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# Analysis of the Complete Nucleotide Sequences of Goose and Muscovy Duck Parvoviruses Indicates Common Ancestral Origin with Adeno-Associated Virus 2

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The complete nucleotide sequences of two parvoviruses isolated from goose and muscovy duck were determined. The two virus genomes share 81.9% nucleotide sequence identity, indicating that they are closely related. The coding regions are bracketed by inverted terminal repeats containing palindromes. This is similar to the genome organization of human parvoviruses, adeno-associated virus 2, and B19. Amino acid sequence comparison shows that the closest relative of the goose and muscovy duck parvoviruses is adeno-associated virus 2. This is surprising, because the goose and muscovy duck parvoviruses do not require any helper virus for productive replication, suggesting that adeno-associated virus 2 has been derived from a helper-independent ancestor. © 1995 Academic Press, Inc.

#### INTRODUCTION

Parvoviruses are among the smallest animal viruses and have linear, single-stranded genomes. Parvoviruses have been isolated from vertebrates and invertebrates (Tijssen, 1990). Parvovirus virions are nonenveloped, have icosahedral symmetry, and are 18-26 nm in diameter (Berns, 1990). The virions each contain a single DNA molecule of about 5-6 kilobases in length, in which a long single-stranded coding region is bracketed by shorter terminal palindromic regions capable of folding into hairpin duplexes. The terminal hairpins serve as origins of replication and provide cis signals for packaging (Tattersali and Cotmore, 1990; Carter et al., 1990b), According to Astell (1990), parvoviruses can be classified based on the properties of the terminal hairpins. Type A viruses (e.g., AAV-2 and B19) have inverted terminal repeats containing palindromes, and viruses with type B genomes (e.g., MVM, H1, Lulll, etc.) have palindromes at the left and right termini, the sequences of which are not related. The genome of vertebrate parvoviruses contains two major open reading frames, one encoding for the capsid proteins and the other coding for a family of pleiotropic regulatory proteins (Rhode and Iversen, 1990).

Parvoviruses replicate in the nucleus. Due to the limited number of viral genes, parvovirus replication de-

In the mid-sixties a highly fatal disease of goslings and muscovy ducklings was described (Derzsy, 1967). The causative agent of the disease was classified as a parvovirus (Kisary and Derzsy, 1974). The goose parvovirus (GPV) was described as a small particle of 20-22 nm in diameter with a hexagonal outer appearance. GPV was found to replicate in primary goose embryo fibroblast cells with the formation of Cowdry type A intranuclear inclusion bodies (Kisary and Derzsy, 1974). Another parvovirus isolated from muscovy ducks with clinical signs of Derzsy's disease was shown to be closely related to GPV by Southern hybridization (Zádori et al., 1994). Both GPV and muscovy duck parvovirus (MDPV) replicate autonomously and encapsidate strands of opposite polarities in equal amounts, if they are propagated in embryo (Zádori et al., 1994).

A fair number of parvoviruses have been molecularly cloned and sequenced, including members of the family which parasitize insects and mammals (for references see Rhode and Iversen, 1990; Diffoot *et al.*, 1993; Dumas *et al.*, 1992; Bando *et al.*, 1987). However, there is no information available on the genome organization of avian parvoviruses at the level of nucleotide sequence. Therefore the phylogenetic relationship between avian and the well-characterized mammalian parvoviruses has not been established. We were interested in obtaining the complete nucleotide sequence of GPV and MDPV, because this information would allow us to define the evolutionary relationship between the avian and mammalian parvoviruses. On the other hand, GPV and MDPV

pends on several functions provided by the host cell (Tattersall and Gardiner, 1990). In the case of the members of *Dependovirus*, effective parvovirus replication may require the presence of a helper virus, usually adenovirus or herpesvirus (Carter, 1990).

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differ in several ways. They have different host preferences, there are antigenic differences between them (Kisary, unpublished observations), and the genomes show distinct restriction site patterns (Zádori *et al.*, 1994). Therefore, we also wanted to determine how much GPV and MDPV have diverged.

#### MATERIALS AND METHODS

#### Enzymes and reagents

Restriction endonucleases and T4 DNA polymerase were purchased from MBI Fermentas (Vilnius, Lithuania). For DNA sequencing, we purchased T7 DNA polymerase and reagents from Pharmacia Biotech (Sollentuna, Sweden).

#### Bacterial cells and plasmids

Escherichia coli strains XL1-Blue { $\Delta$ (mcrA)183,  $\Delta$ (mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA, gyrA96, relA1, lac,  $\lambda$ -, [F', proAB, lacl<sup>q</sup>Z $\Delta$ M15, Tn10, (tet')]} and SURE {el4^-(mcrA),  $\Delta$ (mcrCB-hsdSMR-mrr)171, endA1, supE44, thi-1, gyrA96, relA1, lac, recB, recJ, sbcC, umuC:Tn5 (kan'), uvrC, [F' proAB, lacl<sup>q</sup>Z $\Delta$ M15, Tn10, (tet']} from Stratagene (La Jolla, CA) were used for plasmid propagation. For DNA cloning the vector pBluescript II (SK<sup>-</sup>) (Stratagene, La Jolla, CA) was used.

#### Virus propagation and preparation of viral DNA

For molecular cloning, virion DNA was isolated from specific pathogen-free muscovy duck (*Cairina moschata*) and goose (*Anser anser*) embryonated eggs infected with strain FM of MDPV and virulent B strain of GPV as previously described (Zádori *et al.*, 1994). As MDPV and GPV propagated *in embryo* encapsidate both negative and positive DNA strands, we obtained double-stranded nucleic acid.

#### Molecular cloning and sequencing

MDPV virion DNA was digested with either restriction enzyme Sacl or HindIII. Both of these enzymes cut the MDPV genome at a single site. The resulting two DNA fragments were ligated into the EcoRV-Sacl or EcoRV-HindIII site, respectively, of pBluescript II (SK<sup>-</sup>). Upon transformation of the ligation mixture into XL1-Blue competent bacteria, we obtained all of the expected recombinant clones; however, they contained deletions in the inverted terminal repeats (ITRs). In contrast, we were able to obtain recombinant HindIII clones when we prepared competent bacteria from E. coli strain SURE. We also cloned internal EcoRI and PstI fragments into pBluescript II SKT. We generated exonuclease III-mung bean nuclease nested deletions in the clones harboring the Sacl "A" and "B" fragments for sequencing. We also created subclones with restriction endonucleases Hindlll, Stul, Kpnl, and Dral into pBluescript II (SK-). We determined the nucleotide sequences by the dideoxynucleotide chain termination method by applying either T7 DNA polymerase and S<sup>32</sup>-labeled dATP for conventional sequencing gels or fluorescein-labeled primers for automated DNA sequencing with the ALF DNA Sequencer of Pharmacia (Sweden). We used the SK, KS, M13-20, and reverse oligonucleotide primers for DNA sequencing.

GPV virion DNA was cut with *Hin*dIII, and the resulting end fragments, "A" and "C," were ligated to the *Eco*RV– *Hin*dIII site of the vector pBluescript II (SK<sup>-</sup>). The *Hin*dIII "B" fragment was ligated to the *Hin*dIII site of the same vector. We obtained recombinants of correct sites by using the competent bacteria SURE. Internal GPV genomic *Pst*I fragments were also cloned into pBluescript II (SK<sup>-</sup>). We generated exonuclease III–mung bean nuclease nested deletions in the *Hin*dIII clones for sequencing. We determined the nucleotide sequences of the GPV clones as described above for MDPV.

In order to sequence the ITRs of MDPV and GPV, we generated exonuclease III—mung bean nuclease nested deletions and subcloned the terminal *SphI* fragments. The restriction enzyme cuts the ITRs into two fragments.

#### Sequence analysis softwares

For sequence analysis the Wisconsin sequence analysis package version 8 of the Genetics Computer Group (Madison, WI) was employed. Transcription factor recognition sequences were searched with the program Findpatterns in the Transcription Factor Database (Ghosh, 1990).

#### Nucleotide sequence accession number

The complete DNA sequences of MDPV and GPV have been submitted to GenBank and assigned Accession Nos. U22967 and U25749, respectively.

#### **RESULTS**

#### Nucleotide sequence

We succeeded in cloning and sequencing of the entire genomes of GPV and MDPV (Fig. 1). The genome of GPV (5106 bp) is somewhat shorter than that of MDPV (5132 bp). The difference in length between the two genomes can be attributed to short deletions in the ITRs and to a 5-bp deletion in the GPV chromosome shortly after the first stop codon of the capsid gene (from nucleotide 4639 of GPV). By comparing the coding regions of the two genomes, we can say that there is not any deletion; however, there are numerous substitutions. Otherwise, the two genomes show high similarity. They share 81.9% nucleotide sequence identity.

We must note that we experienced difficulties in sequencing the intact "bubble" region (Fig. 1) of the ITRs. We started to sequence the inserts of the plasmids carrying the intact ITRs. We could not obtain unambiguous nucleotide sequences of the plus or the minus DNA

GPV	1	BS <sup>Rep</sup> Y-box BS <sup>Rep</sup> E-box 1 CTCATTGGAGGGTTCGTTCGTACCAGCCAATCAGGGGA.GGGGGAAGTGACGCAAGTTCCGGTCACATGCTTCCGGTC	ATF/CRE	
MDPV	1	****  ****  ****  ****  ****  ****  ****	E-box E-box CACG <b>TGCT</b> TCCGGT <b>CAT</b> GT	
		BSRep . E-box . E-box .MLTF . MLTF. ATF/CREB .	>	
GPV	100	.00 .AGTTCCGGTCACGTG.CTTCCTGTCACGTGTTTCCCGTCACGTGACTTCCCGGTCATGTCACTTCCGCTCACGTGTTTTCC	CGGCTGTT . AGGTTGACCA	194
MDPV	101	01 GACTTCCGGTCATGTGACTTCCCGTGACGTGTCTCCTGTCACGTGACTTCCGGTGACGCACTTCCTTTGATGACGTATTTCC	<u>CGGTT</u> GTCAAGGCTGAT <u>C</u> C	200
anı		"bubble"-region	E-box .	
GPV MDPV		95 CGCGCATGCCGCGGGTCAGCCCCAATAGTTAAGCCGGAAACACGTCACCGGAAGTCACATGACCGGAAGTCACGTGACCC	ATF/CREB	
MDPV	201	01 GCCGCATGCCCCCATCAAAATTAAACCGGAAATACGTCATCAAAGGAAGTGCCTCACCGGAAGTCACGTGACAC		300
GPV	293	E-box ATF/CREB E-box BS <sup>Rep</sup> .  93 .CACGTGACCGGAACT.ACGTCACCGGATGTGCGTCACCGGAAGCATGTGACCGGAACTTGCGTCACTT.CCCCCTCCCCT	Y-box BS <sup>Rep</sup> . GATTGGCTGGTTCGAACGA	389
MDPV	301	E-box E-box < ATF/CREB 01 TCACATGACCGGAAGTCACATGACCGGAAGCACGTGACCGGAACTTACGTCACTTCCCCCCTCCCCTC	•••> <•••< GATTGGCTGGTTCGAACGA	
		trs		
GPV	390	$\begin{array}{ll} & \varphi  & \varphi  & \varphi  \\ & \varphi  & \varphi  \\ & \varphi  & \varphi  \\ & \varphi  & \varphi  & \varphi  \\ &$	NGGARACTTCCGGTTTAGT	489
MDPV	401	••<•••  11 ACGAACCCTCCAATGAGACTCAAGGACACCAGGACTTTTTGCGCGCCAGGAAATGGTGCAATCTAAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGGACTGTATAAAGGACACCAGACCACC	aag <b>cttt</b> tttccggtt <b>gca</b> t	500
		CAP?		
GPV	490	PEP1 90 TCATTCGTTACTCTCCTCAGAGAGAACGGACCTCAGGTCGCAGAGAGGCCCTTTCTAGGCCTCTTCAGATTTCTTCTG	ATAAATTCTATGAAGTTAT	589
MDPV	501	01 TCATTCGTTGCTCTCACAGAGAACGGACCTCAGGTCAGG	ACAAATTCTATGAAGTTAT	600
		c-Fos . c-Myc	, .	
GPV		90 TATTAGATTATCATCGGATATTGATCAAGATGTCCCCGGTCTGTCT		
MDPV	601	01 CATCAGGCTACCCTCGGATATTGATCAAGATGTCCCTGGTTTGTCTTTAACTTTGTAGAATGGCTTTCTACGGGGGTCTGC	JGAGCCCACCGGAATATGG	700
GPV	690	90 AACATGGAGCATGTGAATCTACCGATGGTGACCTTGGCAGAGAAGATCAAGAACATTTTCATACAAAGATGGAATCAGTTC	AACCAGGACGAAACGGACT	789
MDPV		CBP 01 AATATGGAGCATGTGAATCTCCCCATGGTTACTCTGGCAGACAAAATCAAGAACATTTTCATCCAGAGATGGA <u>ACAAAT</u> TCA	•	
GPV	790	90 TCTTCTTTCAACTGGAAGAAGGCAGTGAGTACATTCATCTTCATTGCTGTATTGCCCAGGGCAATGTACGGTCTTTTGTTC1	I <b>C</b> GGGAGATATATGTCTCA	889
MDPV	801	01 TCTTCTTTCAATTGGAAGAAGGCAGTGAGTACATCCATCTGCATTGCTGTATTGCCCAGGGGAATGTGCGATCTTTTGTTCT	r <b>g</b> ggagata <b>c</b> atgtctca	900
			•	
GPV		90 GATAAAAGACTCTATCATAAGAGATGTATATGAAGGGAAACAAATCAAGATCCCCGATTGGTTTGC <u>TATTA</u> CTAAAACCAAG		
MDPV	901	01 AATTAAAGACTCAATTCTGAGAGATGTGTATGAAGGGAAACAGGTAAAAATCCCGGATTGGTTTTC <u>TATAA</u> CTAAAACCAAA CAP?	ICGGGGAGGGCARAATAAG	1000
GPV	990	REP2.  90 ACCGTGACTGCAGCATACATACTGCATTACCTTATTCCTAAAAAGCAACCTGAACTGCAATGGGCCTTTACCAATATGCCTT	PTATTCACTGCTGCTGCTC	1089
		<del></del>		
GPV :	1090	90 TTTGTCTGCAAAAGCGGCAAGAATTGCTGGATGCATTTCAAGAAAGTGATTTGGCTGCCCCTTTACCTGATCCTCAAGCATC	CAACTGTGCCACCGCTTAT	1189
MDPV :	101	01 TATGCCTCCAAAAGAGGCAAGAGTTACTGGATGCTTTTCAGGAAAGTGAGATGAATGCTGTAGTGCAGGAGGATCAAGCTTC	Caactg <b>ca</b> gc <b>t</b> cc <b>c</b> cttat	1200
GPV :	1190	90 TTCCAACAGAGCGCAAAGAACTATAGCAACCTTGTTGATTGGCTCATTGAAATGGGGATAACATCTGAGAAGCAATGGCTC	:ACTGA <b>G</b> AA <b>CCG</b> AGAGAGC	1289
MDPV :	1201	01 TTCCAACAGAGCAGCAAAGAACTATAGCAATCTGGTTGATTGGCTCATTGAGATGGGTATCACCTCTGAAAAACAGTGGCTA	actga <b>r</b> aa <b>tra</b> agagagc	1300

FIG. 1. Complete nucleotide sequences of GPV and MDPV. The nucleotide sequences of the plus strands are shown. The differences between the two sequences are in boldface. The regions corresponding to the inverted terminal repeats (ITRs), except those of the "bubble regions," are underlined. The putative Rep recognition sequences (BS<sup>Rep</sup>) and the transcription factor binding sites (Y-box, E-box, ATF/CREB, MLTF, GC-box, c-Fos, c-Myc, CBP, and AP-1) are double underlined. The vertical arrows indicate the putative terminal resolution sites (trs). Vertical lines (| | | ) indicate start and stop codons of the putative proteins REP1, REP2, VP1, VP2, and VP3. The promoters p9, p19, and p41 are underlined. The marks and "CAP" designate putative transcription start sites. The putative polyadenylation and splice donor and acceptor sites (\*) are also indicated.

GPV 1290 TACAGRAGCTTTCAAGCAACTTCTTCAAATAATAGACAAGTGAAAGCTGCACTGGAAAATGCCCGTGCTGAAATGTTATTGACAAAGACTGCAACTGATT 1389 1390 ACCTGATAGGAAAAGACCCTGTCCTGGATATAACTAAGAATAGGGTCTATCAAATTCTGAAAATGAATAACTACAACCCTCAATACATAGGAAGTATCCT 1489 MDPV 1401 ATTTGATTGGAAAAGACCCAGTTCTGGACATTACTAAAAATCGGATCTATCAAATTCTGAACTTGAATAACCCTCAATATCTAGGGAGCGTCCT 1500 1490 GTGCGGCTGGGTGAAGAGAGAGTTCAACAAAAGAAACGCCCATATGGCTCTACGGACCTGCCACCGGGAAGACCAACATTGCAGAAGACTATTGCCCAT 1589 MDPV 1501 ATGCGGATGGGTGAAAAGAAATTCAACAAAAGAAATGCCATATGGCTCTACGGACCTGCGGAACACACATAGCCGAGGCTATTGCCCAT 1600 1590 GCTGTACCCTTCTATGGCTGTGTTAACTGGACTAATGAGAACTTTCCTTTTAATGATTGTGTTGATAAAATGCTGATTTGGTGGGAGGAGAAAATGA 1689 MDPV 1601 GCTGTACCCTTCTATGGCTGTTAACTGGACTAATGAGAACTTCCCATTTAATGACTGCGTTGATAAAATGCTTATATGGTGGGAGGAAAAATGA 1700 GPV 1 690 CTAATAAGGTTGTTGAATCTGCAAAAGCAATTTTGGGAGGGTCTGCTGCCGGGTAGACCAGAAATGTAAAGGATCTGTTTTGTATTGAACCTACTCCTGT 1789 GPV 1790 AATTATTACTAGTAATACTGATATGTTTAGATGGCAACTCTACTACAATGGAACATAGAATACCATTAGAGGAGCGTATGTTTCAAATTGTC 1889 GPV 1890 CTATCACATAAATTGGAGCCTTCTTTTGGAAAAATTTCTAAAAAAGGAAGTCAGAGAATTTTTCAAATGGGCCAATGACAATCTAGTTCCTGTTGTGTCTC MDPV 1901 CTTTCCCATAAGCTGGAAGCAAATTTTGGAAAAATTTCAAAAAGGGGCTAAAAGAGTTTTTCAAATGGG<u>CCAAT</u>GATAATCTTGTTCCAGTAGTTTCT<u>TG</u> 2000 GPV 2001 AGTTCAAAGTCCCTACGAATGAACAAACCAAACTTACTGAGCCCGTTCCTGAACGAGCGAATGAGCCTTCCCGAGCCTCCTAAGATATGGGCTCCACCTAC 2100 CAP? p41? CPV 2090 TAGGGAGGAGTTAGAAGAGCCTTTTAAGAGCCAGCCCAGAATTGTTCTCATCAGTCGCTCCAATTCCTGTGACTCCTCAGAACTCCCTGAGCCTAAGAGA 2189 p41 MDPV 2101 TAGGGAGGAGCTAGAGGAGATATTAAGAGCGAGCCCTGAGCTCTTTCCTTCAGTTGCTTCCCTTCCCGTCCCGGACACATCTCCCTAAGAGAAAAGAAA 2200 Splice donor 2190 AGCAGGAACAATTACCAGGTACGCCTGCGCTTTGCATACTTATGACAATTCTATGGATGTTATTGAATGTTATGGAATGTGAATGTATGGAATGTCTGAATCTTCCTGAAT 2289 GPV MDPV 2201 ACCCGTGGGGAGTATCAGGTACGCTGTGCTATGCACAGTTTAGATAACTCTATGAATGTTTTTGAATGCCTGGAGTGTGAAAGAGCTAATTTTCCTGAAT 2300 2290 TTCARCCTCTGGGMGAAAATTATTGTGATGAACATGGGTGGTATGATTGTGCTATATGTAAAGAGTTGAAAAAATGAACTTCCMGAAAATTGATCATCTTT 2389 MDPV 2301 TTCAGAGTCTGGGTGAAAACTTTTGTAATCAACATGGGTGGTATGATTGTGCATTCTGTAATGAACTGAAAGATGACATGAAATTGAACATGTTTT 2400 Splice acceptor 1 Splice acceptor 2 VPI 2390 TGAGCTTGATGATGCTGAAAATGAACAATAAGATGAGCTCAAAGCAGATATGTCTACTTTTTTAGATTCTTTTTGAAGAGTGGTATGAGACTGCAGCCGCC 2489 Ш Ш MDPV 2401 TGCTATTGATGATATGGAGAATGAACAATAAAGGTGATTCAAAATAGGTATGTCTACTTTTTTAGAGAAATTTGAAGACTGGTATGAGACTGCAGCCGCA 2500 

FIG. 1 — Continued

strands corresponding to the "bubble" region. Therefore, we generated subclones of the intact ITRs by cutting them in half with restriction endonuclease *SphI*. The restriction endonuclease *SphI* cuts the GPV and MDPV ITRs at nucleotide 202 and 4907, and 208 and 4924, respectively. The subclones obtained this way gave un-

ambiguous sequence data for both the plus and minus DNA strands by using the suitable pBluescript oligonucleotide primers. The analysis of the sequences of the subclones revealed that the sequencing difficulties in the complete ITR-containing clones were caused not by the high GC content of the "bubble" region, but by the inter-

2590 GGGGCTTTGTACTTCCTGGCTATAAGTATCTTGGGCCTGGTAACGGCCTGGATAAAGGCCCACCTGTCAATAAGGCGGACAGCGTCGCGCTTGAACACGA 2689 MDPV 2601 GGGCTTTGTACTTCCTGCTATAAGTATGTTGGGCCTGGTAACGGCCTTGATAAAGGGCCACCTGTCAATAAAGCGGACAGCGTCGCGCTTGAGCACGA 2700 TATA-box? MDPV 2701 TAAMGCGTACGACCAGCAGCTCAAGGCAGGAGACACCCCTATATAAAATTTAAGCACGCAGATCAAGAATTTATAGATAATCTGCAAGGTGATACCTCC 2800 2790 TTCGGAGGTAATCTTGGAAAGGCTGTATTTCAGGCCAAAAAAGGTATCTTAGAGCCATTTGGCCTAGTAGAAGATCCTGTCAACACGCACCTGCAAAAA 2889 Ш 2890 AMANTACAGGGAAGCTTACTGACCATTACCCGGTAGTTAAGAAGCCTAAACTTACCGAGGAAGTCAGTGCGGGAGGTGGTAGCAGTGCCGTACAAGACGG 2989 **GPV** MDPV 2901 AGAGTAGTGGAAAACTAACAGATCACGACCCTATAGTAAAGAAGCCTAAATTATCTGAAGAAAACTCCCTTCACCTAGTAATAATGTGGAGGAGAAGCAAG VP3 3101 GGTAATGCCTCAGGAAATTGGCATTGCGATTCCCAATGGCTGGGAGACACAGTCATTACCAAGACTACAAGAACCTGGGTCCTGCCAAGCTACAACAACC 3200 3190 ACATCTACAAAGCAATTACCAGCGGAACCTCTCAAGATGCAAATGTCCAGTATGCAGGATACAGTACCCCCTGGGGGTACTTTGATTTCAACCGCTTCCA 3289 3201 ACATGTACCAAGCCATCACMAGCGGAACMACCCAGACTCAAATMCCCAMTATGCTGGATACAGCACCCCTGGGGGTACTTTGATTTCAACMGATTCCA 3300 CPV 3290 CTGCCACTTCTCCCCTAGAGACTGGCAGAGACTTATCAACAACCATTGGGGAATCAGACCCAAGTCTCTTAAATTCAAGATCTCAATGTCCAAGTCAAA 3389 MDPV 3301 CTGCCATTTCTCTCCAAGAGACTGGCAGAGACTCATCAACAACCATTGGGGGATTAGACCGAAAGCACTCAAATTCAAGATATTCAATGTGCAAGTTAAA 3400 3390 GAAGTCACAACGCAGCATCAGACAAAGACCATTGCAAACAATCTCACCTCAACAATTCAAGTCTTTACGGATGATGAGCATCAACTCCCGTATGTCCTGG 3489 3401 GAAGTCACGACGACGACGACGACAAAGACTATTGCTAACAACCTTACCACTCTACAATCCAGATATTCACGGATAATGAACACCAGCTGCCCTATGTTCTGG 3500 3490 GCTCGGCTACGGAAGGCACCATGCCGCGTTCCCGTCGCATGTCTATGCCCTGCCGCAGTACGGCTACTGCACAAATGCACCACCAGCAGAATGGAGCACC 3589 3590 CTTCAATGACCGTAGTGCATTCTACTGCTTAGAGTACTTCCCTAGTCAGATGCTAAGAACAGGCAACAACTT<u>TGAGTTCA</u>CATTTGACTTTGAAGAAGAT 3689 3601 ATTCAATGACAGAACTGCCTTCTATTGCTTAGAGTACTTCCCCAGTCAGATGCTCAGAACAGGGAATAATTT<u>TGAATTCAGT</u>TTTGAAGTATGAAGAAGTT 3700 MDPV 3701 CCCTTCCATAGCATGTTCGCTCATTCACAGGATTTAGACAGGCTAATGAATCCTCTCCTAGATCAGTACCTGTGGAATTTCTCTGAGGTTAATGGTGCA 3800 3790 CAAATGCTCAATTTAAAAAGGCTGTGAAAGGGGCTTATGGCACCATGGGCCGCAATTGGCTCCCAGGACCTAAATTCCTGGATCAAAGAGTTAGGGCCTA 3889 MDPV 3801 GGAATGCACAGTTCAAAAAAAGCTGTGAAAGGACCATTTGGTGCAATGGGGACAAATTGGCTTCCAGGACCGAAACTTCTAGACCAAAGGGTAAGAGCATA 3900

FIG. 1 -- Continued

conversion between the "flip" and "flop" orientation. The interconversion took place while the full-length ITR clones were propagated in *E. coli*, in spite of the fact that we used the strain SURE, which is deficient in the major recombination genes. Thus, even if we started DNA purification from a single, newly transformed colony,

we obtained a mixture of plasmids carrying the "bubble" region in both "flip" and "flop" orientation, as judged by the *SphI* restriction enzyme cleavage patterns (data not shown). Also, about 30% of the *SphI*-generated subclones contained the opposite orientation of the region compared to that of the rest of the analogous clones. In Fig.

```
MDPV 3901 CACTGGAGGAACAGATAACTATGCGAACTGGTCAATCTGGAGTAAAGGTAAAGGTTTTTCTTAAAGACAGAGAGTATCTCCTGCAACCAGGTCCAGTA 4000
    3990 TCACCTACTTACACAGAAGCOGAGGCTTCCAGCCTTCCAGCTCAAAATATTTTAGGGATAGCTAAAGATCCATACAGATCAGGCAGCACTACAGCAGGAA 4089
MDPV 4001 GCTACTACAGATACAGAAGATCAGGCTTCCAGTGTACCGGCTCAAAAGATAATAGGAATTGCAAAAGACCCCTACAGGTCAGGCAGTACTCTGGCAGGAA 4100
    4090 TAAOTGACATTATGGTCACGGAAGAACAAGAAGTAGCACCTACAAATGGAGTAGGGTGGAAACCATATGGTAGGACTGTAACGAATGAACAAAACACTAC 4189
MDPV 4101 TTTCMGACATCATGGTAACAGATGAGCAAGAAATAGCACCAACTAACGGTGTAGGGTGGAGACCTTATGGATTGACCGTAACCAAACGAACACAAC 4200
    GPV
MDPV 4201 AACAGCTCCTACAAATGCTGAGCTAGAAGTACTGGGAGCACTACCTGGCATGGTCTGGCAGAACAGAGATATTTACCTGCAGGGTCCTATATGGGCTAAA 4300
    4290 ATACCGAAGACTGATGGTAAATTCCATCCTTCTCCGAATCTEGGAGGATTTGGCCTGCACAATCCACCACCGCAGGTGTTGATCAAGAATACACCACTGC 4389
GPV
    4301 ATACCCAAAACAGATGGGAAACCCCATCCTTCTCCAAACCTGGGAGGTTTTGGTCTCCATAATCCACCTCCCCAGGTCTTTATTAAAAATACTCCTGTTC 4400
    4390 CTGCAGACCCTCCAGTAGAATAGGTGCACCAGAAGTGGAATTCCTACATAACCCAGTACTCTACGGGCCAGTGTACAGTAGAGATGGTGTGGGAGCTGAG 4489
GPV
    MDPV
    4490 AAAAGAGAATTCAAAGAGATGGAACCCAGAAATCCAGTTCACCAGTAATTTCAGTAACAGAACAAGCATAATGTTTGCACCTAATGAAACTGGTGGATAT 4589
MDPV
    4501 AAAAGAAAACTCCAAGAGATGGAACCCTGAGATCCAATTTACCAGTAACTTTGGAAATAGAACAACTACTATGTTTGCTCCAAATGAGACTGGAGGCTAT 4600
                                                  STOP
    AP-1 STOP<sub>VP</sub> . polyA [ITR<sub>p</sub>⇒ GC-box 4590 GTAGAAGATAGATTGATAGACAGATATCTAAATCTGAAATCTGTAAAT....TCTGTGTAAAAATTCAAATAAAGCACTTCCTGGCGCGCAAAATAT 4684
                                                  Ш
                                                                               Tita
MDPV 4601 GTAGAAGATAGGCTGATTGGTACCAGGTATT<u>TGACTCAG</u>AATCTG<u>TAA</u>AT<del>TTCTGTATTCTTTGAT</del>TTTA<u>AATAAACCATTTCCTGGCGCGCAAAAAGT</u> 4700
                      trs
                                        BSRep
                                                                               BS<sup>Rep</sup>
                                                    Y-box
                                                                                      E-box
GPV
    .GGGGGAAGTGACGCAAGTTCCGGT
                                                                                      ACATGCTTCCGGTGAC 4783
                                                                      ATF/CREB < ***
    4701 CCTGCTGTCCTTGAGTCTCATTGGAGGGTTCGTTCGTACCAGCCAATCAGGGGAGGGGGAAGTGACGTAAGTTCCGG
                                                                                              CGGTCAC 4800
                         BS<sup>Rep</sup>
                 ATF/CREB
                                                                                       ATF/CREB
                                                            MLTF
                                                                          MLTF
                                       CTTCCTGTCACGTGTTTCCCGTCACGTGACTTCCGGTCATG.
ATF/CREB
GPV
    4784 GCACATCCGGTGACGT.AGTTCCGGTCACGTG
                                                                              .TGACTTCCGGTGACGTGTTTCCGG 4879
MDPV 4801 GTGCTTCCGGTCATGTCACTTCCGGTCATGTCACTTCCGGTCACGTGTCTCTGTCACGTGACTTCCGGTCACGTCACTTCCATGACGTATTTCCGG 4900
                         ..... bubble region ......
                         GC-box
                                                                ATF/CREB
                                                                                MLTF
                                                                                                MLTF
    4880 CTTAACTATTGGGCTGACCGCGCATGCGCGTGGTCAACC.....TAACAGCCGGAAACACGTCACC
                                                                          .GGAAGTCACATGACCGGAAGTCACGTGA 4970
GPV
                                  GC-box
    4901 TT....GTCAAGGCTGATCGGCCCCCGATCTCCCATGAAATTAAACCGGAAATACGTCATCAAAGGAAGT.GCGTCACCGGAAGTCACGTGA 4995
                                      BSRep
                                                                              BS<sup>Rep</sup>
                                           ATF/CREB
                E-box
                                           .ACCTCACCGGATGTCCGTCACCGGAAGCATGTGACCGGAACTTCCGTCACTT
                                                                                            CCCCCTCCC 5067
                             CACGTGACCGGAACT
                                                        E-box
               ATF/CREB
                                            F. = 00 X
                                                                                  ATF/CREB
MDPV 4996 CAGGAGACACGTGACCGGAAGTCACATGACCGGAAGTCACATGACCGGAAGCACGTGACCGGAAGCACGTGACCGGAAGCACTTACGTCACTTCCCCCCTCCC 5095
                        BS<sup>Rep</sup>
    5068 CTGATTGGCTGGTTCGAACGAACGAACCCTCCAATGAGA 5106
                    ***> <****
MDPV 5096 CTGATTGGCTGGTTCGAACGAACGAACCCTCCAATGA
```

FIG. 1 - Continued

1 we show the more frequent forms of the ITRs regarding the "flip" and "flop" configurations.

#### Structure of the terminal palindromes

The sequence analysis revealed that the left and the right ends of the GPV and MDPV genomes consist of

identical inverted repeats that are 444 and 457 nucleotides in length, respectively. The distal 407 nucleotides of GPV and 418 nucleotides of MDPV can fold up to form a U-shaped double-stranded hairpin structure. The hairpins of the GPV and MDPV genomes consist of a 181- and a 186-bp stem without mismatches and a "bub-

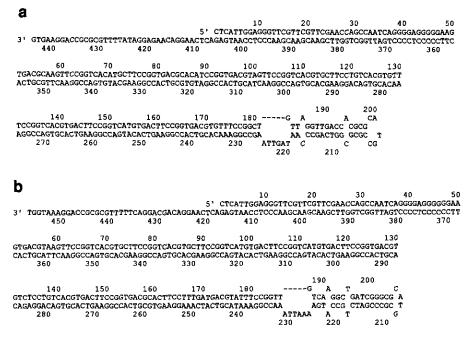


FIG. 2. Structure of the left ITRs of (a) GPV and (b) MDPV genomes. The positive strand is shown.

ble" region (Fig. 1) of 44 and 45 bp, forming an arrow-like structure as drawn in Fig. 2. The restriction endonuclease *SphI* cuts in the "arrowhead," thereby forming a quasi-symmetrical center. The shape and size of the ITRs of GPV and MDPV are reminiscent of those of human parvovirus B19 (Deiss *et al.*, 1990).

The ITR also contains numerous short repeats of 4–10 nucleotides in length. For example, the heptanucleotide "TTCCGGT" or its degenerated form is repeated 18 times in the ITR of GPV. Many other repeated motifs match the recognition sequences of known transcription factors (Fig. 1).

We identified the putative terminal resolution sites (trs) by analogy with other parvoviruses (Astell, 1990) at nucleotides 407 and 4700 of GPV and nucleotides 418 and 4716 of MDPV (Fig. 1).

### Assignment of coding domains

There are two major open reading frames (ORFs) in both the GPV and the MDPV genomes. Interestingly, the two ORFs are located in the same reading frame. A diagram of the stop codons and ATG codon in each of the three reference frames of GPV and MDPV plus-strand DNA is shown in Fig. 3. When the minus-strand DNA was analyzed, like that in all of the vertebrate parvoviruses studied, no ORFs of significant sizes were found.

By analogy with the genomic organizations reported for other parvoviruses, and also by the amino acid homologies observed for the putative proteins, we assign the left ORF of GPV and MDPV to code for the nonstructural proteins, while the right ORF codes for the three capsid proteins VP1-3 (see below).

## Analysis and comparison of the putative nonstructural proteins

We compared the amino acid sequences of the largest putative proteins coded by the left ORFs of GPV and MDPV to one another and to the nonstructural proteins of other parvoviruses. We found that the polypeptides encoded by the left ORFs of GPV and MDPV show high similarity and that the amino acid sequences share 90.6% identity. To our surprise, the closest relative of GPV and MDPV among other parvoviruses is the human parvovirus AAV-2. By using the GAP program of the GCG computer program package, we found 61.7% similarity between the left ORF encoded polypeptide of GPV and AAV-2, and the similarity is 61.8% between the same proteins of MDPV and AAV-2. Therefore, we designated the GPV and MDPV nonstructural proteins REP in analogy with the homologous proteins of AAV-2. On the basis of the homology between GPV, MDPV, and AAV-2 (Fig. 4), we assume that there are two potential translational start sites for the REP proteins. The first ATG initiates the largest putative nonstructural protein REP1 at nucleotide 537 of GPV and at nucleotide 548 of MDPV. The second ATG that initiates the putative REP2 polypeptides is located at nucleotide 1065 of GPV and at nucleotide 1076 of MDPV (Fig. 1). The left ORF is terminated with an ocher codon at nucleotide 2418 of GPV and at nucleotide 2429 of MDPV (Fig. 1). We looked for the presence of conserved protein sequence motifs of known function in the putative REP polypeptides of GPV and MDPV. We found the "P loop" ATP/GTP binding motif (GXXXXGK[TS], Saraste et al., 1990), which is also highly conserved among parvovirus and papovavirus polypeptides (Astell et al., 1987). It is worth noting that the amino acid sequence of

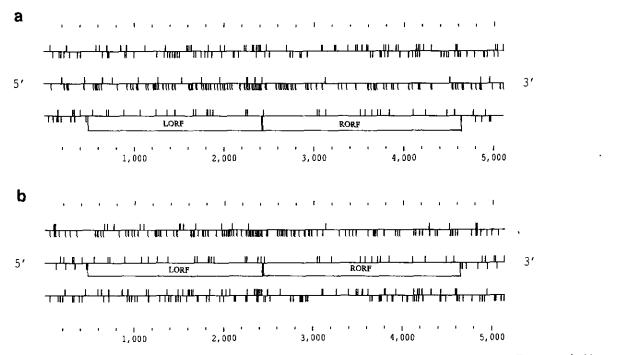


FIG. 3. Diagram of the open reading frames (ORFs) of the genomes of (a) GPV and (b) MDPV. The plus strands are shown. The top vertical bars indicate ATG codons, and the bottom bars indicate stop codons. The left ORFs (LORF) and right ORFs (RORF) are shown by open blocks.

this motif (GPATTGKT) is identical in GPV, MDPV, and AAV-2 (Fig. 4). As shown in Fig. 5, a region spanning an active-site tyrosine, which is implicated in the covalent attachment of the murine parvovirus MVM nonstructural

protein to the 5' end of the genome during replication (Cotmore et al., 1995), is conserved in GPV, in MDPV, and also in each of the vertebrate parvoviruses. A putative zinc binding motif, which was recognized in the car-

```
MDPV
       1 MAPSRPLQISSDKFYEVIIRLPSDIDQDVPGLSLNFVEWLSTGVWE..PTGIWNMEHVNLPMVTLADKIKNIFIQRWNQFNQDETDFFF.QLEEGSEYIH 97
        1 MALSRPLQISSDKFYEV1IRL#SDIDODVPGLSLNFVEWLSTGVWE..PTGIWNMEHVNLPMVTLAEKIKNIFIQRWNOFNQDETDFFF.QLEEGSEYIH
 GPV
       1 MP......GFYEIVIKVPSDLDGHLPGISDSFVMMVAEKENELPPDSDMDLNLIEQAPLTVAEKLQRDFLTEWRRVSKAPEALFFVQFEKGESYFH
AAV-2
MDPV
      98 LHCCIAQGNVRSFVLGRYMSQIKDSIIRDVYEGKQVKIPDWFSITKTKR..GGQNKTVTAAYILHYLIPKKQPELQWAFTNMPLFTAAALCLQKRQELLD 195
       98 LHCCIAQGNYRSFYLGRYMSQIKDSIIRDYYEGKQIKIPDWFAITKTKR..GGQNKTYTAAYILHYLIPKKQPELQWAFTNMPLFTAAALCIQKRQELLD 195
AAV-2
      91 MHVLVETTGVRSMVLGRIFLSQIREKLIQRIYRGIEPTLPNWFAVTKTRNGAGGGNKVVDBCYIPNYLLPKTQPELQWANTNMRQYLSACLMLTERERLVA 190
MDPV 196 ...AFQESENDA VVQBDQASTAAPLI SNRAAKNYSNLVDWLIEMGITSEKQWLTENKESYRSFQATSSNNRQVKAALENARAEMLLTKTATDYLIGKDPV 292
  GPV 196 ...AFQESDLAAPLPDPQASTVAPLISNRAAKNYSNLVDWLIEMGITSEKQWLTENRESYRSFQATSSNNRQVKAALENARAEMLLTKTATDYLIGKDPV 292
AAV-2 191 QHLTHVSQTQBQNKENQNPNSDAPVIRSKTSARYMELVGNLVDKGITSEKQWIQEDQASYISFNAASNSRSQIKAALDNAGKIMSLTKTAPDYLVGQQPV 290
 MDPV 293 LDITKNRIYOLLKINNYNPOYYGSVLCGWVKREFNKRNAIWLYGPATTGKTNIAEATAHAVPFYGCVNWTNENFPFNDCVDKMLIWWEGKMTNKVVESA 392
  GPV 293 LDITKNRVYQILKMNYNPQYIGSILCGWVKREFNKRNAIWLYGPATTGKTNIAEAIAHAVPFYGCVNWTNENFPFNDCVDKMLIWWEEGKMTNKVVESA 392
AAV-2 291 EDISSNRIYKILELNGYDPQYAASVFIGWATKEFGKRNTIWLEGPATTGKTNIAEAIAHTVPFYGCVNWTNENFPFNDCVDKMVIWWEEGKMTAKVVESA 390
MDPV 393 KAILGGSAVRVDQKCKGSVCIEPTPVIITSNTDMCMIVDGNSTTMEHRIPLEERMFQIVLSHKLEGNFGKISKKEVKEFFKWANDNLVPVVSEFKVPTNE 492
  GPV 393 KAILGGSAVRVDQKCKGSVCIEPTFVIITSNTDMCMIVDGNSTTMEHRIPLEERMFQIVLSHKLEPSFGKISKKEVKEFFKWANDNLVPVVSEFKVRTNE 492
AAV-2 391 KAILGGSÆVRVDQKCK.SSÆQIDPTPVIVTSNTÆNCÆVTDGNSTTFEHQOPLODRMFÆFÆLTRÆLDHDFGKVTKQEVKDFFÆVÆÆDHVVÆVÆHEFÆVÆKGG 490
MDPV 493 QTKLTEPVPERANEPSEPPKIWAPPTREELEEILRASPELFASVAPLPSSPDTSPARKATRGEYQVR..CAMHSLONSMNVFECLECERANFPEFQSLGE 592
  GPV 493 QTNLPEPVPERANEPREPKIWAPPTREELEELLRASPELFSSVAPIPVTPQNSPEPKRSRNNYQVR..CALHTYDNSMDVFECMECEMANFPEFQPLGE 592
AAV-2 491 AKKRPAP SDADISEPKRVRESVAQPSTSDAEASINYADRYQNKCSREVGHRIMLFPCRQCERHNONSNICFTHGGK.....DCLECFPVS..ESQPVSV 590
 B19 594
                                                                        VRGLPVCCVQHINNSGGGLGLCPBCINVGAMYNGVKFR 624
 MDPV 591 .....NFCNgHGWYD......CAFCNELKDDMMEIEHVFAIDDMENEQ 627
 GPV 591 .....NYCONHOWYD......CATCKELKNELAEIEHVFELDDAENEQ
AAV-2 583 VKKAYQKLCYTHHIMG. KVPDACTACD.LVNVD......LDDCIFEQ
  B19 625 EFTPDLVRCSCHVGASNPFSVLTCKRCAYLSGLQSF......VDYE
```

FIG. 4. Amino acid sequence alignment of REP proteins of GPV, MDPV, and AAV-2 (Srivastava et al., 1983) and the carboxyl terminus of the nonstructural protein of B19 (Shade et al., 1986). The region conserved in every vertebrate parvovirus nonstructural protein is underlined. The amino acids, which are not conserved in the four parvoviruses, are boldface and italic. The NTP binding motif is indicated bŷ. The cysteins and histidins of the putative zinc fingers are marked with \*.

MVM	193	tkka	yvkcvlfgnmiayyfltkkki	217
CPV	195	tkkd	ytkmvhfgnm <i>i</i> ay <b>y</b> flt <b>k</b> kki	219
FPV	195	tkkd	ytkmvhfgnmiayyfltkkki	219
H-1	193	tkkd	yvkcvlfgnmiayyflskkki	217
LuIII	193	tkka	yvkcvcfgnmvay <b>y</b> fltkkki	217
PPV	194	tkkd	yvkmthfgnmiayyflnkkrk	218
GPV	142	tktk	rggqnktvtaa.yilh <b>y</b> lipkkqp	168
MDPV	142	tktk	rgggnktvtaa.yilh <b>y</b> lipkkqp	168
AAV2	135	tktr	ngagggnkvvdec.yipn <b>y</b> llpktqp	163
B19	126	tkgk	yfrdgeq.fienylm.kkip	147
BPV	63	kcrk	angtlvaqaingtefitr <b>y</b> mlp <mark>knrk</mark>	92

FIG. 5. Amino acid sequence alignment of a region of parvovirus nonstructural proteins [minute virus of mice (MVMi), Astell *et al.*, 1986; canine parvovirus (CPV), Parrish *et al.*, 1988; feline panleukopenia virus (FPV), Carlson *et al.*, 1985; H-1, Rhode and Paradiso, 1983; Lulll, Diffoot *et al.*, 1993; porcine parvovirus (PPV), Vasudevacharya *et al.*, 1990; AAV-2, Srivastava *et al.*, 1983; B19, Shade *et al.*, 1986; bovine parvovirus (BPV), Chen *et al.*, 1986] implicated in the covalent attachment to the 5' end of parvovirus DNA. A conserved tyrosine is indicated by boldface letters and with \*. A highly conserved isoleucine is shown by boldface type. The lysine-rich regions flanking the conserved Tyr are boxed.

boxyl terminus of the AAV-2 REP proteins (Carter *et al.*, 1990a), can also be found in the REP polypeptides of GPV, MDPV, and B19 (Fig. 4). It is interesting that all of these viruses posses type A genomes, regarding their terminal hairpins. This amino acid sequence motif is missing from the nonstructural proteins of bovine parvovirus and the parvoviruses belonging to the rat virus-like group.

By comparing the amino acid sequences of parvovirus proteins, we found that B19 and bovine parvovirus are evolutionary closer to GPV and MDPV than they are to the rat virus-like group (data not shown).

# Analysis and comparison of the putative capsid proteins

Both GPV and MDPV virions contain three capsid proteins. Le Gall-Reculé and Jestin (1994) reported that the MDPV capsid proteins have apparent molecular masses of 91, 78, and 58 kDa as determined by SDS-PAGE. Our data are somewhat different; we observed by SDS-PAGE three MDPV capsid proteins with molecular masses of 85, 70, and 58 kDa (data not shown). We also determined the apparent molecular masses of the GPV capsid proteins, and we found that they have  $M_r$  87,000, 72,000, and 60,000 (data not shown). The analysis of the right ORF of GPV revealed two ATG codons, from which the translation of the capsid proteins VP1 and VP3 could initiate. The calculated molecular masses for VP1 and VP3 are 81.3 and 60.0 kDa. Thus, by analogy with the translation initiation of the AAV-2 capsid proteins and by the observed 72-kDa molecular mass, we presume that the translation of the capsid protein VP2 initiates from an ACG codon at nucleotide 2874. The calculated molecular mass for GPV VP2 is 65.0 kDa, which is between the  $M_r$ of VP1 and VP3. The same is true for MDPV. We found two ATGs, one for VP1 and the other for VP3, but VP2 could initiate only from an ACG triplet at nucleotide 2885. Thus, the calculated molecular masses for MDPV VP1, VP2, and VP3 are 81.3, 65.0, and 60.0 kDa, respectively. The position of the unusual VP2 initiator is conserved in the sequence of the ORF of the capsid proteins of GPV, MDPV, and AAV-2, providing further support for the hypothesis that the VP2 polypeptides of GPV and MDPV start from an ACG triplet (Fig. 6).

We compared the amino acid sequences of the VP1 polypeptides of GPV and MDPV to each other and to the capsid proteins of other parvoviruses. The VP1 polypeptides of GPV and MDPV share 87.7% amino acid sequence identity. Thus, the capsid proteins are more divergent than the nonstructural proteins, possibly due to the evolutionary pressure by the host immune systems. We found that, like in the case of the REP proteins, the VP1 capsid protein of AAV-2 is the closest relative of waterfowl parvovirus capsid proteins (AAV-2 VP1 shows 70.2% amino acid sequence similarity to GPV VP1 and 70.3% amino acid sequence similarity to MDPV VP1).

Tsao et al. (1991) have determined the three-dimensional structure of canine parvovirus (CPV) by X-ray crystallography. By using computer-aided sequence comparison and the three-dimensional structure of the CPV capsid, Chapman and Rossman (1993) have aligned the capsid protein amino acid sequences of several parvoviruses, among them that of AAV-2. By using this alignment, the probable regions of the AAV-2 capsid protein that are exposed to the surface can be predicted. The homologous regions of the VP3 polypeptides of GPV and MDPV, which are supposedly exposed to the surface of the capsid, are more divergent from each other than from the rest of the molecule (Fig. 6). The largest divergence between the GPV and MDPV capsid polypeptides is located between amino acids 162 and 178, that is, between the start codons of VP2 and VP3 (Fig. 6). We also recognized the conserved "PGY" motif (Chen et al., 1986; Dumas et al., 1992) in both GPV and MDPV VP1 at amino acid 56.

#### Potential regulatory elements

A search for TATA boxes in the GPV and MDPV genomes revealed three potential promoters designated p9, p19, and p41 (see Fig. 1). They precede the start codons of polypeptides REP1, REP2, and VP1, respectively. The p41 promoter of GPV is atypical to some extent, having the nucleotide sequence TTTTAA. We still presume that it could be functional, because its location is analogous to that of the p40 of AAV-2 (Rhode and Iversen, 1990; Carter et al., 1990a). There is a TATA box-like element starting at nucleotide 2732 in the GPV genome and at nucleotide 2743 in the MDPV genome. Whether this TATA box is functional we do not know, especially because it is located downstream of the first ATG of the right ORF.

We identified polyadenylation signals just before the right ITRs of GPV and MDPV at nucleotides 4655 and 4671, respectively. There is also a sequence matching

```
MDDV
       1 MSTFLEKFEDWYETAAASWRHLKAGAPKPKSNQQSQSVSTDRKPQRKDNNRGFVLPGYKYVGPGNGLDKGPPVNKADSVALEHDKAYDQQLKAGDNPYIK 100
 GPV
       1 MSTFLDSFEEWYETAAASWRNLKAGAPQPKPNQQSQSVSPDREPERKDNNRGFVLPGYKYLGPGNGLDKGPPVNKADSVALEHDKAYDQQLKAGDNPYIK 100
       1 MAA. DGYLPDWLEDTLSE......GIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPFNGLDKGEPVNKADAALEHDKAYDRQLDSGDNPYLK 92
AAV-2
MDPV 101 FKHADQEF1DMLQGDTSFGGNLGKAVFQAKKRILEPLGLVEEFVNTAPAKK.......SSGKLTDHD.PIVKKPKLSEEMSPSPSMSGGEASAAA 187
 GPV 101 FNHADQDFIDSLODDQSFGGNLGKAVFQAKKRILEPFGLVEDFVNTAPAKK.......NTGKLTDHY.PVVKKPKLTEEVSAGGGSSAVQDGGAT 187
AAV-2 93 YNHADAEFQERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPCKKRVVEHSPVEPDSSSGTGKAGQQPARKRLNFGGTGDADSVPDPQPLGQP. 192
MDPV 188 TEGSEPVAAPAMAEGGSGA.MGDSAGGADGVGNASGNWHCDSQWLGDTVITKTTRTWVLPSYNNHMYQAITSGTMPDSNTQYAGYSTPWGYFDFNRFHCH 286
 GFV 188 AEGTEPVAASEMAEGGGGA.MGDSSGGADGVGNASGNWHCDSQWMGMTVITKTTRTWVLPSYNNHIYKAITSGTSQDANVQYAGYSTPWGYFDFNRFHCH 286
aav-2 193 paapsglgtnima. Tgsgapmadnnegadgvgnssgnwhcdstwmgdrvittstrtwalptynnhlvkgissgsgasndvqyagystpwgyfdfnrfhch 291
MDPV 287 FSPRDWQRLINNHWGIRPKALKFKIFNVQVKEVTTQDQTKTIANNLTSTIQIFTDMEHQLPYVLGSATEGTMPPFPSDVYALPQYGYCTMHTNQSGARFN 386
 GPV 287 FSPRDWQRLINNHWGIRPKSLKFKIFNVQVKEVTTQDQTKTIANNLTSTIQVFTDDEHQLPYVLGSATEGTMPPFPSDVYALPQYGYCTMHTNQNGARFN 386
AAV-2 292 FSPRDWQRLINNMWGFRPK.RLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFWVPQYGYLTLN...NGSQAV 388
MDPV 387 DRSAFYCLEYFPSQMLRTGNNFEFSFÆFEEVPFHSMFAHSQDLDRLMNPLLDQYLWNFSEVNGGRNA.....QFKKAVKGAFGAMGRNWLPGPKLLDQR 480
 GPV 387 DRSAFYCLEYFPSQMLRTGNNFEFTFDFEEVPFHSMFAHSQDLDRLMNPLVDQYLWNFMEVDSSRNA.....QFKKAVKGAYGTMGRNWLPGPKFLDQR 480
AAV-2 389 GRSSFYCLEYFPSOMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTMTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQR 488
MDPV 481 VRAYSGGTDNYANWSIWSKGNKVFLKDREYLLQPGPVATTHTEDQASSVPAQNIIGIAKDPYRSGSTLAGISDIMVTDEQEIAPTNGVGWRPYGLTVTNE 580
 GPV 481 VRAYTGGTDNYANWNWSNGNKVNLKDROYLLOPGPVSATYTEGEASSLPAONILGIAKDPYRSGSTTAGISDIMVTEEDEVAPTNGVGWEPYGETVTNE 580
AAV-2 489 VSRTSADNAN. SEYS. WTGATKYHLMGROSLVNPGPA. .MASHRODERKFFPQGGVLIFGKQGSERTNVDIEKVMITDEREIRTINPVATEQYGSVSTNL 584
MDPV 581 QNTTTAPTNAELEVLGALPGMVWQNRDIYLQGPIWAKIPKTDGKPHPSPNLGGFGLHNPPPQVFIKNTPVPADPPLEYVMQKWNSYITQYSTGQCTVEMV 680
 GPV 581 QNTTTAPTSSDLDVLGALPGMVWQNRDIYLQGPIGAKIPKTDGKFHPSPNLGGFGLHNPPPQVFIKNTPVPADPPVEYVBQKWNSYITQYSTGQCTVEMV 680
AAV-2 585 QRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLNGGFGLKHPPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIB 684
MDPV 681 WELRKENSKRWNPEIQFTSNFGNRTSTMFAPNETGGYVEDRLIGTRYLTQNL 732
 GPV 681 WELRKENSKRWNPEIQFTSNFSNRTSIMFAPNETGGYVEDRLIGTRYLTQNL 732
AAV-2 685 WELQKENSKRWNPEIQYTSWYNKSVNVDFTVDTMGVYSEPRPIGTRYLTENL 736
```

FIG. 6. Amino acid sequence alignment of the VP proteins of GPV, MDPV, and AAV-2. The reported sequence of the capsid gene of AAV-2 was corrected according to Trempe and Carter (1988) and Ruffing et al. (1994). The first amino acids of VP2 and VP3 are indicated by arrows. The amino acids, which are different in the three-capsid proteins, are boldface and italic. The conserved motif "PGY" is marked by \*. The regions supposedly exposed to the surface of the capsid are underlined.

the consensus polyadenylation signal starting at nucleotide 1703 in the MDPV genome that is missing from the GPV chromosome. We found a potential splice donor site, which is in analogous position with that of AAV-2 (Trempe and Carter, 1988), at nucleotide 2207 of GPV and nucleotide 2218 of MDPV. Two alternative splice acceptor sites can be found at nucleotides 2423 and 2455 of GPV and nucleotides 2434 and 2466 of MDPV (Fig. 1).

We made a computer search for the recognition sequences of known transcription factors in the genomes of GPV and MDPV. These motifs for DNA binding proteins can be found at highest densities in the ITRs (see Fig. 1), implicating that they might have some role in regulating the transcription or replication of the GPV and MDPV genomes.

#### DISCUSSION

As intact virus can be rescued from a recombinant plasmid carrying a full-length clone of the MDPV nucleotide sequence shown in Fig. 1, the sequence appears to represent the biologically fully active genome (Zádori *et al.*, manuscript in preparation). The nucleotide sequence of GPV also seems complete, by comparing it to that of MDPV.

There is some heterogeneity at the termini of the ge-

nomes. We observed that in our sequence the right ITR is one nucleotide longer (GPV) or shorter (MDPV) relative to the left ITR. This heterogeneity at the end bases could be due to cloning artifacts or it may result from a flexible terminal resolution site. A 1- to 2-base heterogeneity at either end of the AAV-2 genome has been described previously (Fife et al., 1977). It has also been reported that the site where the NS1 protein of MVM cuts its substrate can be flexible in vitro (Cotmore and Tattersall, 1994). Minor products of the in vitro trs endonuclease reaction carried out by the purified REP78 (Im and Muzyczka, 1992; Owens and Carter, 1992; Chiorini et al., 1994b) and REP68 proteins (Snyder et al., 1993) can also be observed; however, these minor products may have been the result of some contaminations.

While other authors experienced difficulties in sequencing the ITRs of parvoviruses B19 and JcDNV because of GC compression (Deiss et al., 1990; Dumas et al., 1992), we found that interconversion between the "flip" and "flop" orientations can also cause a problem in obtaining correct sequence data. The observation that "flip-flop" interconversion can occur even in bacterial cells which are deficient in the major recombination genes indicates that the ITRs are strong recombination hot spots. It also warns that caution should be taken while propagating similar parvovirus DNA ends in bacteria, because false results could be obtained if one disre-

gards the possible different biological activities of the ITRs with "flip" and "flop" orientation, for example, in an in vitro replication assay. This "flip-flop" interconversion in bacterial cells is indeed artifactual, but it can take place during the replication of a double-stranded circular plasmid, thereby suggesting that "hairpin transfer" type replication is not essential for this process. Probably recombination can also produce ITRs with "flip" and "flop" orientation during parvovirus replication in the native host.

The NS1 polypeptide binds to a [ACCA]<sub>2</sub> motif 17 nucleotides apart from its endonucleotic cleavage site (Cotmore et al., 1995). The same sequence motif in the left ITR in similar positions can be found in parvovirus genomes of the members of the rat group-like parvoviruses (see the ITR sequences in Astell, 1990). The AAV-2 REP68 protein binds to a GCTC repeat motif 16 nucleotides apart from the trs (Chiorini et al., 1994a; McCarty et al., 1994). There is a similar tetranucleotide repeat motif 12 nucleotides apart from the putative trs of the GPV and MDPV genomes (Fig. 1). Taking into account the high similarity among the GPV, MDPV, and AAV-2 REP proteins and the conserved position of the tetranucleotide motifs, it can be hypothesized that the REP1 proteins of GPV and MDPV bind to this GTTC-containing repeat (BSRep, Fig. 1). An experimental test is required to substantiate this hypothesis.

By comparison to the consensus splice acceptor site, acceptor site 2 (see Fig. 1) is in a more favorable context than acceptor site 1 (Alberts *et al.*, 1989). The possible translation products of the processed mRNA spliced to acceptor site 2 are VP2 and VP3. As VP3 is the major capsid protein of GPV and MDPV, it is likely that splice acceptor site 2 is indeed preferred *in vivo*, in a similar manner as in the case of AAV-2 (Trempe and Carter, 1988).

Our search for transcription factor binding sites has revealed several interesting motifs. We found among others sequences matching E box and Y box elements, as well binding sites of transcription factors MLTF and ATF/ CREB (Anthony-Cahill et al., 1992; Mitchell and Tjian, 1989; Chodosh et al., 1986). The protein factors recognizing these elements have been implicated in the regulation of transcription or genomic replication of the parvoviruses AAV-2 (Chang et al., 1989) and MVM (Gu et al., 1995; Cotmore and Tattersall, 1994). We have obtained preliminary data that the MDPV left ITR is able to activate transcription in a Saccharomyces cerevisiae transcription assay system in vivo, indicating that the factors binding to this region are evolutionary conserved (R. Stefancsik, unpublished observations). As the E boxes, Y boxes, MLTF, and ATF/CREB recognition sequences (ie in the ITRs, there might also be cellular factors recognizing these elements in the native host of GPV and MDPV.

The high similarity between the GPV and MDPV genomes suggests that they did not diverge from their common ancestor long ago on an evolutionary time scale.

The density of nucleotide differences show peaks in the p9 and the "bubble" region. This could be due to differences in the celfular factors which regulate the life cycles of the two viruses.

Our results demonstrate that the closest relative of GPV and MDPV is the human parvovirus AAV-2. This is rather surprising, because neither GPV nor MDPV requires helper virus for effective replication. Our data suggest that AAV-2 may have evolved from an ancestral virus which replicated helper independently. The observations that in special circumstances AAV-2 can replicate without helper virus provides further support to this hypothesis (Yakobson *et al.*, 1987).

As the evolutionary divergence is smaller between AAV-2 and the waterfowl parvoviruses than between, e.g., AAV-2 and human parvovirus B19, the data presented in this paper limit the value of the hypothesis of Bando et al. (1987), who proposed that parvoviruses have evolved in a host-dependent manner. One must take into account the possibility of vertical transfer of parvoviruses between different host species when analyzing evolutionary relationships. Thus, some parvoviruses can be closer to each other on a phylogenetic tree than their respective hosts. Our protein comparison data also suggest that Astell's (1990) classification of parvoviruses based on the properties of the terminal hairpins may represent an evolutionary relationship among the viruses falling into the same category; therefore, it would be useful to incorporate these categories into the current taxonomy of parvoviruses.

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