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RAPID COMMUNICATION

The Canine Minute Virus (Minute Virus of Canines) Is a Distinct Parvovirus That Is Most Similar to Bovine Parvovirus

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We characterized the genome and proteins of the canine minute virus (the minute virus of canines (MVC)). The genome sequence showed MVC to be an autonomous parvovirus encoding a large nonstructural protein 1 gene, a smaller nonstructural protein, and overlapping VP1 and VP2 protein genes. The virus was most closely related to bovine parvovirus (BPV), with which it was 43% identical at the DNA sequence level, while the NS1 and VP1 proteins were 33.6 and 41.4% identical to those of BPV, respectively. Spliced messages of the NS1 gene transcripts were detected by RT-PCR. VP1 and VP2 proteins were detected in purified capsids, as were modified versions of each protein, and VP3 was also found in full capsids.

Introduction. The canine minute virus, also known as the minute virus of canines (MVC), was first described as an isolate from a healthy dog in the U.S.A. (*1*). That virus was grown in the Walter Reed canine (WRC) cell line and had the properties of a parvovirus, including small size (20–21 nm diameter), and the presence of virions in infected cell nuclei (*1–3*). The MVC appeared antigenically and genetically distinct from the canine parvovirus type-2 (CPV) based on antibody cross reactivity and restriction enzyme analysis of the viral DNA (*3*), but its genomic structure and relationship to other parvoviruses has not been described. The distribution of MVC and its association with canine diseases are also not well understood. Serological testing suggests that MVC is widespread in dogs in the U.S.A., with over 50% positive samples in the studies reported (*1, 4*). Although it is likely that most infections with MVC are subclinical, diseases associated with virus infection include fetal infections leading to reproductive failure and neonatal respiratory disease (*2, 5, 6*). The virus may also be associated with some cases of enteritis in puppies or older dogs (*1*). However, MVC is highly restricted in its tissue culture replication; there are few sensitive diagnostic tests widely available, and the true incidence of the virus infection or its associated diseases are therefore not known. Parvoviruses are widespread pathogens of vertebrate and invertebrate animals and are the cause of many different diseases. They replicate through a linear DNA replicative form (RF) and encapsidate a single-stranded DNA genome of either a single polarity, or from either DNA strand. Parvovirus capsids are assembled from between two and four overlapping capsid proteins that are generated by alternative splicing of one or two viral mRNAs, or by use of alternative start codons in one message (*7*).

Results. DNA sequence and analysis. The almost complete sequence of the genome of MVC was obtained from plasmid clones or directly from viral RF DNA and that covered 5097 bases (Fig. 1; GenBank Accession No. AF495467). We did not obtain the sequences of the very 3′ and 5′ ends of the genome, but by restriction analysis of the RF DNA with *Xho*I (nt 470 in the sequence determined) and *Bsa*MI (nt 5014 in the sequence determined), we determined that the sequence obtained extended to within 140 bases of the extended 3′ end and 150 bases of the extended 5′ end, suggesting a total genomic length of about 5390 bases. Electrophoretic analysis of the RF DNA digested with *Xho*I and *Bsa*MI showed that the turn-around and extended forms of the 3′ end differed in length by about 80 bases, and those of the 5′ end differed in length by about 75 bases, indicating that those palindromes contained about 160 and 150 bases, respectively (results not shown).

Comparing the MVC sequence with those of other parvoviruses showed that the most closely related virus

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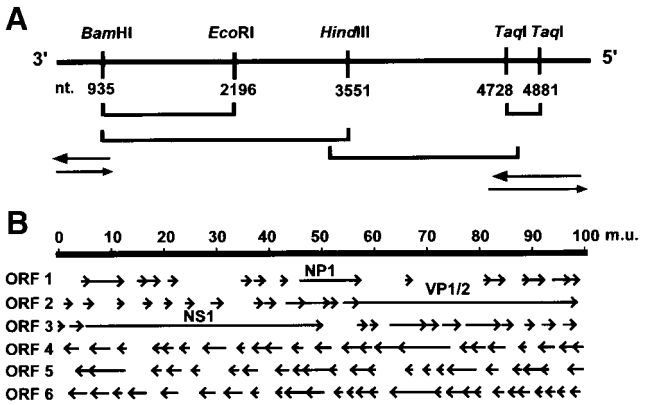

FIG. 1. (A) Cloning and sequencing the MVC genome. The central portions of the genome were cloned into plasmids from the RF DNA or from PCR products, while the 3′ and 5′ terminal sequences were determined directly from the viral RF DNA. The sequence has been deposited in GenBank (AF495467). (B) ORFs within the MVC sequence shown as arrows. Labels indicate the ORFs homologous with NS1, NP1, or VP1 and VP2 genes of the BPV.

was the bovine parvovirus (BPV), with which it shared 43% DNA sequence identity. When a conserved region within the translated sequence of the NS1 gene was compared to the equivalent sequence from other parvoviruses, MVC was again closely related to BPV, and the next closest relatives were the erythroviruses (human B19 and simian parvoviruses) (Fig. 2). MVC was quite distinct from canine parvovirus and feline panleukopenia virus and the other viruses in the rodent virus-related clade.

Large open reading frames (ORF) within the left and right halves of the genome encoded proteins homologous to the NS1 and the VP1 and VP2 proteins, respectively, of other parvoviruses, while a shorter ORF in the middle of the genome overlapped with the sequence of the NS1 protein, and that was similar to the predicted NP-1 protein of BPV (Figs. 1 and 3) (*8*). The NS1 sequence likely initiated at a methionine codon at nt 429 in the sequence and encoded a 717 residue protein with a predicted molecular weight of 81.9 kDa, which shared 32.6% identity with the NS protein of BPV (Fig. 3A). The right-hand ORF encoded VP1 and VP2 proteins of 78.9 and 63.9 kDa, respectively, which initiated at methionine codons in the same reading frame, at nts 2933 and 3329 in the sequence (Fig. 3B). The 21.7-kDa protein encoded by a third ORF was 39% identical to the NP-1 protein predicted from the BPV sequence (Fig. 3C) (*8*). The first 188 nts of the NP1 ORF overlapped with the C-terminal sequence of NS1.

Analysis of mRNAs by RT-PCR. We used RT-PCR to isolate spliced mRNA products from MVC-infected cells and then cloned the products and examined their sequences. Two different pairs of PCR primers were used which would flank the introns described for many parvoviruses (Fig. 4). For the RT-PCR spanning the region around the beginning of the VP1 ORF between nts 2601 and 3346 (Fig. 4, primer pair 2 and 3), the only product detected was the intact 745-bp sequence, and no clones were identified that would represent splices of these sequences. For the RT-PCR spanning the 368- to 3346-nt region of the genome (primer pair 1 and 3 in Fig. 4), the predicted full-sized 2978-nt product was detected, as well as products that appeared to be about 1100 nts and smaller than 400 nts (Fig. 4). Clones isolated from the middle-sized product had two splices that fused two sequences within NS1 and part of the NP1 ORF. Smaller products showed at least two sequences—in one 26 residues from the N-terminus of NS1 were fused to the VP1 ORF, while the other fused the NS1 ORF to an alternative ORF overlapping that of NP1. Whether the NS1–VP1 fusion is formed in any quantity was not determined, but it is possible that those represent the origin of the alternative forms of VP1 recovered from the viral capsids (Fig. 5).

Protein Analysis. Purified MVC capsids contained a number of protein forms, with a minor and major form representing the VP1 and VP2 proteins (Fig. 5). The major form of VP1 had an estimated size of 81 kDa, while the VP2 forms were 67 and 63 kDa. VP3 (61 kDa) was present in the full virus capsids, likely resulting from cleavage of VP2 at arginine 19 in the VP2 sequence, similar to that reported for MVM and CPV (*9*). Western blotting of the proteins from MVC-infected cells using a

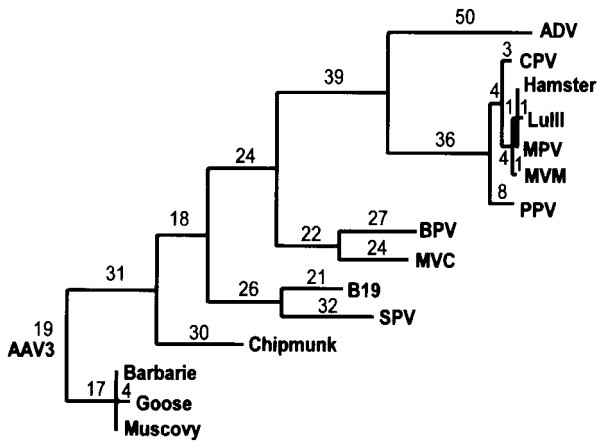

FIG. 2. A phylogeny showing the relationship of MVC to other members of the family *Parvovirinae*, including Aleutian mink disease virus (ADV), canine parvovirus (CPV), hamster parvovirus (hamster), LuIII, mouse parvovirus (MPV), minute virus of mice (MVM), porcine parvovirus (PPV), BPV, MVC, human parvovirus B19 (B19), simian parvovirus (SPV), chipmunk parvovirus (chipmunk), barbarie duck parvovirus (barbarie), goose parvovirus (GPV), and Muscovy duck parvovovirus (Muscovy). The tree was rooted using adenoassociated virus (AAV) as an outgroup. A conserved sequence of 149 residues of the MVC NS1 was aligned with the sequences of the other viruses indicated and the most parsimonious phylogeny determined using the program PAUP 3.1.1. The single tree determined is shown; the numbers shown indicate the number of residues on each branch.

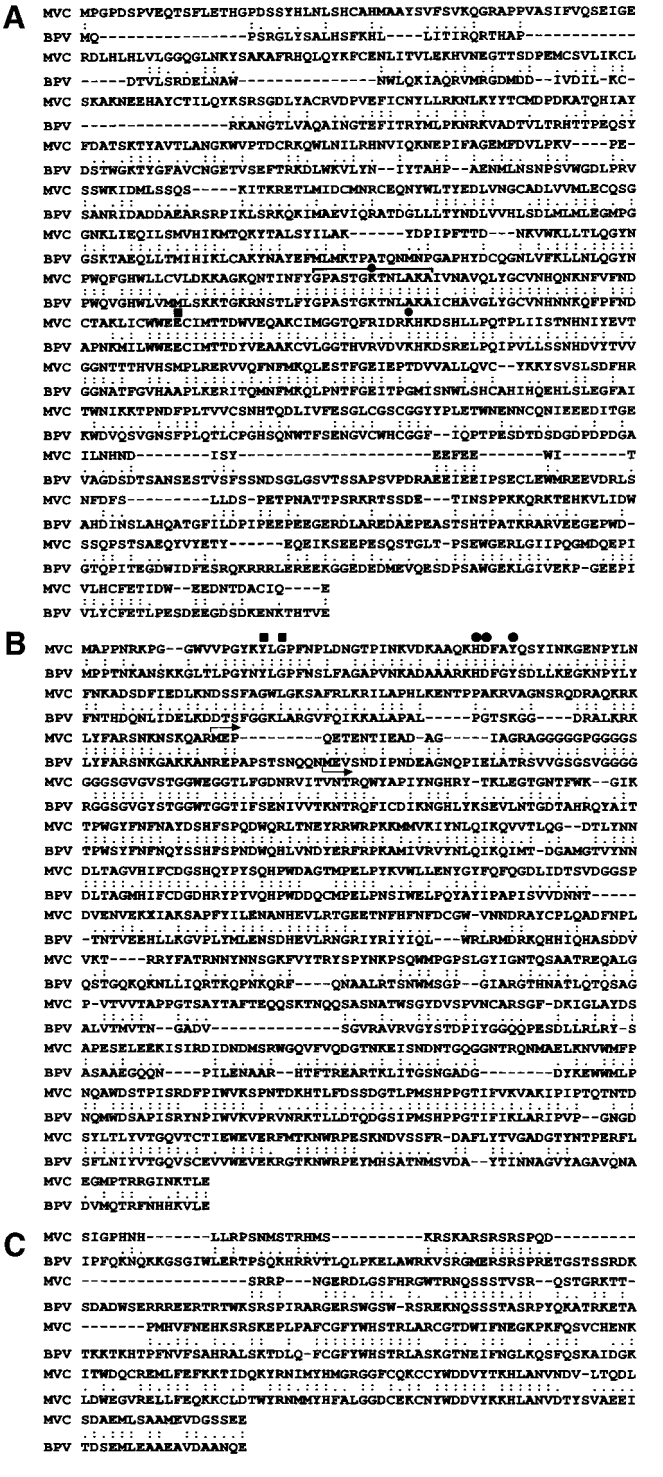

FIG. 3. Alignments of the MVC protein sequences (top) with those of BPV (bottom). (A) The NS1 ORF. Within the conserved central region of the protein, the nucleotide-binding site required for DNA nicking identified in the MVM NS1 sequence is marked by the overlining. Conserved residues that in MVM have been associated with nucleotide binding or ATPase activity are indicated by circles and squares, respectively. (B) The VP1/VP2 ORF. The conserved phospholipase A2-specific residues in that sequence are indicated. Closed circles indicate the active site motif (HDXXY) and the closed squares indicate the Ca^{2+} binding loop residues (YXGXG). The arrow indicates the predicted start of the VP2 sequence. (C) The NP-1 ORF.

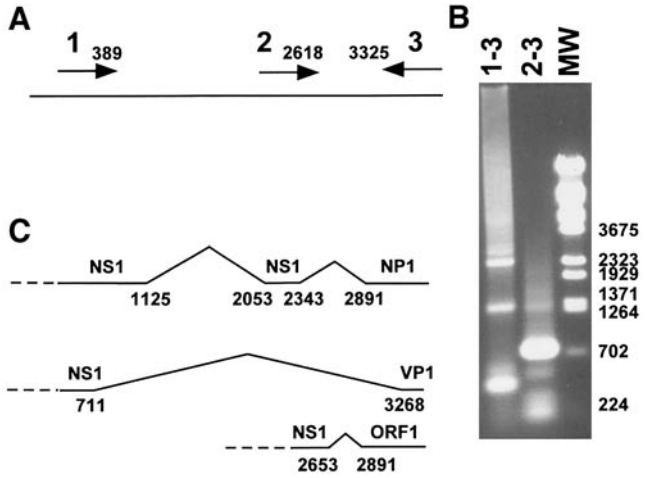

FIG. 4. The RT-PCR analysis of viral mRNA transcripts. (A) The RT-PCR reactions performed are diagrammed, showing the three primers that were used to amplify the messages. Numbers indicate the nucleotide in the genome complementary to the 3′ end of each primer. (B) The RT-PCR products detected in an agarose gel. Size of the molecular weight standards in base pairs. (C) Spliced forms identified in the PCR reactions, as determined from products cloned into plasmids and sequenced. The nucleotides at the junctions of the different products are given.

postinfection dog serum showed the VP2 protein, as well as a protein band of about 80 kDa which could represent the VP1 and NS1 proteins, which are close to this size and would likely be recognized by the postinfection serum (Fig. 5B).

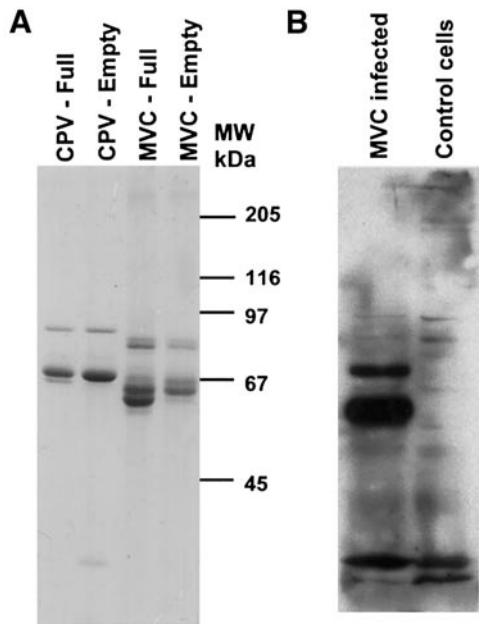

FIG. 5. (A) Proteins of purified full and empty MVC particles detected by Coomassie blue staining, compared to those of CPV full and empty capsids. Size standards are shown in kDa. (B) Proteins from infected or uninfected WR cells electrophoresed on a 10% acrylamide gel, transferred to a nitrocellulose membrane, and then probed with antibodies from an SPF dog that had been infected with MVC.

Discussion. The MVC has been associated with several different diseases of fetal and neonatal puppies, and by serological testing the virus appears to be widespread in dogs, most likely causing subclinical infections in many infected animals (*1–6, 10*). Here we show that MVC is a distinct member of the *Parvoviridae* which is most closely related to the bovine parvovirus, although it shares only 43% identity in DNA sequence with that virus. MVC was only distantly related to the well-characterized CPV, as has been suggested by serological testing and by restriction enzyme analysis of the viral DNA (*3*).

The genetic structure of MVC was typical of a parvovirus, with large ORFs encoding the NS1, VP1, and VP2 proteins, along with a small ORF that partially overlapped with the NS1 gene that was equivalent to the NP1 protein of BPV (*8, 11*). The function of the NP1 protein is unknown, and there is no obvious homology with any protein in the data bases. The NS1 protein sequence had a conserved core that contained the sequences identified as being involved in the nucleotide binding and DNA-nicking activities of the protein (*12, 13*) (Fig. 3A). Sequences identified in the MVM NS1 as a metal coordination site (histidines 127 and 129), and to be part of the active site (tyrosine 210), could not be clearly identified in the NS1 of MVC or BPV. Conserved sequences that are associated with the active site of a phospholipase A2 activity of the VP1 unique region of several parvoviruses were present in the MVC and BPV sequences, suggesting that that was a highly conserved function of the protein (Fig. 3B) (*14*).

The mRNA transcripts identified by RT-PCR between primer sites around nts 390 or 2618 and 3325 showed a variety of possible mRNA forms producing a variety of proteins (Fig. 4). We cannot define the significance of those spliced products, but they appear similar to those described for the Aleutian mink disease virus and the human parvovirus B19, where a variety of spliced products have been reported, but for most of which the functions have not been defined (*15–17*). The two capsid proteins appear similar to the VP1 and VP2 proteins of the other parvoviruses, but no evidence was found of a spliced product within the 2618- to 3325-nt region, and the two proteins are likely encoded by a single message with translation initiating at two ATG codons to give VP1 and VP2.

The evolution and relationships of the parvoviruses is only partially understood (*18, 19*). Among the viruses of vertebrates there appear to be at least three distinct clades, which include adenoassociated viruses which are more closely related to the avian-derived viruses, the rodent virus-related viruses which include canine parvovirus and minute virus of mice, and the erythroviruses which include B19 and similar viruses from primates, as well as the more distantly related BPV which is now grouped with MVC. However, MVC is still only distantly related to the other viruses, indicating that it diverged in the distant past.

Materials and Methods. Virus growth, capsid, and DNA purification. The GA3 isolate of MVC (*5*) was grown in thinly seeded Walter Reed canine cells (*1*). Replicative form DNA was harvested from infected cells 2 days after virus inoculation by modification of the method of Hirt (*20*). The cells were lysed into 10 mM Tris–HCl (pH 7.4) 10 mM EDTA, 0.6% SDS; then 20 mg/ml of Pronase was added and incubated for 2 h at 37°C. The lysate was made up to 1 M NaCl, incubated on ice, and then centrifuged at 80,000 *g* for 1 h. The supernatant was collected, ethanol precipitated, made up into 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, and extracted with phenol and then with chloroform. The RF DNA was purified from a 1% agarose gel by spin column extraction.

To prepare virus for protein analysis, WRC cells were inoculated with virus for 1 h at 37°C. After 4 days of incubation, the cells were frozen and thawed twice. Capsids were pelleted from the culture supernatant at 80,000 *g* for 3 h; the pellet was sonicated for 2 min, and then the virus was banded in 10 to 40% sucrose gradients at 90,000 *g* for 6 h. Proteins in each gradient fraction were electrophoresed in 10% SDS–polyacrylamide gels (*21*), and the gels stained with Coomassie blue. Purified full and empty capsids of canine parvovirus were included for comparison. To examine the proteins in infected cells, lysates of infected cells were electrophoresed in 10% PAGE and then transferred to nitrocellulose membranes and incubated with a postinfection dog serum. The blot was incubated with goat anti-dog IgG conjugated with horseradish peroxidase and then with Super-Signal substrate (Pierce Chemical, Rockford, IL).

DNA Cloning and Sequencing. The strategy for cloning and sequencing the viral genome is diagrammed in Fig. 1A. The RF DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Taq*I (*3*). DNA fragments were cloned into the vector pGEM3Z (Promega, Madison, WI); sequences were obtained with SP6 and T7 primers from the flanking regions in the plasmids. Sequences were determined in both orientations by automated sequencing with a variety of specific primers. A 1177-base region between the *Hin*dIII site and a *Taq*I fragment was amplified from virus DNA by PCR and cloned into the vector pGEM-T easy (Promega), and three clones sequenced. To obtain sequences near the ends of the genome, purified viral RF DNA (100 ng/μl) recovered from infected cells was sequenced directly using specific primers annealing near the ends of the sequences obtained from the cloned DNA fragments, and sequences were also obtained directly from the RF DNA using reverse primers. The viral genomic sequence was analyzed using the program DNAStar. The translated open ORFs identified were aligned with those of BPV, the most closely related sequence in GenBank.

A highly conserved region of the NS1 gene (residues 332 to 481, equivalent to residues 412 and 562 of the MVMp NS1 sequence) was aligned with the homologous regions of other parvoviruses, and the phylogenetic relationships were determined using the branch-and-bound algorithm of the program PAUP 3.1.1.

RT-PCR and Sequencing of Viral Messages. RT-PCR was used to examine for possible spliced products in the viral RNAs. Primers prepared were from positions 368–389 (5′-GCAAAGAGCACTGGGCGGTATT-3′), 2601–2618 (5′-ACCTCTTCCTGCGTTCTG-3′), and 3346–3325 (5′-AGTTTCCTGCGGTTCCATCCTA-3′). RNA was isolated using the RNAgents kit (Promega); then cDNA was prepared from the total RNA using specific primers and the Access RT-PCR kit (Promega). The PCR products were run on a 1% agarose gel. To determine the origins of specific products, the amplified DNAs in different bands were isolated from the agarose gel and ligated into the pGEM-T Easy plasmid. Then plasmids were sequenced using the T7 and SP6 primers in the vector.

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