JOURNAL OF VIROLOGY, Oct. 1994, p. 6476–6486
0022-538X/94/$04.00+0

Vol. 68, No. 10

# Molecular Characterization of a Newly Recognized Mouse Parvovirus

LISA J. BALL-GOODRICH* AND ELIZABETH JOHNSON

*Section of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut 06520-8016*

Received 31 May 1994/Accepted 1 July 1994

**Mouse parvovirus (MPV), formerly known as orphan parvovirus, is a newly recognized rodent parvovirus distinct from both serotypes of minute virus of mice (MVM). Restriction analysis of the MPV genome indicated that many restriction sites in the capsid region were different from those of MVM, but most sites in the nonstructural (NS) region of the genome were conserved. MPV resembled MVM in genome size, replication intermediates, and NS proteins. Replication intermediates in infected cells were the same for MPV and MVM, including packaging of the 5-kb minus (V) strand. Furthermore, the MPV NS proteins were the same size as and present at the same ratio as the MVM(i) proteins in infected cells. Cloning and sequencing of the MPV genome revealed a genome organization closely resembling that of MVM, with conservation of open reading frames, promoter sequences, and splice sites. The left terminal hairpin was identical to that of MVM(i), but the right terminus was not conserved. Also, the MPV genome was unique in that it contained 1.8 copies of the terminal repeat sequence rather than the 1 or 2 copies found in other parvoviruses. The predicted amino acid sequence of the NS proteins of MPV and MVM(i) were nearly identical. In contrast, the predicted amino acid sequence of the capsid proteins of MPV was different from sequences of other parvoviruses. These results confirm that MPV is a distinct murine parvovirus and account for the antigenic differences between MPV and MVM.**

Autonomous parvoviruses, including minute virus of mice (MVM), rat virus, canine parvovirus (CPV), and LuIII, are nonenveloped icosahedral viruses that replicate in vertebrates without the aid of a helper virus (12). In general, parvoviruses show a high degree of host species specificity. Additionally, parvoviruses require, but are unable to induce, cell division in order for viral replication to occur (12). As an in vivo correlate, these agents are primarily teratogenic, causing fetal and neonatal abnormalities by destroying specific cell populations rapidly dividing during development. In contrast, severe infection does not ensue in mature animals, but the block may be overcome by inducing proliferation of appropriate tissues. The lack of S-phase transition in mature tissues undoubtedly explains some resistance to infection, but developmentally regulated component(s) and the differentiated state of the host cell are also important for viral replication.

The molecular biology of several parvoviruses has been studied in detail. Parvoviruses have a linear single-stranded DNA (ssDNA) genome of approximately 5 kb, which is bracketed by short terminal palindromes involved in viral replication. Replication occurs through monomer- and dimer-length duplex DNA intermediates, and the monomer-length ssDNA is encapsidated. Most autonomous parvoviruses have different palindromic sequences at each end of the viral genome and package predominantly the minus (V) strand. LuIII has different termini, but packages both plus and minus strands in equivalent amounts (7).

MVM, the best-characterized murine parvovirus, contains two large and several smaller open reading frames (ORFs) on the plus (C) strand (3). There is a promoter upstream from each of the two large ORFs, P4 at genome map unit (m.u.) 4 and P38 at m.u. 38 (24). All transcripts terminate at the right end of the genome at m.u. 95 (10). The P4 transcripts, R1 and R2, encode two nonstructural (NS) proteins by differential splicing of a primary transcript (Fig. 1C). R1 is generated by the removal of a single minor splice between nucleotides 2282 and 2399 and encodes NS1, an 83-kDa protein whose translation terminates prior to the minor splice (11). R2 is generated by removal of both the minor splice and a major splice between nucleotides 510 and 1990 (17, 20) and encodes the 25-kDa NS2 protein (11). At the minor splice in NS2, two possible donor and acceptor sites result in three different carboxy termini. The minor splice also affects the P38 transcripts, R3, that encode the 83-kDa VP1 and the 64-kDa VP2 capsid proteins. VP3 results from protease cleavage of VP2 to a 61-kDa protein found in various amounts in full particles (34).

The two serotypes of MVM exhibit distinct cell tropisms both in vivo and in vitro. MVM(i) is lethal to newborn mice of susceptible genotypes, whereas MVM(p) does not cause clinical disease (8, 18). In vitro, MVM(i) lytically infects T-lymphoid cell lines, while MVM(p) infects fibroblast cell lines (33). These two serotypes defined a mutable genetic component in the viral capsid that determines the capacity of the virus to lytically infect a particular cell type (1, 6, 14).

Recently, a new parvovirus of mice that infects murine cloned T cells was isolated (19). It was initially called mouse orphan parvovirus but is now referred to as mouse parvovirus or MPV-1 (hereafter referred to as MPV). Sera from MPV-infected mice did not inhibit hemagglutination (HA) induced by MVM(p). However, these sera did cross-react with MVM when either a commercially available MVM enzyme-linked immunosorbent assay (ELISA) or an indirect immunofluorescence assay (IFA) with MVM-infected cells was used. Anti-capsid antiserum recognizing both MVM(i) and MVM(p) did not inhibit HA by MPV or react with MPV-infected cells by IFA. In contrast, a commercially available anti-MVM antibody

* Corresponding author. Mailing address: Section of Comparative Medicine, Yale University School of Medicine, P.O. Box 208016, New Haven, CT 06520-8016. Phone: (203) 737-4196. Fax: (203) 785-7499.

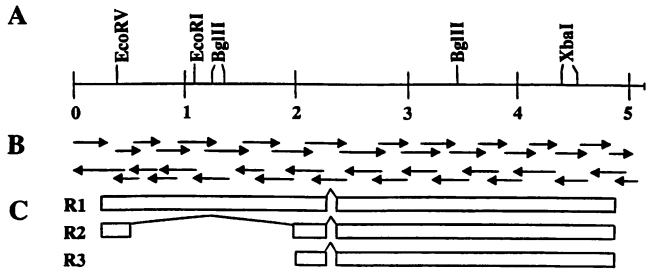


FIG. 1. Cloning and sequencing strategies for MPV and a transcription map of MVM. (A) Restriction sites used for cloning MPV are indicated above the linear genome, and kilobases are indicated below the genome. (B) Sequencing strategy of both strands, using overlapping primers, relative to the genomic map. (C) Transcription map of MVM. mRNA sequences are indicated by blocks, and spliced regions are indicated by individual lines.

known to cross-react with numerous parvoviruses did react with MPV-infected cells. These results indicated that MPV was a rodent parvovirus with conserved NS polypeptides yet was distinct from MVM.

Smith and coworkers (32) demonstrated that sera from MPV-infected mice reacted with MVM(i)-infected cells by IFA, but sera from MVM-infected mice did not react with MPV-infected cells. Additionally, they determined that MPV is equally infectious for adult and neonatal mice. Unlike MVM(i), MPV infection of mice <24 h old did not result in lethal infection.

We have characterized MPV at the molecular level to obtain a better understanding of its relationship to other autonomous parvoviruses. Replicating DNA was examined in parallel with MVM by neutral and alkaline gels, and the strandedness of the single-stranded form was determined. Because of the serological cross-reactions (19, 32), the NS proteins of MPV and MVM(i) were compared by Western immunoblotting. The entire MPV genome was cloned and sequenced. Analysis of the sequence confirmed that MPV is most similar to the rodent parvoviruses yet is distinct from all known parvoviruses.

## MATERIALS AND METHODS

**Cell lines.** L3 cells, a cytolytic $CD8^+$ T-cell clone permissive for MPV, were maintained as described previously (19). Briefly, L3 cells were maintained in Dulbecco's modified minimal essential media containing 5% heat-inactivated fetal calf serum, 10 mM morpholinopropanesulfonic acid, $5 \times 10^{-5}$ M 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, additional amino acids, and 10 U of interleukin-2 per ml (EL4 cell supernatant was the source of interleukin-2). 324K is a clone of simian virus 40-transformed human newborn kidney cells, and Hyb1/11-iD5 is a subclone of the somatic cell hybrid line Hyb1/11 (33). The latter two cell types were maintained as described previously (33).

**Generation and titration of viral stocks.** MPV stock was generated by infecting L3 cells at a low multiplicity of infection [MOI] (0.005 to 0.001 50% infectious dose [$ID_{50}$] per cell). Infected cultures were harvested when 80 to 90% of the cells were positive by IFA using mouse MPV convalescent serum. Cells were centrifuged, medium was harvested and stored at −20°C, and the pellet was resuspended in 1/10 the culture volume of Tris-EDTA (pH 8.7). After freezing and thawing three times, the cell debris was removed and the supernatant was stored at −20°C. Both the medium and cell pellet virus stocks were titered on L3 cells to determine the $ID_{50}$. Replicate cultures containing $10^5$ freshly seeded L3 cells were inoculated with serial 10-fold dilutions of viral stock. Six days after inoculation, cells in each well were harvested, resuspended in phosphate-buffered saline (PBS), and spotted onto slides (Cel-line Associates, Newfield, N.J.). Slides were air dried, fixed in acetone at −20°C, and stained as described previously (30), using convalescent serum from MPV-infected mice and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody. The last dilution with fluorescence determined the $ID_{50}$ titer. MVM(i) viral stock was the first passage after transfection of the infectious clone pMVM(i), and stocks were made and titered as previously described (5).

**Infections and preparation of viral DNA.** MPV DNA was prepared for cloning by a modified Hirt protocol (15) using $7 \times 10^6$ L3 cells infected at an MOI of 0.05. When approximately 80% of the cells were viral antigen positive by IFA (4 to 5 days postinfection), they were pelleted and rinsed with PBS. If total cellular DNA was being prepared, the cell pellet was frozen at −80°C until processed as described previously (6). MVM(i) DNA was prepared from a 24-h parasynchronous infection at an MOI of 2 in 324K cells as described previously (6).

**DNA analysis by Southern blotting.** MPV Hirt DNA and the MVM(p) genome [excised from a full-length clone of MVM(p) and gel purified] were digested with the restriction enzymes indicated in Fig. 2. Aliquots of each DNA were electrophoresed through 0.8% agarose in 1× Tris-borate-EDTA. DNA was transferred to GeneScreenPlus (Dupont, Wilmington, Del.) or Hybond N+ (Amersham, Arlington Heights, Ill.) nylon membranes by standard Southern protocol and incubated at 80°C for 2 h. $^{32}P$-labeled random-primed probes (specific activity of $10^8$ cpm/μg of DNA) were synthesized by the protocol of the manufacturer (GibcoBRL, Gaithersburg, Md.), using full-length MVM(p) as the template. Membranes were prehybridized and hybridized at 65°C, using standard procedures. Blots were washed at 65°C in 5× Denhardt's solution–2× SET (1× SET is 160 mM NaCl, 30 mM Tris [pH 8.0], and 2 mM EDTA), in 2× SET–0.5% sodium dodecyl sulfate (SDS), and in 0.1× SET–0.1% SDS and then exposed to Kodak XAR film (Eastman Kodak, Rochester, N.Y.).

Riboprobes were synthesized and hybridized as described previously (6), using the MPV clone pRVXB 36 linearized with either *Sac*I or *Hin*dIII to generate both plus- and minus-strand-specific riboprobes with specific activities of $2 \times 10^7$ cpm/μg of RNA.

DNA was electrophoresed on a 0.8% alkaline agarose gel (28) and transferred to Hybond N+ as described above. The DNA was detected with a random-primed MPV probe (gel-purified *Eco*RV-*Xba*I [bp 384 to 4340] fragment) with a specific activity of $10^8$ cpm/μg of DNA.

**Cloning of the MPV genome.** The coding sequences of MPV were cloned by using the *Eco*RV site (bp 384 in the MPV genome; restriction sites shown in Fig. 1A) and *Xba*I site (cuts at both bp 4340 and bp 4529, but only the bp 4340 site was used in the cloning). MPV Hirt DNA was digested with *Eco*RV and *Xba*I. *Hin*dIII, which does not cut the MPV genome, was added to the digest to reduce the background of clones containing cellular DNAs. The pBluescript II KS+ vector (Stratagene, La Jolla, Calif.) was digested with *Eco*RV and *Xba*I, treated with calf intestinal phosphatase, and ligated with the insert. The ligation mix was transformed into *Escherichia coli* JM109, and colonies were screened by restriction digestion and Southern hybridization using an MVM probe. Two clones, pRVXB 36 and pRVXB 45, that contained the MPV sequences were recovered.

The left terminus of MPV was cloned from purified mono-

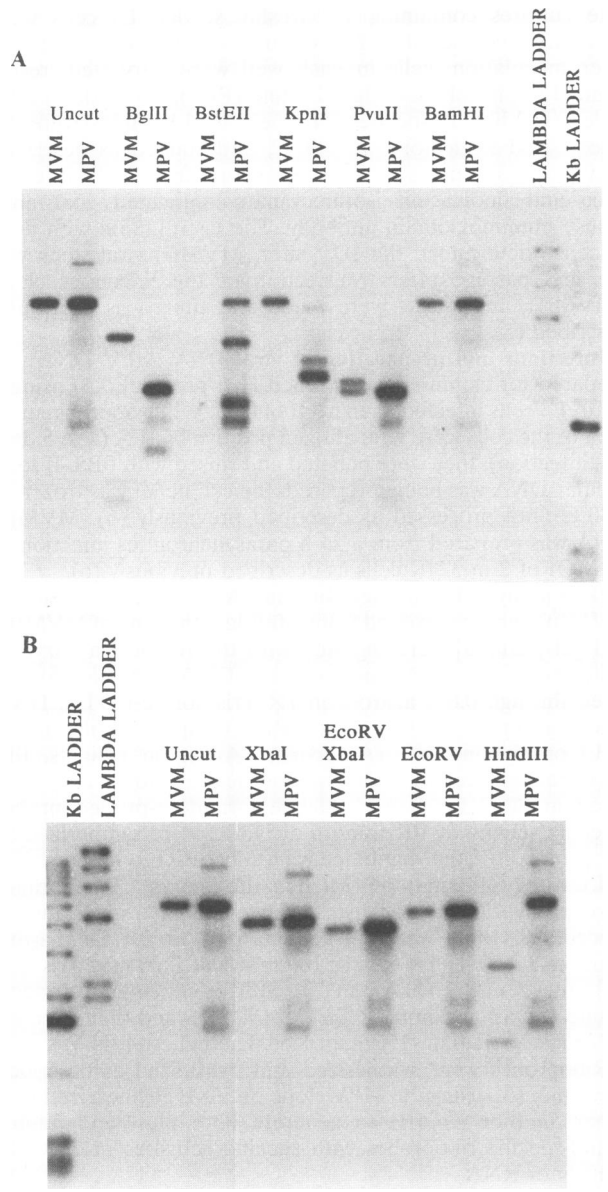


FIG. 2. Restriction analysis of MPV compared with MVM(p). MVM(p) genome, purified from the plasmid pSP65, and MPV Hirt DNA were digested with the indicated restriction enzymes. The digests were fractionated on a 0.8% agarose gel, transferred to nylon, and analyzed by using a random-primed MVM(p) probe. A random-primed kilobase ladder and lambda DNA digested with *Hin*dIII served as size markers.

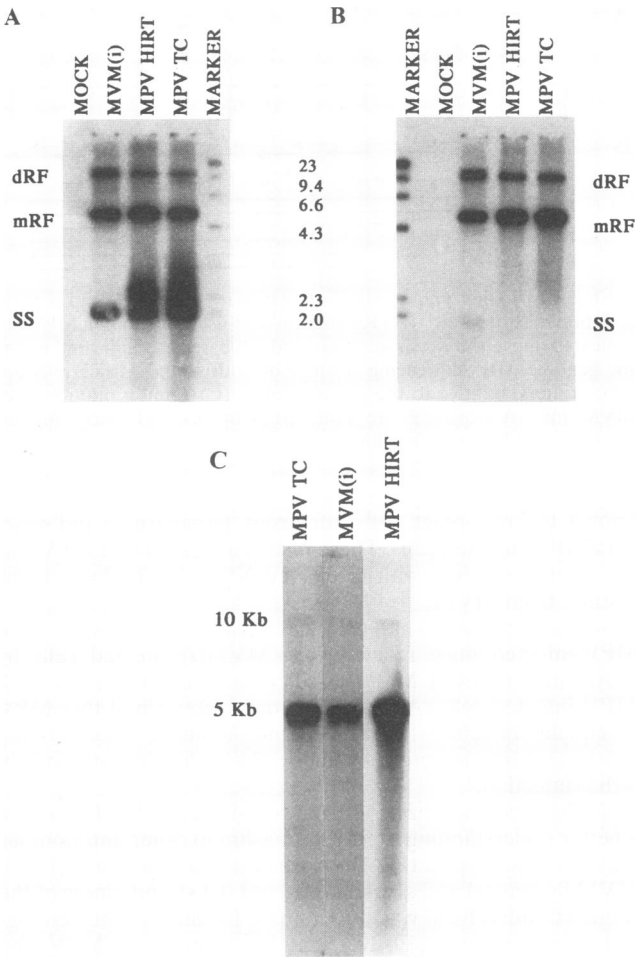


FIG. 3. Comparison of MPV replication intermediates with those of MVM(i). (A and B) MPV total cell and Hirt DNAs were compared with MVM(i) total cell DNA on a 1.4% agarose gel. The DNA was transferred to a nylon membrane and analyzed with plus-sense (A) or minus-sense (B) MPV riboprobes. The various replicative forms, dRF and mRF, as well as newly synthesized ssDNA (SS) are indicated. The marker was random-primed lambda DNA digested with *Hin*dIII. (C) MPV total cell DNA from a 24-h infection of L3 cells at an MOI of 7.5 and MPV Hirt DNA were compared with MVM(i) total cell DNA. Species of 5 and 10 kb are indicated. Markers were as described for Fig. 2.

mer replicative form (mRF), using *Bam*HI linkers and the internal *Eco*RI site at nucleotide 1087. mRF DNA from a Hirt preparation was gel purified by using β-agarose digestion (New England Biolabs, Beverly, Mass.) of low-melting-point agarose, treated with DNA polymerase I Klenow fragment, and ligated with *Bam*HI linkers. The DNA was cut to completion with *Bam*HI and then with *Eco*RI, and linkers were removed by Microcon 100 (Amicon, Inc., Beverly, Mass.) filtration. The insert DNA was ligated to pBluescript II KS+ digested to completion with *Eco*RI and *Bam*HI. The ligation mix was transformed into *E. coli* JM109, and the colonies were screened by filter hybridization (28) with gel-purified fragments derived from the *Eco*RV-to-*Bgl*II (bp 383 to 1246) and *Bgl*II-to-*Xba*I (bp 3451 to 4342) fragments. Four intact left terminal clones were recovered.

The right terminus of MPV was cloned by using the *Bgl*II site at bp 3449 (*Bgl*II cuts at bp 1244, 1355, and 3449 in MPV) and *Hin*dIII linkers at the right terminus. The vector, pBluescript II KS+, was digested with *Hin*dIII and *Bam*HI, a compatible end for *Bgl*II. Hundreds of transformants were screened as described above, and numerous left termini were recovered. Only one right terminus clone contained most of the hairpin, although it was deleted of 69 nucleotides compared with the MVM(i) terminus.

**Detection of NS1 and NS2.** L3 cells or parasynchronous 324K cells were infected for 24 h at MOIs of 2.5 $ID_{50}$ (MPV) and 1 PFU [MVM(i)] per cell. Cells were harvested as

described previously (6), and extracts were electrophoresed on an SDS–12% polyacrylamide gel. Western immunoblotting was performed as instructed by the manufacturer (Hoeffer Scientific, San Francisco, Calif.), and proteins were detected by using the protocol and reagents included in the ECL detection kit (Amersham, Arlington Heights, Ill.). Rabbit antiserum specific for the common N terminus of NS1 and NS2 (11) was diluted 1:2,500, and horseradish peroxidase-conjugated anti-rabbit antibody for detection was diluted 1:10,000. Bio-Rad (Hercules, Calif.) low-range prestained SDS-polyacrylamide gel electrophoresis molecular weight markers were included on each gel.

**Sequencing of MPV-1.** Sequencing of the MPV genome was performed either by using the modified T7 DNA polymerase (Sequenase version 2.0; U.S. Biochemical, Cleveland, Ohio) as instructed by the manufacturer for dideoxy sequencing of double-stranded DNA or by the DNA Sequencing Facility at the W. M. Keck Foundation Biotechnology Laboratory at Yale School of Medicine, using PCR sequencing and an automated sequencer apparatus. The strategy for sequencing of the genome, outlined in Fig. 1B, encompassed 97% of the genome in both directions. Both internal clones (pRVXB 36 and 45), two different left-end clones, and seven different right-end clones (only one of which contained the hairpin sequences) were sequenced. Because the most complete right terminal clone is deleted, we deduced the final 69 nucleotides from those found on the opposite strand and used the end of MVM as the terminus endpoint.

The MPV sequence was analyzed with the Genetics Computer Group (GCG) analysis programs on the VAX at Yale School of Medicine. The program used is indicated in each relevant figure legend, and the parameters were default settings. The sequences used for comparison were those of MVM(i) (2, 27), MVM(p) (3), LuIII (13), H-1 (26), CPV (25), and porcine parvovirus (PPV) (36).

**Nucleotide sequence accession number.** The sequence reported has been submitted to GenBank and assigned accession number U12469.

## RESULTS

**Restriction analysis of MPV.** Restriction digests of MPV Hirt DNA and MVM(p) genome-length DNA were compared by Southern blot analysis using a full-length MVM DNA probe (Fig. 2). The uncut MPV lanes showed the replicative forms found in parvovirus-infected cells (10-kb dimer replicative form [dRF], 5-kb mRF, and single-stranded form, which migrates at approximately 2 kb). The single-stranded form is still present after digestion with the various restriction enzymes. *Bam*HI did not digest either genome, *Bgl*II and *Pvu*II digestions yielded different restriction patterns for MVM and MPV, and *Kpn*I digested only MPV (Fig. 2A). Although *Bst*EII only partially digested the MPV DNA and the MVM DNA denatured under the reaction conditions, two products identical in size to the expected MVM products were evident in the MPV lanes. The hybridization of the MVM probe was stronger for the 1.89-kb left-end (NS region) *Bst*EII fragment of MPV than for the 3.26-kb right-end fragment. Conversely, *Hin*dIII digested MVM but not MPV DNA (Fig. 2B). The *Eco*RV site at bp 383 and the *Xba*I site at bp 4342 (MVM numbering) were conserved between MVM and MPV. Restriction sites identified by this analysis were used to clone the MPV coding sequences and both termini as described in Materials and Methods.

**Replication of MPV.** To verify that MPV replication in L3 cells is similar to MVM replication in 324K cells, we compared replicative forms from infected cell cultures by Southern blot analysis (Fig. 3). A plus-strand riboprobe prepared from cloned MPV detected dRF, mRF, and minus single-stranded form of both MPV and MVM(i) (Fig. 3A). A minus-strand MPV riboprobe easily detected dRF and mRF (Fig. 3B). A faint band of plus single-stranded MVM(i) was detected with the minus-strand probe, but the MPV lanes had no detectable plus single-stranded form. When duplicate samples were examined on a denaturing agarose gel (Fig. 3C), only a 5-kb band and a faint 10-kb band were detected. This confirmed that the 5-kb single-stranded form of MPV migrates at approximately 2 kb when it is not denatured.

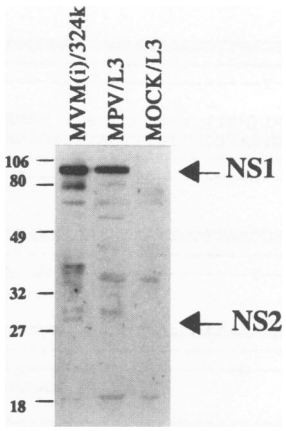


FIG. 4. Western blot analysis of nonstructural proteins. Extracts from $10^5$ MVM(i)- or MPV-infected cells were run on an SDS–12% polyacrylamide gel and analyzed by Western blotting. Nonstructural proteins were detected with rabbit anti-N-terminus polyclonal antiserum and the ECL detection kit. NS1 and NS2 are indicated, as are sizes (in kilodaltons) of the markers.

**Homology between MPV and MVM NS1 and NS2 proteins.** Serologic tests (19, 30) and restriction enzyme analysis suggested a strong similarity between the nonstructural genes of MPV and MVM(i). A polyclonal antiserum recognizing the NS1 and NS2 proteins of MVM was used to compare NS protein synthesis in MPV- and MVM(i)-infected cell extracts by Western immunoblotting (Fig. 4). The antibody recognized MPV NS1 and NS2 proteins, and the sizes and quantities of these two proteins were similar to those of NS proteins made by MVM(i)-infected cells.

**Sequence comparison of MPV and MVM(i).** MPV clones were sequenced by the strategy outlined in Figure 1B. The nucleotide sequences of MPV, including the MVM-defined promoter sequences, splice donors and acceptors, and polyadenylation signals, were compared with those of MVM(i) and LuIII as shown in Fig. 5. The P4 and P38 promoter sequences, including the TATA, CAAT, and TAR regions, were conserved between MPV and MVM. Sequences around both the major and minor splice junctions were conserved between MPV and MVM. The left terminus of MPV was identical to that of cloned MVM(i), but the right terminus contained 11 nucleotide changes from MVM(i) and 21 changes from LuIII. MPV differed from all murine parvoviruses in the tandem repeats found between the capsid coding sequences and the right terminus. The MVM(p) and H-1 genomes contain two copies of this sequence, whereas MVM(i) and LuIII genomes have only one copy. Because of a small deletion in one of two repeats, MPV contains 1.8 copies. A unique A-T-rich region, thought to be involved in the packaging of both strands, is

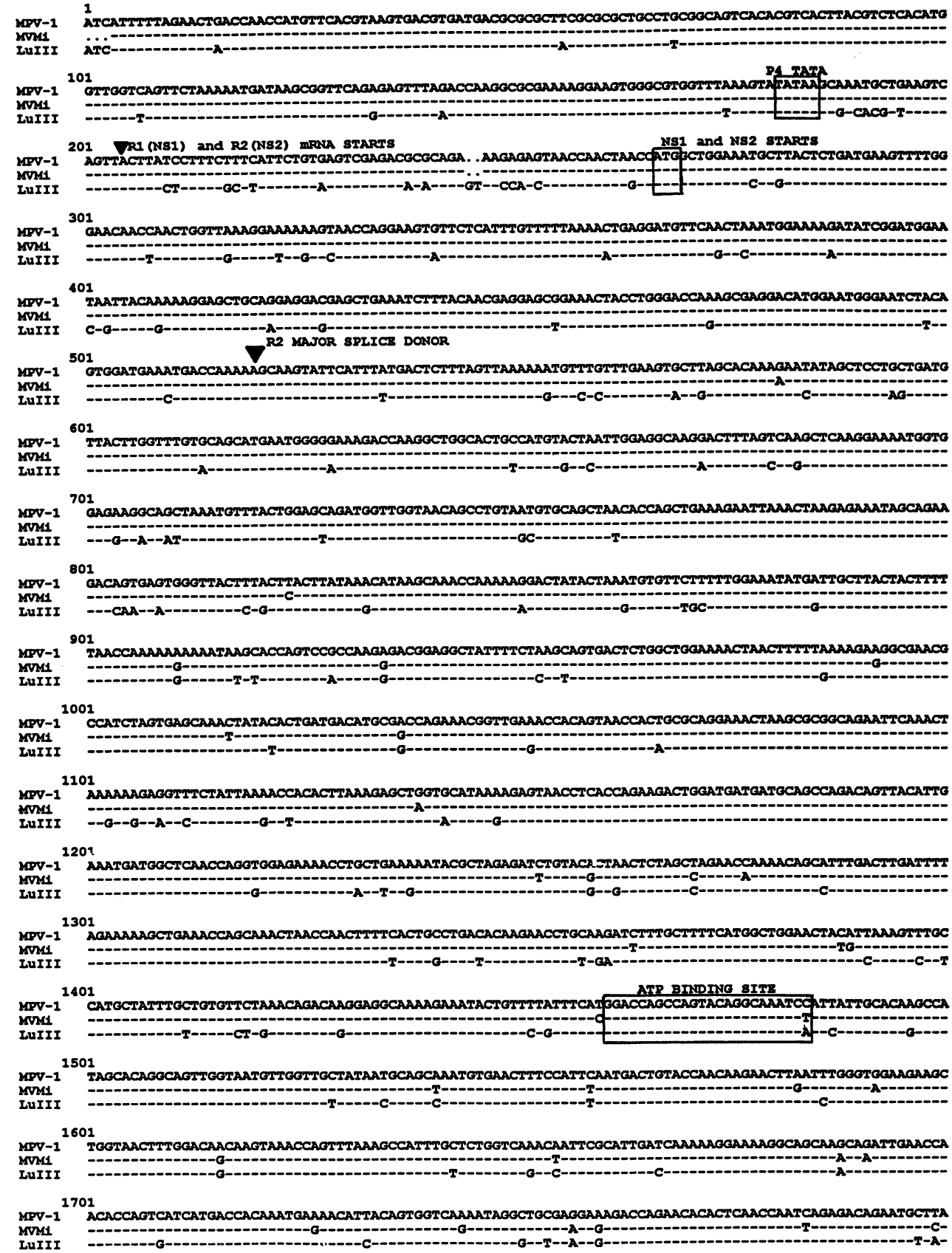


FIG. 5. Sequence comparison of MPV, MVM(i), and LuIII. Dashes indicate nucleotides identical to those of MPV, and dots indicate spaces inserted for maximal sequence alignment. Nucleotide sequences conserved among parvoviruses, the LuIII A-T-rich region, and the right-end direct repeats are boxed. An asterisk near the 3′ end of the first repeat indicates the 12-nucleotide deletion found in MPV. Arrows indicate mRNA initiation sites, splice donors, and acceptors. The GCG programs Pileup, Pretty, and Publish were used to generate the alignment.

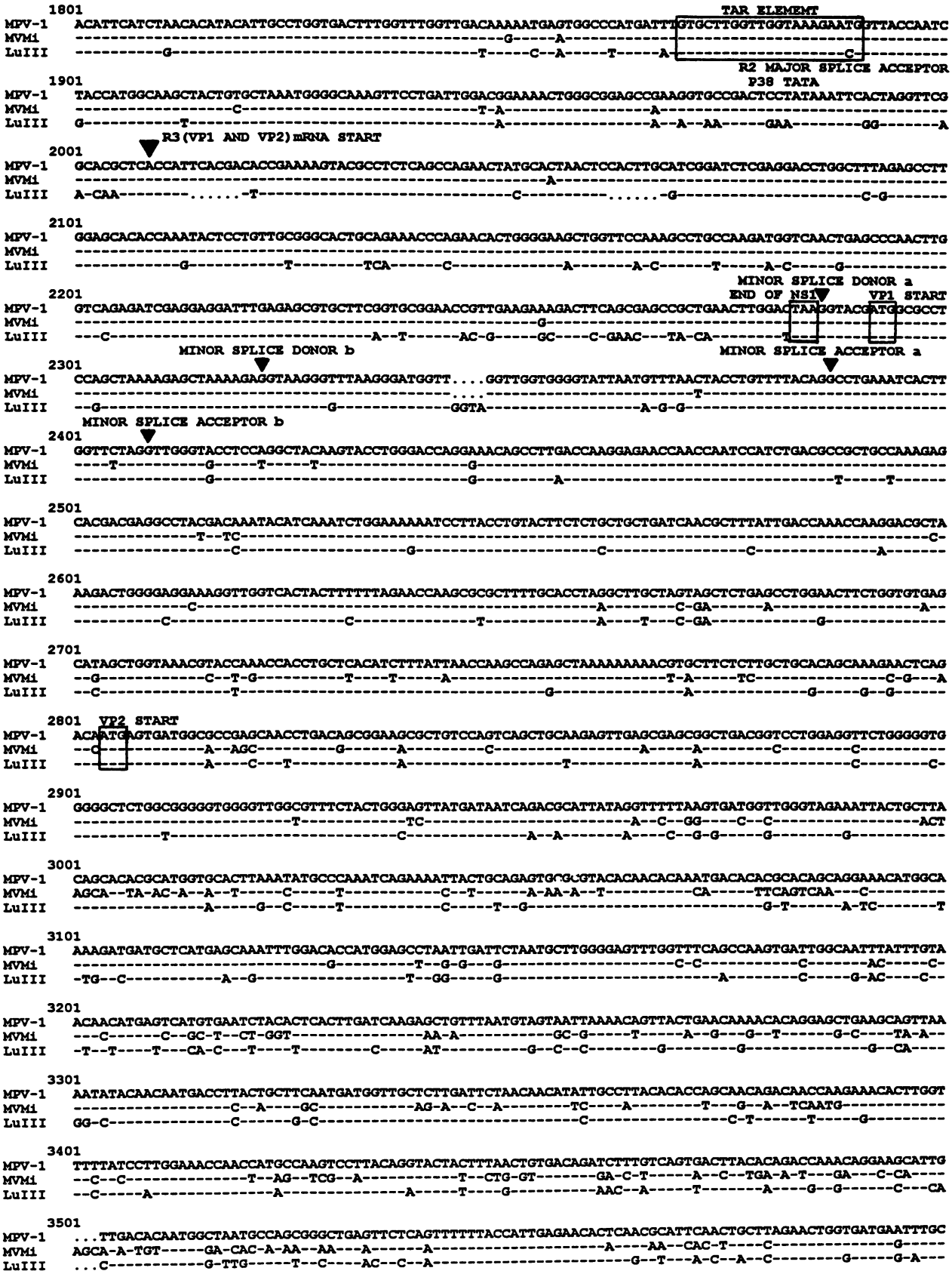


FIG. 5—*Continued.*

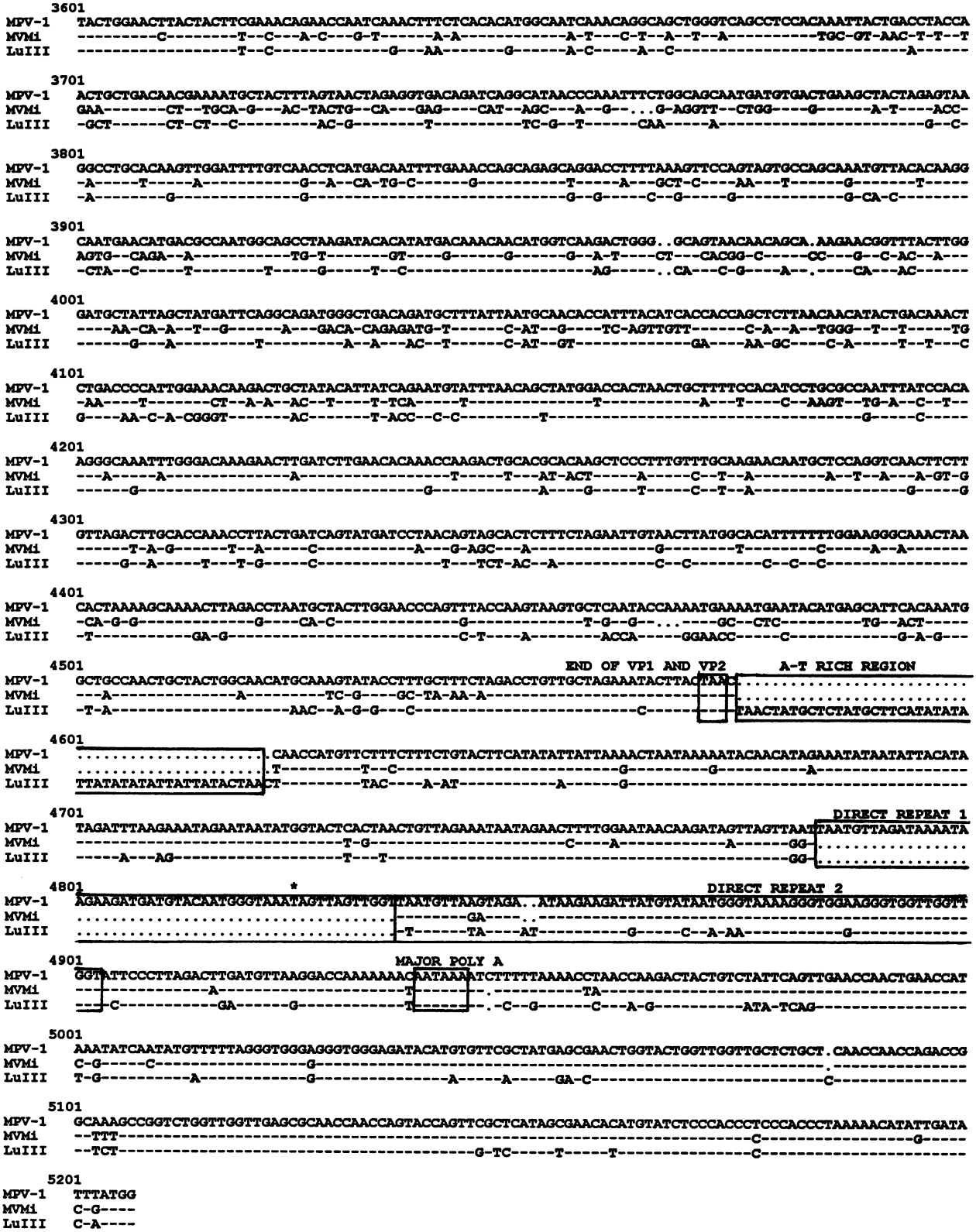


FIG. 5—*Continued.*

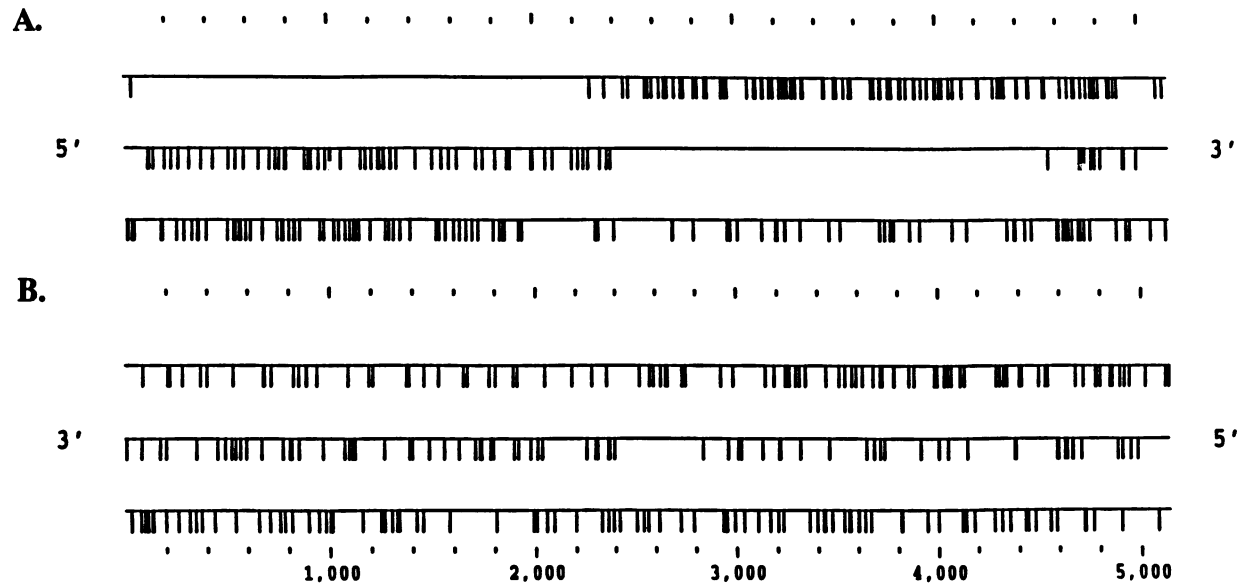


FIG. 6. Translational stops in the MPV genome. Stop codons in all three reading frames of the plus (A) and minus (B) strands of the viral genome are indicated by lines.

found within the 5′ noncoding region of LuIII (13), but it is not found in either strain of MVM or in MPV.

Like MVM, the plus strand of MPV (Fig. 6A) contained two large and several smaller ORFs, whereas the minus strand (Fig. 6B) contained no sizable ORFs. The nucleotide sequences of MPV and MVM(i) were compared with those of five other autonomous parvoviruses (Table 1). MVM(i) and MVM(p) were most identical (96.8%), and MPV was almost equally identical to MVM(i), MVM(p), and LuIII (89%). On the basis of the nucleotide and transcription signal identity between MPV and other parvoviruses, MPV NS1 and NS2 were assigned to the large left ORF, and VP1 and VP2 were assigned to the large right ORF.

The NS1 ORFs were translated for MPV and the other autonomous viruses, and their amino acid sequences were compared (Table 2). The NS1 amino acid sequences were highly conserved among the rodent parvoviruses, with greater than 95% similarity and 91% identity. In contrast, CPV and PPV had less than 85% similarity and 74% identity with either MVM(i) or MPV. MVM(i) NS1 was more identical to MPV (99.3%) than to MVM(p) (97.8%). When the NS2 proteins of MPV were compared with those of MVM(i), there were no coding changes in the common N-terminal region and only one coding change between the major and minor splice. The two predominant carboxy termini of MPV NS2 were identical to those of MVM(i) and MVM(p), but the minor form was extended as in MVM(p) and contained two coding changes from MVM(i) and three coding changes from MVM(p) (Table 3).

The amino acid sequence of VP1, the largest capsid gene, was less conserved among parvoviruses (Table 4). MVM(i) and MVM(p) have a high degree of similarity (99%) and identity (98%), but MPV was only 87% similar and 77% identical to either MVM strain. MPV VP1 was most identical (86%) and similar (91%) to LuIII. The amino acid similarity and identity were much lower between either mouse virus and CPV or PPV.

An alignment of VP1 sequences (Fig. 7) revealed higher conservation within the VP1 unique region (155 N-terminal amino acids) than the homology within the VP1-VP2 common region. Thus, MPV and MVM(i) or MVM(p) were 92.3% identical and MPV and LuIII were 93.5% identical in the VP1 unique region. Pockets of divergence, bracketed by pockets of homology, were obvious in the VP1-VP2 common region.

TABLE 1. Percent nucleotide identity between MVM(i) or MPV and other autonomous parvoviruses[a]

| Virus strain | % Nucleotide identity | |
|---|---|---|
| | MVM(i) | MPV |
| MPV | 89.3 | 100 |
| MVM(i) | 100 | 89.3 |
| MVM(p) | 96.8 | 88.3 |
| LuIII | 84.1 | 88.5 |
| H-1 | 81.1 | 80.5 |
| CPV | 63.4 | 64.8 |
| PPV | 63.3 | 63.3 |

[a] Determined by the GCG GAP sequence alignment program.

TABLE 2. Percent NS1 amino acid similarity and identity between MVM(i) or MPV and other autonomous parvoviruses[a]

| Virus strain | MVM(i) | | MPV | |
|---|---|---|---|---|
| | % Similarity | % Identity | % Similarity | % Identity |
| MPV | 99.9 | 99.3 | 100 | 100 |
| MVM(i) | 100 | 100 | 99.9 | 99.3 |
| MVM(p) | 98.7 | 97.8 | 98.5 | 97.6 |
| LuIII | 95.5 | 92.5 | 95.7 | 93.1 |
| H-1 | 95.5 | 91.4 | 95.4 | 91.4 |
| CPV | 84.4 | 73.9 | 84.4 | 74.0 |
| PPV | 80.1 | 70.6 | 80.0 | 70.4 |

[a] Determined by the GCG Best Fit alignment program.

TABLE 3. Carboxy termini of NS2 in MVM(i), MVM(p), and MPV

| MVM(i) | MVM(p) | MPV |
|---|---|---|
| PEITWF | PEITWF | PEITWF |
| YDGAS | YDGAS | YDGAS |
| LGASWL | LGASWLQVPGTREQP | LGTSRLQVPGTRKQP |

## DISCUSSION

Molecular analysis confirms that MPV is distinct from previously recognized murine parvoviruses. MPV resembles MVM in some basic characteristics, including replication intermediates, genome size, and NS proteins. However, significant differences from MVM are found in the capsid genes, the region of the genome important for parvoviral tropism.

Replicating DNA from MPV-infected L3 cells had the same intermediate forms found in MVM-infected cells, including dRF, mRF, and ssDNA. MVM(i) virions contain 99% minus strand and 1% plus strand, and DNA from MVM(i)-infected cells reflects a similar proportion of plus and minus single-stranded forms. We were able to detect the small amount of plus ssDNA present in the MVM(i)-infected cells, although the major single-stranded form was minus strand. Only minus ssDNA was detected in MPV-infected cells, indicating that MPV also packages primarily minus-strand DNA during infection. Alkaline gel electrophoresis of the samples confirmed the 5-kb length of the single-stranded form, which migrates at 2 kb on a neutral agarose gel.

As expected, the anti-N-terminus antiserum that recognizes MVM NS1 and NS2 reacted with MPV NS1 and NS2. MPV NS proteins were similar in size to those of MVM(i). Furthermore, MPV and MVM(i) expressed the same ratio of NS1 to NS2, whereas previous studies demonstrated that MVM(p) expresses more NS2 than does MVM(i) in infected cells (6). The role of the NS1/NS2 ratio during infection has not been determined, but it is interesting that both MPV (16) and MVM(i) are more efficient than MVM(p) at replicating in mice (8, 18).

Restriction analysis indicated differences between MPV and MVM at the nucleotide level. Restriction sites in the NS coding region of MVM were conserved in MPV, but most sites in the capsid coding region were either different or absent. Additionally, the MVM probe hybridized better to MPV fragments from the NS coding region than to capsid coding region fragments. This was especially evident with the *Bst*EII digestion of MPV, in which there was stronger hybridization signal to the 1.89-kb left-end fragment than to the 3.26-kb right-end fragment. These results suggest a higher degree of identity to MVM in the nonstructural region of the MPV genome than in the capsid region.

The coding region of MPV was more readily cloned than the termini. Multiple intact left termini were recovered, but only one right-terminus clone contained most of the hairpin sequence. We have constructed an intact viral genome from the coding region and two terminal clones, and we are currently testing its infectivity in L3 cells. Although the MPV right terminus has 69 nucleotides deleted, it is reasonable to expect this clone to be infectious. The CPV infectious clone is truncated similarly, and it is repaired during replication (21).

Sequencing indicated that MPV had similar nucleotide identity with MVM(i), MVM(p), or LuIII, a human cell line contaminant of questionable origin (29). The pattern of MPV ORFs, splice junctions, and promoters was almost identical to that of MVM. This implies a similar pattern of transcription for MPV and MVM(i) during infection. The 3′ end of the MPV genome, including the terminal, coding, and noncoding sequences, was almost identical to the 3′ end of MVM(i). In contrast, the 5′ end of the MPV genome, including coding, noncoding, and terminal sequences, was quite different from those of MVM(i), MVM(p), and LuIII. The sequence identity between MPV and LuIII did not include the A-T-rich noncoding region in LuIII, which is hypothesized to be necessary for packaging both strands (13). Additionally, the 5′ terminus had 11, 8, and 21 nucleotide changes, respectively, from MVM(i), MVM(p), and LuIII. Also, MPV contained 1.8 copies of the sequence repeated near the right-hand palindrome in MVM(p), albeit with nucleotide changes from MVM. This slight deletion was found in multiple clones examined and is unique among the parvoviruses, which have either one [MVM(i) and LuIII] or two (H-1, feline parnleukopenia virus, and CPV) complete copies in their genomes.

A comparison of the NS proteins demonstrated that MVM(i) NS1 is more identical to MPV than to MVM(p). This finding correlates with both MVM(i) (8, 18) and MPV (16) replicating more efficiently than MVM(p) in vivo. The VP1 comparison revealed an unexpected similarity of amino acid sequence between MPV and LuIII. MPV was 85.6% identical and 91.2% similar to LuIII, a higher level of identity than was found between MPV and either MVM serotype. Although this level of identity is much lower than the homology between the two MVM serotypes (98.4%), it will be interesting to determine if serologic or functional characteristics are shared by LuIII and MPV. Chapman and Rossmann (9) reported a similar alignment of multiple parvovirus VP2 proteins, including localization of VP2 amino acid sequence on the crystallized structure of the CPV virion. By extrapolation, most pockets of diversity between MPV and other murine parvoviruses are at or near positions determined to be surface amino acids.

Serologic differences between MVM and MPV can be explained by the sequencing results. Generic serologic tests such as IFA and ELISA measure reactivity to both NS and VP antigens. Thus, the cross-reactivity of sera from infected mice is attributed to the high level of amino acid identity between the NS proteins of MVM(i) and MPV. In contrast, HA inhibition measures reactivity to VP antigens. Lower levels of amino acid identity in the VP1 and VP2 capsid proteins, particularly in regions thought to be surface epitopes of the virion (9, 35), explain the lack of cross-reactivity between MPV and MVM by HA inhibition.

The origin of MPV is unknown, but retrospective testing of banked sera indicates that MPV and MVM have been infecting mouse colonies for more than two decades (31). Our data and previous results (19, 32) support the conclusion that MPV could not be identified earlier because the confirmatory assay

TABLE 4. Percent VP1 amino acid similarity and identity between MVM(i) or MPV and other autonomous parvoviruses[a]

| Virus strain | MVM(i) | | MPV | |
|---|---|---|---|---|
| | % Similarity | % Identity | % Similarity | % Identity |
| MPV | 86.7 | 77.0 | 100 | 100 |
| MVM(i) | 100 | 100 | 86.7 | 77.0 |
| MVM(p) | 99.2 | 98.4 | 86.8 | 77.4 |
| LuIII | 87.5 | 77.3 | 91.2 | 85.6 |
| H-1 | 84.0 | 72.8 | 81.5 | 72.1 |
| CPV | 72.6 | 56.6 | 72.6 | 57.2 |
| PPV | 69.7 | 54.6 | 68.7 | 54.7 |

[a] Determined by the GCG Best Fit alignment program.

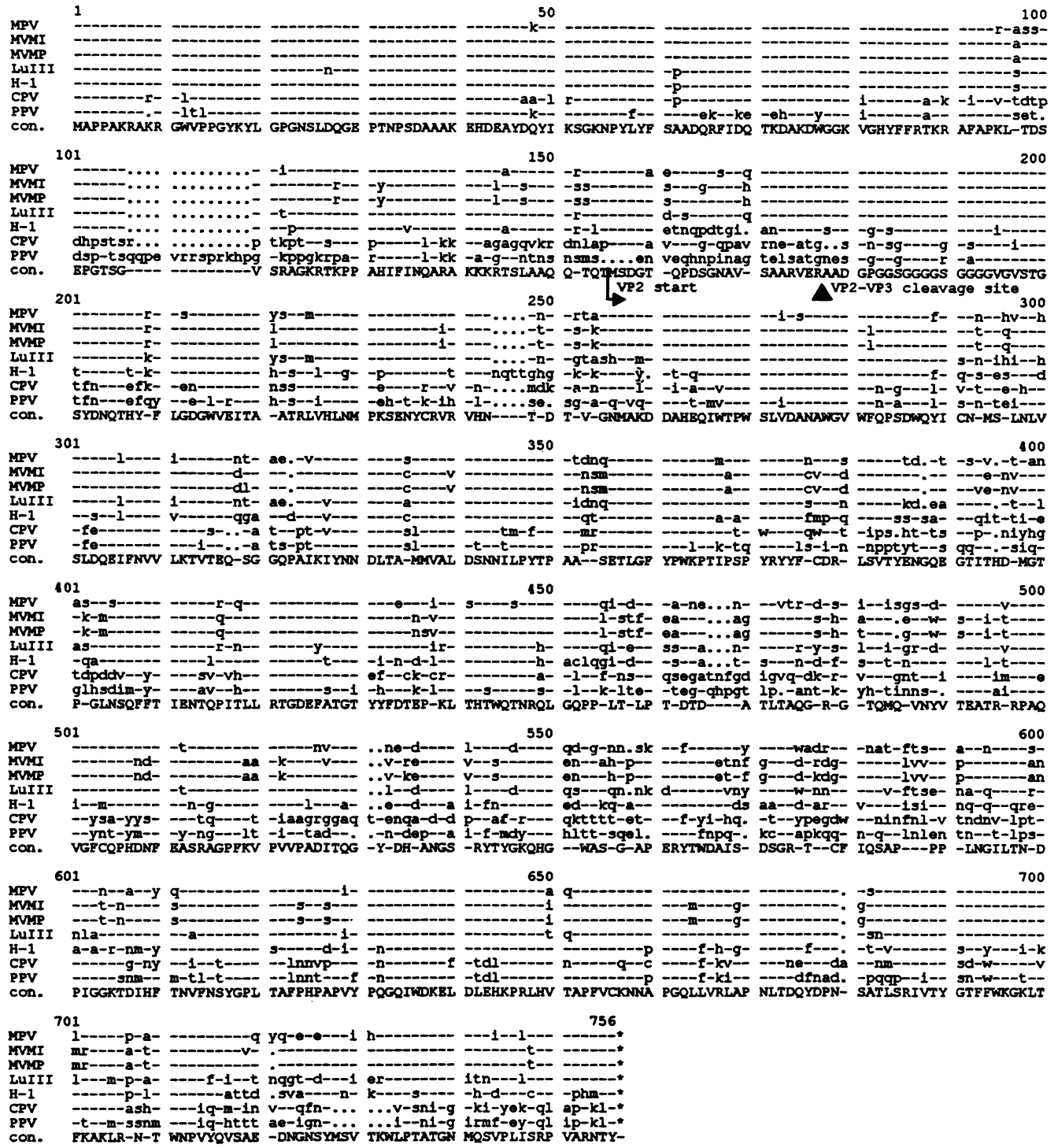


FIG. 7. VP1 amino acid alignment for autonomous parvoviruses. Dashes indicate amino acids identical to those of the consensus sequence, and dots indicate spaces inserted for maximal sequence alignment. The internal VP2 start codon and the VP2-to-VP3 cleavage sites are indicated. The sequences were aligned by the GCG programs Pileup and Pretty.

for parvoviruses was the MVM HA inhibition test. Therefore, MVM and MPV appear to be distinct viruses, both of which have been infecting mouse colonies for many years. A less likely possibility is that MPV originated by a recombination event between LuIII and MVM to generate a hybrid virus. Our data do not support this hypothesis for several reasons. First, there are far more differences in the nucleotide and coding sequences of LuIII and MPV than are found between parvoviruses thought to originate from a common ancestor (22). Second, examination of CPV isolates by Parrish and coworkers

(23) indicates that autonomous parvoviruses mutate by individual nucleotide changes rather than by recombinational events.

Mutational events may influence the evolution of mouse parvoviruses. A second MPV (MPV-2) was recently identified by direct examination of tissues from naturally infected mice (32). MPV-2 is similar to MPV-1 in the capsid coding region of the genome, but several silent and coding changes distinguish it from MPV-1 (4). Both MPVs asymptomatically infect adult mice and cause persistent infection (16).

Collectively, these findings expand our understanding of the molecular biology of MPV and its relationship to other autonomous parvoviruses. The differences demonstrated in the capsid region among parvoviruses of mice may determine virulence, tropism, and duration of infection. The availability of characterized clones will facilitate the definition of viral determinants involved in the pathogenesis of infection.

## ACKNOWLEDGMENTS

This research was supported by grant RR00393 from the National Institutes of Health and by funding from the Charles River Foundation.

We thank Susan Cotmore, Robert Jacoby, Maureen McKisic, and Abigail Smith for critical reading of the manuscript. We also thank Frank Paturzo for technical support.

## REFERENCES

1. **Antonietti, J.-P., R. Sahli, P. Beard, and B. Hirt.** 1988. Characterization of the cell type-specific determinant in the genome of minute virus of mice. J. Virol. **62**:552–557.
2. **Astell, C. R., E. M. Gardiner, and P. Tattersall.** 1986. DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. **57**:656–659.
3. **Astell, C. R., M. Thomson, M. Merchlinsky, and D. C. Ward.** 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. Nucleic Acids Res. **11**:999–1018.
4. **Ball-Goodrich, L. J.** Unpublished data.
5. **Ball-Goodrich, L. J., R. D. Moir, and P. Tattersall.** 1991. Parvoviral target cell specificity: acquisition of fibrotropism by a mutant of the lymphotropic strain of minute virus of mice involves multiple amino acid substitutions within the capsid. Virology **184**:175–186.
6. **Ball-Goodrich, L. J., and P. Tattersall.** 1992. Two amino acid substitutions within the capsid are coordinately required for the acquisition of fibrotropism by the lymphotropic strain of minute virus of mice. J. Virol. **66**:3415–3423.
7. **Bates, R. C., C. E. Snyder, P. T. Banerjee, and S. Mitra.** 1984. Autonomous parvovirus LuIII encapsidates equal amounts of plus and minus DNA strands. J. Virol. **49**:319–324.
8. **Brownstein, D. G., A. L. Smith, R. O. Jacoby, E. A. Johnson, G. Hansen, and P. Tattersall.** 1991. Pathogenesis of infection with a virulent allotropic variant of minute virus of mice and regulation by host genotype. Lab. Invest. **65**:357–364.
9. **Chapman, M. S., and M. G. Rossmann.** 1993. Structure, sequence, and function correlations among parvoviruses. Virology **194**:491–508.
10. **Clemens, K. E., and D. Pintel.** 1987. Minute virus of mice (MVM) mRNAs predominantly polyadenylate at a single site. Virology **160**:511–514.
11. **Cotmore, S. F., and P. Tattersall.** 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. **58**:724–732.
12. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33**:91–174.
13. **Diffoot, N., K. C. Chen, R. C. Bates, and M. Lederman.** 1993. The complete nucleotide sequence of parvovirus LuIII and localization of a unique sequence possibly responsible for its encapsidation pattern. Virology **192**:339–345.
14. **Gardiner, E. M., and P. Tattersall.** 1988. Mapping of the fibrotropic and lymphotropic host range determinants of the parvovirus minute virus of mice. J. Virol. **62**:2605–2613.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–359.
16. **Jacoby, R. O., E. A. Johnson, L. J. Ball-Goodrich, A. L. Smith, F. X. Paturzo, and M. D. McKisic.** Unpublished data.
17. **Jongeneel, C. V., R. Sahli, G. K. McMaster, and B. Hirt.** 1986. A precise map of splice junctions in the mRNAs of minute virus of mice, an autonomous parvovirus. J. Virol. **59**:564–573.
18. **Kimsey, P. B., H. D. Engers, B. Hirt, and C. V. Jongeneel.** 1986. Pathogenicity of fibroblast- and lymphocyte-specific variants of minute virus of mice. J. Virol. **59**:8–13.
19. **McKisic, M. D., D. W. Lancki, G. Otto, P. Padrid, S. Snook, D. C. Cronin II, P. D. Lohmar, T. Wong, and F. Fitch.** 1993. Identification and propagation of a putative immunosuppressive orphan parvovirus in cloned T cells. J. Immunol. **150**:419–428.
20. **Morgan, W. R., and D. C. Ward.** 1986. Three splicing patterns are used to excise the small intron common to all minute virus of mice RNAs. J. Virol. **60**:1170–1174.
21. **Parrish, C. R.** 1991. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. Virology **183**:195–205.
22. **Parrish, C. R.** 1990. Emergence, natural history, and variation of canine, mink, and feline parvoviruses. Adv. Virus Res. **38**:403–450.
23. **Parrish, C. R., C. F. Aquadro, M. L. Strassheim, J. F. Evermann, J.-Y. Sgro, and H. O. Mohammed.** 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J. Virol. **65**:6544–6552.
24. **Pintel, D., D. Dadachanji, C. R. Astell, and D. C. Ward.** 1983. The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. Nucleic Acids Res. **11**:1019–1038.
25. **Reed, A. P., E. V. Jones, and T. J. Miller.** 1988. Nucleotide sequence and genome organization of canine parvovirus. J. Virol. **62**:266–276.
26. **Rhode, S. L. I., and P. R. Paradiso.** 1983. Parvovirus genome: nucleotide sequence of H-1 and mapping of its genes by hybrid-arrested translation. J. Virol. **45**:173–184.
27. **Sahli, R., G. K. McMaster, and B. Hirt.** 1985. DNA sequence comparison between two tissue-specific variants of the autonomous parvovirus, minute virus of mice. Nucleic Acids Res. **13**: 3617–3633.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Siegl, G.** 1976. The parvoviruses. Virology monographs, vol. 15. Springer-Verlag, New York.
30. **Smith, A. L.** 1983. Response of weanling random-bred mice to inoculation with minute virus of mice. Lab. Anim. Sci. **33**:37–39.
31. **Smith, A. L.** Personal communication.
32. **Smith, A. L., R. O. Jacoby, E. A. Johnson, F. Paturzo, and P. N. Bhatt.** 1993. In vivo studies with an "orphan" parvovirus of mice. Lab. Anim. Sci. **43**:175–182.
33. **Tattersall, P., and J. Bratton.** 1983. Reciprocal productive and restrictive virus-cell interactions of immunosuppressive and prototype strains of minute virus of mice. J. Virol. **46**:944–955.
34. **Tattersall, P., and A. J. Shatkin.** 1977. Sequence homology between the structural polypeptides of minute virus of mice. J. Mol. Biol. **111**:375–394.
35. **Tsao, J., M. S. Chapman, M. Agbandje, W. Keller, K. Smith, H. Wu, M. Luo, T. J. Smith, M. G. Rossmann, R. Compans, and C. R. Parrish.** 1991. The three-dimensional structure of canine parvovirus and its functional implications. Science **251**:1456–1464.
36. **Vasudevacharya, J., S. Basak, R. V. Srinivas, and R. W. Compans.** 1989. Nucleotide sequence analysis of the capsid genes and the right-hand terminal palindrome of porcine parvovirus, strain NADL-2. Virology **173**:368–377.