and WU20 to WU25, formed large, lytic plaques on RAW cell monolayers similar to those formed by MNV1.CW3 (Fig. 5; also data not shown). Like MNV1.CW3, these MNVs caused significant

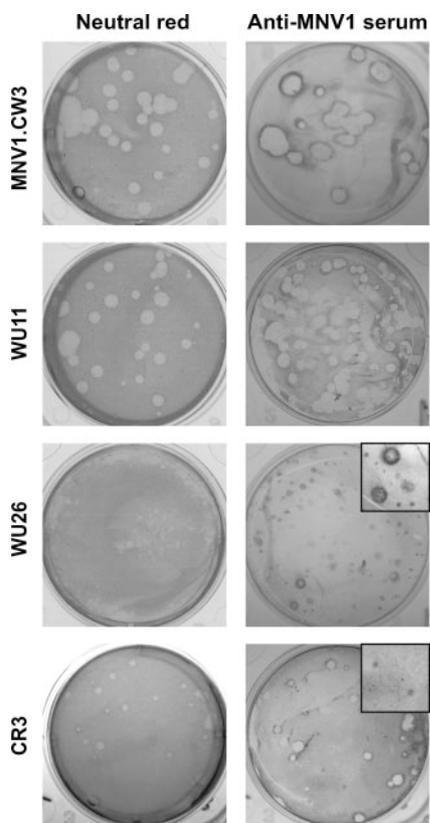


FIG. 5. Many new MNVs exhibited altered plaque morphology. RAW cell monolayers were infected with dilutions of WU11, WU26, CR3, or MNV1.CW3, passage 4, and processed by plaque assay. Plaques were visualized with neutral red staining or fixed with acid alcohol, incubated with a 1:5,000 dilution of rabbit polyclonal serum raised against MNV1 (78), and visualized using the rabbit immunoglobulin Vectastain Elite ABC reagent and a 3,3'-diaminobenzidine kit (Vector Laboratories, Inc., Burlingame, CA). Pictures of wells with dilutions showing individual plaques were taken.

CPE during amplification in RAW cells and had titers of 10^6 to 10^7 PFU/ml. The rest of the new MNVs did not form plaques of the type observed for MNV1.CW3 and did not cause significant CPE during amplification. The plaques were often

diffuse and/or small, making them difficult to detect using a neutral red stain of the RAW cell monolayer (Fig. 5; also data not shown). All plaques formed by the new MNVs were visualized using a rabbit polyclonal serum that was raised against MNV1 virions (78) (Fig. 5; also data not shown), demonstrating serological cross-reactivity between all MNV strains examined and MNV1. This observation supports the findings of a previous study demonstrating the cross-reactivity of MNV1, UM2, UM3, and UM4 by an indirect fluorescent-antibody assay (32).

Serological analysis comparing neutralizing antibody responses between highly divergent strains suggested that MNVs may comprise a single serotype. Volunteer challenge studies and immune capture electron microscopy have previously identified human noroviruses that do not cross-protect and cross-react, respectively (42, 43, 48, 80). However, more recent studies using antibodies raised against virus-like particles have demonstrated broad cross-reactivity between even distantly related human noroviruses (28, 38). Due to the lack of a tissue culture system and animal model, the ability of human noroviruses to cross-neutralize cannot be determined. We took advantage of the ability of MNV1.CW3 to form plaques on RAW cell monolayers to evaluate the ability of other MNV strains to neutralize MNV1. Immune serum against each of the following MNVs was raised: MNV1.CW3, WU11, CR1, CR3, CR6, and CR7. The viruses chosen for antiserum production were closely or distantly related to MNV1.CW3 (Fig. 3A and C). All of the antisera were able to bind MNV1.CW3 virions (Fig. 6A), showing that all MNV strains examined cross-reacted with MNV1. Significant differences in binding were seen for antisera raised against MNV1.CW3 and CR7 (Fig. 6A). Possible explanations for these differences are altered kinetics of seroconversion in vivo and/or differences in the affinities for MNV1 virions in vitro. Despite these differences, all of the antisera were able to neutralize MNV1.CW3 in vitro (Fig. 6B). The ability of antisera raised against even distantly related MNV strains to neutralize MNV1.CW1 suggested that MNVs may comprise a single serogroup.

MNV1.CW3 was cleared from wild-type mice. Since MNVs in a single genogroup exhibited different growth in vitro (Fig.

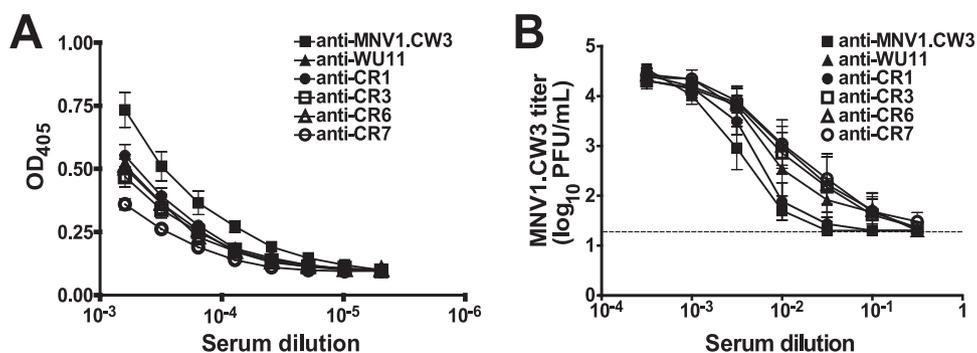


FIG. 6. Serological analysis suggested that MNVs comprise a single serotype. (A) Sera raised against other MNV strains reacted with MNV1.CW3. Antisera were raised against WU11, CR1, CR3, CR6, CR7, and MNV1.CW3, passage 4. A serial dilution of each antiserum was incubated with purified virions of MNV1.CW3, passage 5, and reactivity was measured by an ELISA. Significant differences in the reactivity of the anti-MNV1.CW3 serum ($P < 0.03$) and the anti-CR7 serum ($P < 0.04$) were detected by the paired t test. Error bars represent the standard error of the mean of results for three independently generated antisera for each virus. OD₄₀₅, optical density at 405 nm. (B) Sera raised against other MNV strains neutralized MNV1.CW3. A serial dilution of each antiserum was incubated with 3×10^4 PFU of MNV1.CW3, passage 4. The limit of detection of the plaque neutralization assay is represented by a dashed line. No significant differences in neutralization were detected by analysis of variance ($P = 0.9465$). Error bars represent the standard error of the mean of results for three independently generated antisera for each virus.

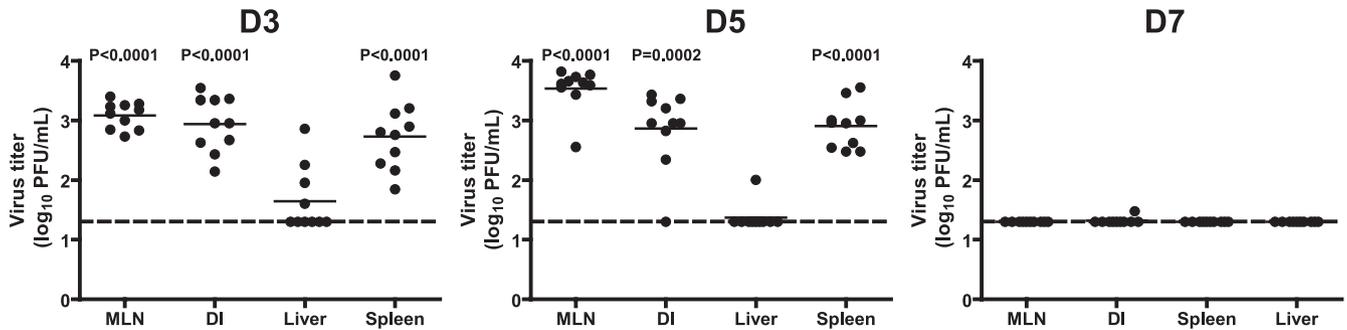


FIG. 7. MNV1.CW3 was rapidly cleared from wild-type mice. C57BL/6 mice were orally inoculated with 3×10^7 PFU of MNV1.CW3, passage 5. Tissue samples were harvested at 3 (D3), 5 (D5), and 7 (D7) days after inoculation. Virus titers were measured by plaque assays. Significant differences in mean titers were detected by the Mann-Whitney test. DI, distal ileum.

5; data not shown), we examined the growth of several MNVs *in vivo*. Previous studies demonstrated that MNV1.CW1 does not persistently infect wild-type CD-1 mice (31, 32). However, as demonstrated above, MNV1.CW1, passage 3, is attenuated in STAT1^{-/-} mice *in vivo*. Therefore, wild-type C57BL/6 mice were orally inoculated with 3×10^7 PFU to examine the kinetics of MNV1.CW3 infection. Significant virus titers in the mesenteric lymph nodes (MLN), distal ilea, and spleens were detected at both 3 and 5 days after inoculation (Fig. 7). However, virus titers above the limit of detection (20 PFU/ml) were not found in any organ at 7 days after inoculation. These data agree with the results of a previous study demonstrating the clearance of MNV1.CW3 from the duodena, spleens, and livers of wild-type 129S6/SvEv mice by day 7 after inoculation (56). These data demonstrate that although MNV1.CW3 was able to acutely infect wild-type mice, it was rapidly cleared.

Several new MNVs persistently infected wild-type mice. Since many of the new MNVs did not form easily detectable plaques on RAW cell monolayers (Fig. 5; also data not shown), a TCID₅₀ assay with RAW cells was used to investigate the ability of five new MNVs, WU11, CR1, CR3, CR6, and CR7, to infect C57BL/6 mice by the oral route. Due to the low titers of some of these viruses, mice were inoculated with 300 TCID₅₀s. Even at this low dose, significant virus titers in the MLN were detected at 3 days after inoculation with CR3, CR6, or CR7 (Fig. 8A). Significant virus titers in the distal ilea were also detected at 3 and 7 days after inoculation with CR3. No significant virus titers in the MLN or distal ilea were detected at day 3 or day 7 after inoculation with MNV1.CW3, WU11, or CR1 or at day 35 after inoculation with any virus tested (Fig. 8A).

Due to the toxicity of tissue samples to RAW cells, TCID₅₀ analysis is not very sensitive and does not permit the analysis of fecal material. To analyze tissue samples by a more sensitive assay and to determine whether the new MNVs were shed from C57BL/6 mice, the number of genome copies in feces was measured using qPCR. Significant numbers of viral genome copies in the feces were detected at 3, 7, and 35 days after oral inoculation with CR3, CR6, and CR7 and at 7 and 35 days after inoculation with CR1 (Fig. 8B). Significant numbers of viral genome copies in the MLN and distal ilea, but not in the duodena, lungs, livers, or spleens, were also detected at 35 days after inoculation with CR1 or CR3 (Fig. 8C). The discrepancy

between the CR1, CR3, CR6, and CR7 titers measured by the TCID₅₀ assay and the numbers of viral genome copies may be due to the high limit of detection of the TCID₅₀ assay (200 TCID₅₀s/ml). Alternatively, this discrepancy may be due to a ratio of genome copies to infectious doses that is greater than 1. Interestingly, the same mice that were shedding virus at day 35 (those inoculated with CR1, CR3, CR6, or CR7) also exhibited a robust seroconversion at day 35 (Fig. 8D). In contrast, mice inoculated with MNV1.CW3 or WU11 had undetectable levels of MNV antibodies at day 35. One possible explanation for this observation would be delayed seroconversion for these viruses after inoculation with a low dose of virus. These experiments demonstrated that long-term shedding of MNVs can occur and suggested that the site of persistent MNV replication is the MLN and/or the distal ileum. These data extend the observations of a previous study that demonstrated the presence of UM2, UM3, and UM4 genomes in the MLN, jejunum, spleens, and feces of CD-1 mice at 8 weeks after infection (31).

DISCUSSION

Although MNV is widespread in research colonies in the United States and Canada and has been isolated from a research colony in Germany (GenBank accession number DQ911368) (32), only five full-length sequences have been reported to date. In this study, we describe the isolation and characterization of 21 new MNV isolates from two geographically separate research colonies. Although all 26 full-length MNV genomes were 87% or more similar at the nucleotide level, a phylogenetic analysis revealed 15 distinct MNV strains comprising a single genotype and genogroup. The demonstration of cross-neutralization between highly divergent MNV strains suggested that the MNV genogroup is a single serotype.

Asymptomatic, persistent infection may promote the emergence of new norovirus strains. The isolation of the original MNV strain from the brains of immunocompromised mice (39) may have altered the biology of this virus *in vivo*. In this study, we demonstrated that several new MNV strains isolated from the feces of mice persistently infected wild-type mice. Like several MNV strains that were previously isolated from the MLN (31), CR1 and CR3 were detected in the MLN and intestines and were shed in feces weeks after inoculation. How-

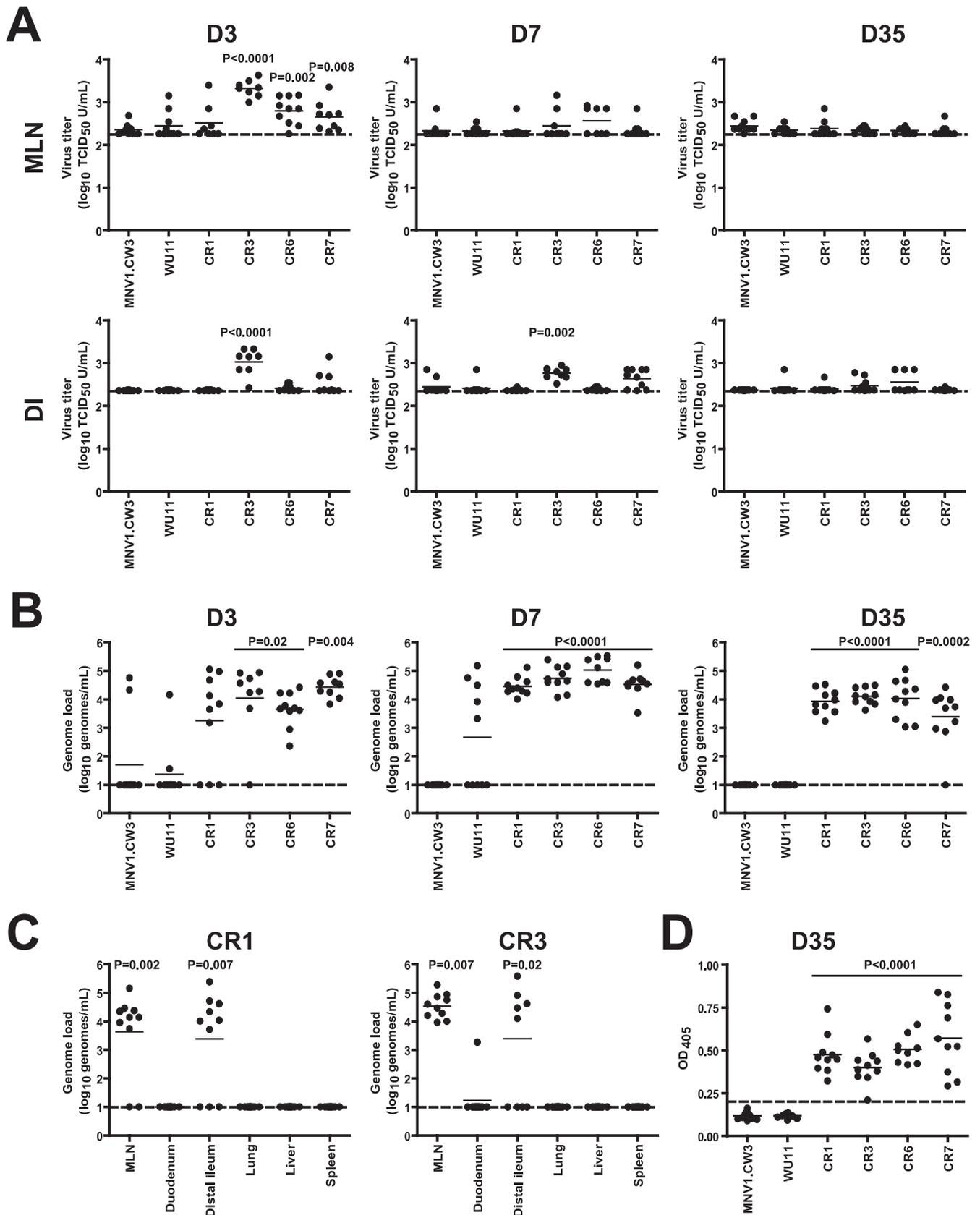


FIG. 8. Several new MNV strains persistently infected wild-type mice. C57BL/6 mice were orally inoculated with 300 TCID₅₀s of WU11, CR1, CR3, CR6, CR7, or MNV1.CW3, passage 4. Tissue samples were harvested at 3 (D3), 7 (D7), and 35 (D35) days after inoculation. (A) Virus titers in the MLN and distal ilea (DI) were measured by the TCID₅₀ assay. The limit of detection of the TCID₅₀ assay is represented by a dashed line.

ever, not all MNV strains isolated from feces persisted in wild-type mice; WU11 was not shed in feces at 35 days after inoculation. Overt symptoms of clinical disease were not observed in wild-type mice after inoculation with either persistent or nonpersistent strains of MNV. Long-term human norovirus shedding by immunocompromised patients has been documented previously (17, 41, 52, 58). The recent demonstration that children of less than 6 weeks of age can shed noroviruses for 6 weeks (57) raises the intriguing possibility that persistent infection with these viruses may be more common than is presently appreciated. The ability of MNVs to persist in immunocompetent mice in the absence of clinical symptoms supports this idea. Indeed, persistent norovirus infection may permit the accumulation of genetic changes necessary for the emergence of new norovirus strains. Future studies using the MNV model will examine the role of persistent infection in norovirus evolution.

Homologous recombination may lead to the emergence of new norovirus strains. Recombinant human noroviruses have been identified as the cause of norovirus outbreaks worldwide (14, 27, 34, 40, 62, 74). However, the molecular mechanisms that promote norovirus recombination have not been determined experimentally. In this study, we report evidence for recombination at several breakpoints within ORF2 in several MNV genomes. Recombination within ORF2 has also been reported for human noroviruses, although the predominant breakpoint detected in recombinant human norovirus genomes is at the ORF1-ORF2 junction (61, 69). Additional studies using coinfection with genetically distinct MNVs will be needed to map the recombination hot spot(s) in the MNV genome. Coinfection with multiple human noroviruses after the consumption of shellfish and the cocirculation of multiple human noroviruses during outbreaks have been reported previously (7, 9, 37, 67, 77). However, the role of recombination in the evolution of new norovirus strains is not understood, as some recombinant noroviruses do not spread widely and cause only sporadic cases of gastroenteritis (1). The recovery of genetically distinct MNV isolates from the feces of an individual mouse and the cocirculation of multiple MNV strains in individual isolator cages (K. S. Henderson, unpublished observations) argue that the MNV model can be used to study the role of recombination in norovirus evolution in vivo.

Identification of an additional ORF in the MNV genome and a novel region of nucleotide conservation in ORF2. The alignment of 26 full-length MNV genomes uncovered regions of conservation at both the nucleotide and amino acid levels. The conservation at the 5' end of the genome and at the ORF1-ORF2 junction was predicted, since the genome organization of MNV is shared with human noroviruses (71, 79). However, in this study, we detected an additional ORF, ORF4, in all sequenced full-length MNV genomes. ORF4 overlaps ORF2

and is predicted to encode a 214-amino-acid protein. A similar ORF that overlaps the VP1 coding region is present in genogroup I sapoviruses, although the production of a protein from this ORF has not been demonstrated. Future studies will assess whether an ORF4 protein is expressed during MNV replication in culture and/or in vivo and will determine the role of this putative protein in MNV biology. In this study, we also identified a region of complete conservation between nucleotides 5401 and 5447 of ORF2. Perhaps this region, like other regions of nucleotide conservation in the norovirus genome, has a role in norovirus replication. Alternatively, this region may correspond to part of a conserved domain found in both the capsid protein VP1 and the putative ORF4 protein. The conservation of amino acids in two overlapping protein products would severely limit the possibility of synonymous substitutions. Future studies will aim to elucidate the role of this region in MNV biology.

Classification of MNVs. Molecular analyses of partial sequences in conserved regions of the human norovirus genome have previously been used successfully to diagnose and type human norovirus infection. The regions commonly used for these analyses are region A (the *pol* gene coding for NS7), region B (the 3' end of ORF1), region C (the 5' end of ORF2), and region D (the 3' end of ORF2) (2, 3). More recently, the ORF1-ORF2 junction has been used for human norovirus outbreak analysis (8, 36). The extreme level of conservation of the ORF1-ORF2 junction in all sequenced full-length MNV genomes suggests that this region should be used to diagnose MNV infection. Indeed, it was used successfully in this study to design qPCR primers and probes that detected six MNV strains. This study demonstrated that the use of partial sequences is inadequate for a phylogenetically robust classification of MNV strains due to the high degree of similarity among MNV genomes and the potentially confounding factor of recombination. Therefore, we recommend the use of full-length genome sequences for the classification of MNVs. Furthermore, we propose that in order for a new isolate to be categorized as a new MNV strain, the full-length genome should be compared to previously sequenced genomes. We speculate that the identification of a new isolate as an MNV strain within the present MNV genogroup will require divergence of greater than 3% from the genome of a reported strain at the nucleotide level. The biological relevance of classifying MNVs as different strains remains to be determined. We further propose that to designate a new MNV genotype within the present MNV genogroup, a comparison of VP1 sequences must show a distinct genetic cluster that does not overlap with that of genotype I.

In conclusion, the isolation and characterization of 21 new MNV isolates provide a detailed molecular map for future studies of MNV biology. Studies to identify molecular deter-

Significant differences in mean titers compared to those of MNV1.CW3, passage 4, were detected using the Mann-Whitney test. (B) The number of genome copies in a single fecal pellet was measured using qPCR. The limit of detection of qPCR is represented by a dashed line. Significant differences in mean numbers of genome copies compared to those of MNV1.CW3, passage 4, were detected using the Mann-Whitney test. (C) The numbers of genome copies in various tissues at 35 days after inoculation were measured using qPCR. (D) The seroconversion of mice inoculated with MNV was measured by an ELISA. The background optical density at 405 nm (OD_{405}) is represented by a dashed line. Significant differences in mean OD_{405} compared to that of MNV1.CW3, passage 4, were detected using the Mann-Whitney test.

minants of persistence and recombination have awaited the development of a reverse genetics system for MNV. Such a system has recently been developed (75), providing an opportunity to define the importance of the conserved and divergent MNV sequences observed in the present study. The inability of phylogenetic analysis to predict the *in vitro* and *in vivo* biological properties of even closely related MNV strains within a single genogroup suggests that caution is advisable in drawing conclusions about the properties of human noroviruses based solely on genetic classification.

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