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Evolution of the Feline-Subgroup Parvoviruses and the Control of Canine Host Range In Vivo

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A related group of parvoviruses infects members of many different carnivore families. Some of those viruses differ in host range or antigenic properties, but the true relationships are poorly understood. We examined 24 VP1/VP2 and 8 NS1 gene sequences from various parvovirus isolates to determine the phylogenetic relationships between viruses isolated from cats, dogs, Asiatic raccoon dogs, mink, raccoons, and foxes. There were about 1.3% pairwise sequence differences between the VP1/VP2 genes of viruses collected up to four decades apart. Viruses from cats, mink, foxes, and raccoons were not distinguished from each other phylogenetically, but the canine or Asiatic raccoon dog isolates formed a distinct clade. Characteristic antigenic, tissue culture host range, and other properties of the canine isolates have previously been shown to be determined by differences in the VP1/VP2 gene, and we show here that there are at least 10 nucleotide sequence differences which distinguish all canine isolates from any other virus. The VP1/VP2 gene sequences grouped roughly according to the time of virus isolation, and there were similar rates of sequence divergence among the canine isolates and those from the other species. A smaller number of differences were present in the NS1 gene sequences, but a similar phylogeny was revealed. Inoculation of mutants of a feline virus isolate into dogs showed that three or four CPV-specific differences in the VP1/VP2 gene controlled the in vivo canine host range.

Understanding the processes leading to variation and evolution of animal viruses is important, as such variation can lead to changes in antigenicity, host range, or pathogenic potential and can determine the success of viruses in new or altered hosts or environments. The evolution of many viruses has been examined by analysis of genome sequences. RNA viruses and retroviruses have highly error prone polymerases involved in replication, and the error rates are sufficiently high (10^{-3} to 10^{-5} errors per nucleotide per replication cycle) for the viruses to exist as quasispecies of related sequences (7, 16, 18). The retention of those mutations may differ widely, however, and the resulting natural evolution differs. Viruses such as retroviruses, papovaviruses, herpesviruses, and hepatitis viruses that require intimate contact between individuals for transmission also tend to be divided into geographically isolated genotypes (3, 19, 26, 30, 33, 34, 44, 46, 54, 56, 66). More readily transmitted viruses which spread efficiently, such as influenza A viruses in mammals, picornaviruses, or epidemic vesicular stomatitis virus, may become rapidly distributed over wide geographic regions and in some cases undergo more directed evolution (20, 23, 25, 32, 42, 45, 47, 50, 51, 59, 68, 69). However, those same viruses in endemic associations with their hosts (such as avian influenza viruses and vesicular stomatitis virus in endemic foci) may undergo little and apparently random variation.

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Less is known about the evolution of DNA viruses. Those viruses which replicate by using cellular DNA polymerase are assumed to have error rates similar to cellular DNA mutation rates, 10^{-8} to 10^{-9} per nucleotide per replication (49), although there may be a variation in rate that is related in a poorly understood way with the size of the viral genome (10). Other error rates may be present for viruses which encode their own DNA polymerases. The evolution of several double-stranded DNA viruses has been examined. Human papillomaviruses, polyomaviruses, or herpesviruses form persistent or reactivating-latent infections and require close contact between individuals for transmission (9, 15, 19, 24, 34, 44, 46, 49). The sequence analysis of those viruses showed isolation of virus genotypes both geographically and within different ethnic groups. The molecular clock of DNA virus evolution is poorly defined, and it has often been assumed that the virus genotypes diverged at the same times as the hosts or human population groups, and separations of viruses by thousands or millions of years have been predicted to explain the sequence diversity seen (15, 29, 34, 46, 49).

The host ranges and natural associations of viruses with particular animals are generally considered to be well defined and not subject to variation. However, occasionally geographically restricted viruses may gain access to new populations of susceptible hosts, or viruses may mutate to extend their host ranges and infect previously resistant hosts.

In this study, we examined a group of closely related parvoviruses of carnivores to define the evolution of a single-stranded DNA virus with a small genome, compare its evolution with that of similarly sized RNA viruses, and also examine the evolution of host range. Feline panleukopenia virus (FPV), mink enteritis virus (MEV), and canine parvovirus (CPV) have been classified within the feline parvovirus subgroup, among the autonomous parvoviruses (53). Viruses isolated from dogs are named CPV, while other viruses have generally been

named for their hosts of origin. FPV has been recognized for many years, but CPV emerged in dogs only around 1978 and has since become endemic in dogs throughout the world (reviewed in references 35, 37, and 52). Viruses related to CPV or FPV have been isolated from common raccoons (raccoon parvovirus [RPV]) (38), Asiatic raccoon dogs (*Nyctereutes procyonoides*) (RD), and Arctic foxes (*Alopex lagopus*) (blue fox parvovirus [BFPV]) (67), and there is serological evidence for a similar virus being widespread among red foxes in North America (8).

CPV and the other viruses differ in specific antigenic epitopes and in host range and hemagglutination properties (5, 14, 17, 35, 36, 38, 42, 60, 61). Two antigenic variants of the original CPV strain have been recognized since 1978. The newer virus strains (termed CPV type 2a and CPV type 2b) completely or partially replaced the original CPV type 2 in nature (39, 42, 48). The host ranges of FPV and CPV are complex, and there are differences in the abilities of viruses and their subtypes to replicate in vivo and in vitro. FPV replicated in cultured feline cells and in cats but not in cultured canine cells, although it did replicate to high titer in the canine thymus (61). CPV type 2 isolates replicated in cultured canine and feline cells but not in cats (61), while the CPV type 2a and CPV type 2b strains appear to replicate in cats (14, 31, 62). The feline host range of CPV type 2 was affected by sequences in more than one region of the capsid protein gene, but at least some of the mutations involved were in close proximity in the structure of the capsid (60). The ability of CPV to replicate in canine cells was shown to be determined by two or three sequence differences within the capsid protein gene (1, 5, 17, 36, 41) and a CPV-specific epitope was determined by the presence of an asparagine at VP2 residue 93 (5). Mink and raccoons were susceptible to infection by MEV and FPV but not CPV (2, 43). BFPV isolates from foxes were antigenically more closely related to FPV and MEV than to CPV, and they did not replicate in cultured canine cells (67).

Atomic structures of FPV and CPV capsids have been determined, and it was found that the viruses are assembled from a total of 60 copies of VP1 and VP2 (1, 63). Surface features of the capsid include a raised structure at the threefold axis of symmetry (threefold spike) and depressed regions surrounding the fivefold axis (canyon) and spanning the twofold axis (dimple). Mutations involved in canine or feline host range were found within the threefold spike (1, 5, 60).

In this study, we examined the evolution of CPV, FPV, and similar viruses by analysis of capsid protein and NS1 gene sequences. Phylogenetic relationships between the viruses were defined, and the rates of accumulation of mutations in these sequences were estimated. The changes were correlated with various structural regions of the capsid. Sequences which determine the canine host range in tissue culture cells were tested for their abilities to affect replication in dogs.

MATERIALS AND METHODS

DNA sequences and their analysis. A total of 24 sequences from FPV-related viruses were obtained for analysis (Table 1). The CPV VP1/VP2 sequence was also compared with minute virus of mice, pig parvovirus (PPV), H1 virus, mouse parvovirus, and LuIII virus sequences (Table 1). Published sequences were obtained from GenBank. We obtained further VP1 gene sequences for FPV-d, RD-80, RD-87, BFPV, MEV-b, MEV-d, FPV-23, and FPV-377 after cloning viral replicative-form DNA between *Eco*RI and *Pac*I sites (21 and 91 genome map units [m.u.]) into M13 or plasmid vectors. Clones of CPV-39 covering the entire genome were prepared from replicative-form DNA after blunting the 3′ end of the DNA with the Klenow fragment of DNA polymerase I and cloning with the *Eco*RI site at 21 m.u. Sequences were determined from one strand, either with [^{35}S]dATP and modified T7 DNA polymerase (Sequenase; U.S. Biochemical, Cleveland, Ohio) or by using cycle sequencing and an automated sequencer (Applied Biosystems, Foster City, Calif.). Any ambiguous nucleotides were confirmed by sequencing the complementary DNA strand. Four VP1/VP2 sequences were shorter than complete genes, and those lacked 202 (FPV-Pr, RPV, MEV-a) or 299 (FPV-377) nucleotides from their 5′ ends. The sequence of the NS1 gene of FPV-Carlson contained only the 3′ 1,118 nucleotides (nt) of that gene.

TABLE 1. Sources of DNA sequences used in the analysis reported

Virus isolate	Year of isolation	Country of origin[a]	GenBank accession no.
Minute virus of mice	NA[b]	NA	J02257
H1	NA	NA	X01457
Mouse parvovirus	NA	NA	U12469
LuIII	NA	NA	M81888
PPV	NA	NA	L23427
CPV-d	1978	U.S.	M38245
CPV-128	1979	U.S.	U22186
CPV-Norden	1978	U.S.	M19296
CPV-15	1984	U.S.	M24003
CPV-31	1983	U.S.	M24000
CPV-Y1	1982	Japan	D26079
CPV-39	1984	U.S.	M74849
CPV-133	1990	U.S.	M74852
RD-80 (CPV)	1980	Finland	U22192
RD-87 (CPV)	1987	Finland	U22193
FPV-b	1967	U.S.	M38246
FPV-d	~1964	U.S.	U22189
FPV-Carlson	1966	U.S.	M10824
FPV-193	1970	Australia	X55115
FPV-a	1962	U.K.	M24002
FPV-377	1993	Germany	U22188
MEV-a	1973	U.S.	M23999
MEV-b	1975	U.S.	M24001
MEV-Abashiri	1978	Japan	D00765
MEV-e	1965	U.S.	U22191
MEV-d	~1965	U.K.	U22190
RPV	1979	U.S.	M24005
BFPV	1983	Finland	U22185
FPV-23	1990	U.S.	U22187

[a] U.S., United States; U.K., United Kingdom.
[b] NA, not applicable.

Sequence analysis. Nucleotide (2,256 nt) and translated amino acid (742 residues) sequences of the VP1/VP2 gene were aligned by using the PileUp program in the University of Wisconsin Genetics Computer Group package. The eight FPV, MEV, or CPV complete or partial NS1 gene sequences were also aligned. There were no insertions or deletions among the feline-subgroup parvovirus sequences, and insertions or deletions between those viruses and PPV appeared to be unambiguously placed, as the same reading frame was retained.

Aligned sequences were analyzed by using the program PAUP version 3.1.1 (58). Comparison of LuIII, minute virus of mice, H1, mouse parvovirus, PPV, and CPV VP1 genes confirmed that PPV was the closest related virus (6). The 24 feline-subgroup virus VP1/VP2 gene sequences were analyzed by using the branch-and-bound algorithm to determine the most parsimonious phylogenetic relationships. Repeated analyses were performed with the complete sequences or with the first 299 bases excluded. In some cases, the phylogenies were rooted by using the PPV sequence as an outgroup. Fifteen trees of 110-nt length were generated from the 1,957-nt sequence known for all viruses, and the consensus tree was used as the basis for determining the most likely phylogenetic relationship between the sequences. A resulting tree is shown in Fig. 2A. The analysis of all sequences was repeated for 100 bootstrap repetitions in order to determine the confidence level of the branching order for the phylogeny determined.

The branch-and-bound algorithm was used to analyze the region present in all 24 VP1/VP2 amino acid sequences, and also the 8 NS1 sequences, and the most parsimonious relationships were determined in each case.

Temporal sequence variation and relationships to structure and function. To estimate the rate of accumulation of nucleotide sequence divergence, the number of differences between each sequence in the VP1/VP2 gene phylogeny and the root node, as well as the number between the root and the internal node closest to each sequence, was determined. We are uncertain of the exact dates of collection of some early isolates, although they were most likely collected within 3 years of the dates listed in Table 1. Rates of change were estimated by linear regression of the number of differences for all 24 sequences or were determined separately for the CPV- and FPV-related viruses.

Variant amino acids found within the VP2 sequences of more than one virus were located within the atomic structure of CPV (which is known for VP2

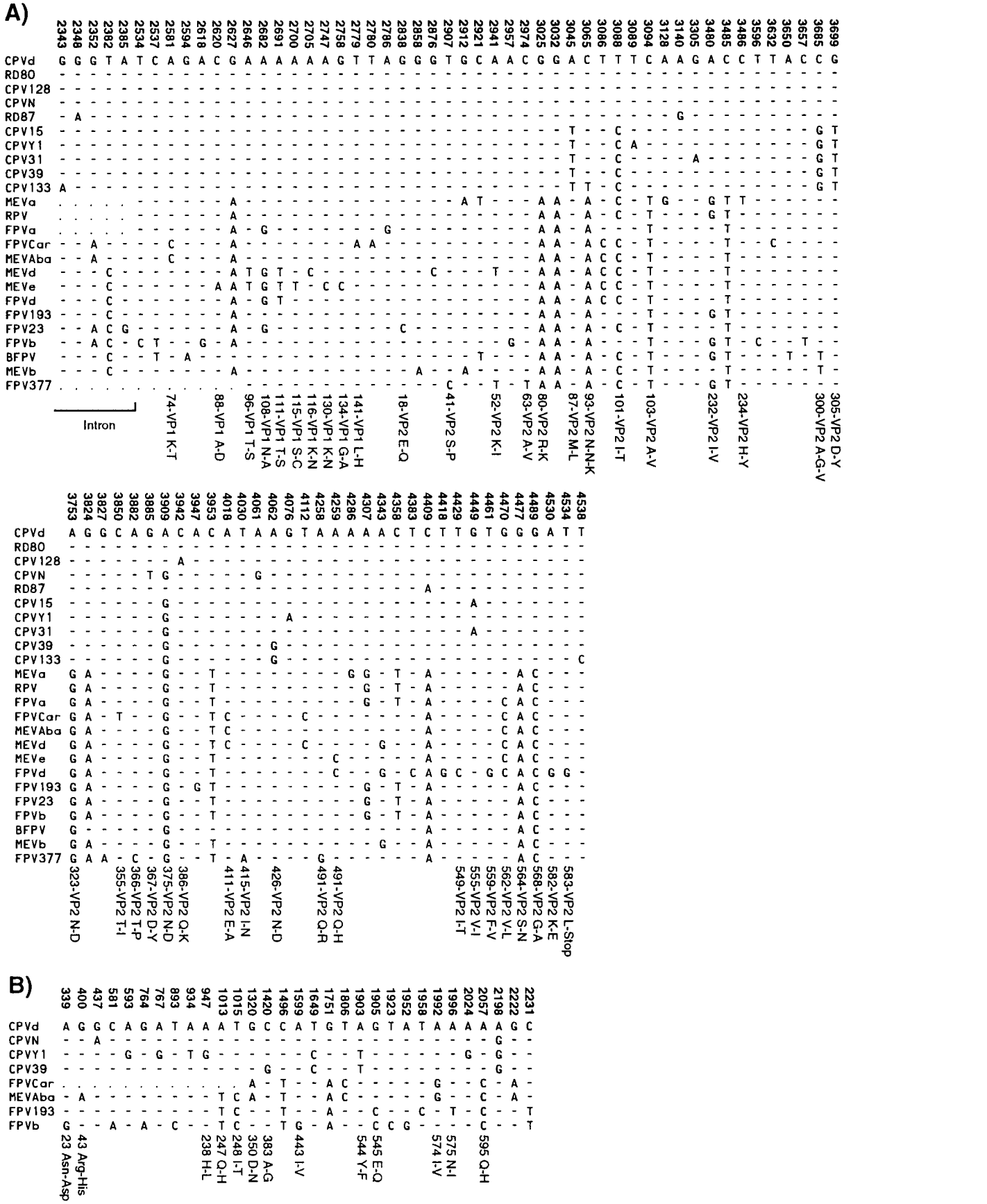
A)
CPVd
RD80
CPV128
CPVN
RD87
CPV15
CPVY1
CPV31
CPV39
CPV133
MEVa
RPV
FPVa
FPVCar
MEVAba
MEVd
MEVe
FPVd
FPV193
FPV23
FPVb
BFPV
MEVb
FPV377
Intron
74-VP1 K-T
88-VP1 A-D
96-VP1 T-S
108-VP1 N-A
111-VP1 T-S
115-VP1 S-C
116-VP1 K-N
130-VP1 K-N
134-VP1 G-A
141-VP1 L-H
18-VP2 E-Q
41-VP2 S-P
52-VP2 K-I
63-VP2 A-V
80-VP2 R-K
87-VP2 M-L
93-VP2 N-N-K
101-VP2 I-T
103-VP2 A-V
232-VP2 I-V
234-VP2 H-Y
300-VP2 A-G-V
305-VP2 D-Y
323-VP2 N-D
355-VP2 T-I
366-VP2 T-P
367-VP2 D-Y
375-VP2 N-D
386-VP2 Q-K
411-VP2 E-A
415-VP2 I-N
426-VP2 N-D
491-VP2 Q-H
491-VP2 Q-R
549-VP2 I-T
555-VP2 V-I
559-VP2 F-V
562-VP2 V-L
564-VP2 S-N
568-VP2 G-A
582-VP2 K-E
583-VP2 L-Stop
B)
43 Arg-His
23 Asn-Asp
247 Q-H
248 I-T
350 D-N
383 A-G
443 I-V
544 Y-F
545 E-Q
574 I-V
575 N-I
595 Q-H
238 H-L

FIG. 1. (A) Variable nucleotides in the sequences of the 24 VP1/VP2 genes compared in this study. Viruses are listed in Table 1. The number of each nucleotide in the complete genome sequence of CPV-d (36) is indicated, as is the coding potential of each change. Amino acids are numbered according to the VP1 or VP2 sequence, as indicated. Nucleotides which are the same as in CPV-d are indicated as dashes, while those that differ are indicated. (B) Variable nucleotides in the eight NS1 genes compared. The number of each nucleotide in the complete genome of CPV-d is indicated, while amino acids are numbered from the translated sequence of NS1. Nucleotides which are the same as in CPV-d are indicated by dashes, while those that differ are indicated.

residues 37 to 584), and the degree of exposure of each residue to the exterior or interior surface of the virus was also estimated from the probe accessibility of the amino acid as described previously (6).

Viruses, cells, and canine in vivo host range. Feline NLFK cells were grown as previously described (36). Prototype strains FPV-b and CPV-d were prepared after transfection of NLFK cells with infectious plasmid clones of those viruses (36). The preparation and in vitro analysis of two recombinant viruses (vBI319 and vBI410) have been described previously (5).

Twelve-week-old specific-pathogen-free dogs were inoculated intramuscularly with between 0.5×10^6 and 1×10^6 PFU of FPV-b, CPV-d, or the recombinant viruses. Animals were euthanized 4.5 days after inoculation, and tissue samples were snap-frozen in liquid nitrogen. Virus titers in tissues were determined in NLFK cells by an immune-staining plaque assay as previously described (41). Virus-infected cells were identified by immunohistochemical staining of tissue sections as described previously (60). Cellular DNA was prepared from the tissues and used in dot blot Southern hybridizations with a probe prepared from an internal 3,000-base region of the viral genome. PCR amplification of viral DNA was performed from total DNA extracts of selected tissues as described previously (60, 61). The DNA amplified was sequenced between 58 and 71 m.u. and between 72 and 83 m.u. after cloning into a plasmid vector.

Nucleotide sequence accession numbers. New sequences determined in this study have been deposited in GenBank under accession numbers U22185 to U22193.

RESULTS

Viruses and DNA sequence analysis. We examined viruses isolated from cats, mink, raccoons, Arctic foxes, Asiatic raccoon dogs, and dogs. Sequences included 20 complete VP1/VP2 genes and 4 that were truncated near their 5′ ends. The NS1 gene sequence of the CPV type 2b isolate (CPV-39) was also determined and compared with those of seven other viruses. The sequences which differed among the VP1/VP2 and NS1 genes of the viruses are shown in Fig. 1.

VP1/VP2 gene. (i) Nucleotide sequences. Fifteen different minimal trees of 110 nt were generated by branch-and-bound analysis of the common 1,957-nt VP1/VP2 gene sequence. A representative phylogeny is shown in Fig. 2A, and the percentages of the minimal trees with the branching order shown are indicated. Essentially the same phylogenetic relationships were revealed by analysis of the complete DNA sequences, including the first 300 nt where those were known. The parsimonious trees generated showed the same overall arrangements of the sequences, and variations were seen primarily in the arrangements of RPV, FPV-a, FPV-193, and FPV-b. As the VP1/VP2 sequences within that cluster differed from each other by between 2 and 5 phylogenetically informative nt, that variation in branching order was most likely not highly significant.

Among the total changes, there were 56 transitions, 31 transversions, and 38 synonymous and 42 nonsynonymous nucleotide sequence differences (Fig. 1A). Of the phylogenetically informative sequence differences, there were 26 transitions, 13 transversions, and 13 synonymous and 22 nonsynonymous changes within the coding region of the gene.

(ii) Amino acid sequences. The VP1/VP2 amino acid sequence analysis revealed phylogenetic relationships similar to those predicted by the VP1/VP2 nucleotide sequences (Fig. 2B). Relationships among the CPV isolates were essentially the same as those of the nucleotide sequences, as differences

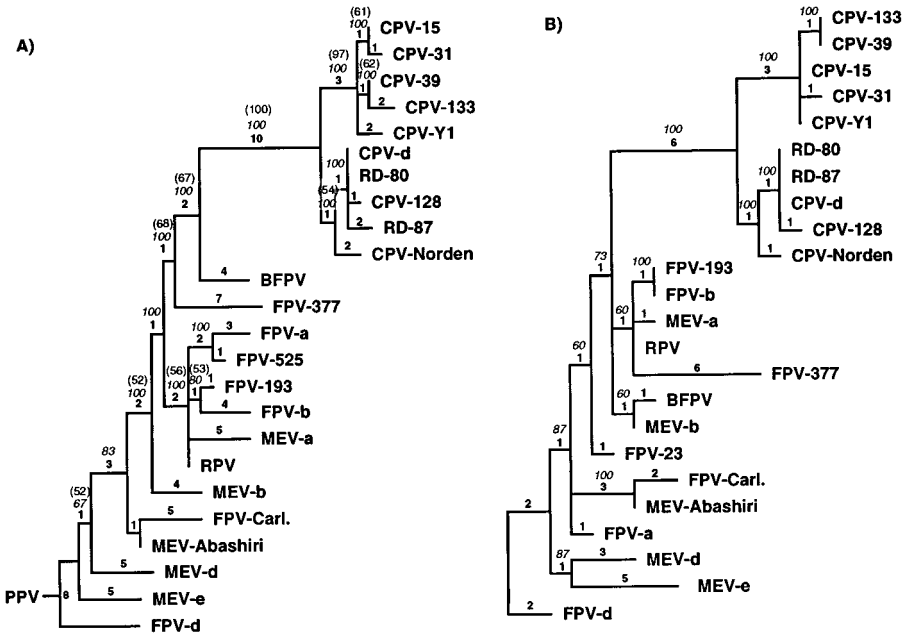

FIG. 2. (A) Phylogenies of the VP1/VP2 gene sequences of 24 isolates of carnivore parvoviruses related to FPV. A representative minimal phylogenetic tree derived from the nucleotide sequences by the branch-and-bound analysis is shown. The number of variant nucleotides in each branch of the phylogeny is indicated. The percentages of minimal trees which had the branching order shown are indicated in italics, and the support for the branching order obtained from the bootstrap analysis is indicated in parentheses where that value was >50%. This tree was rooted by using PPV as an outgroup sequence. (B) Phylogenetic tree derived from the translated amino acid sequences of the region of the VP1 gene which was present for all viruses. The number of changes in each portion of the phylogeny is indicated, and the percentages of the minimal trees which had the branching order shown are indicated in italics. FPV-carl., FPV-Carlson.

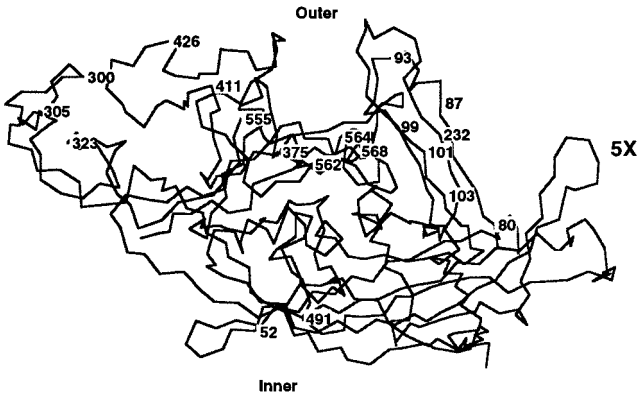

FIG. 3. Locations within the VP2 protein structure of the phylogenetically informative amino acid sequence changes. The view is from the side of the VP2 subunit, with the capsid outer and inner surfaces and the fivefold axis (5×) indicated. The structure shown consists of residues 37 to 584 of the VP2 protein.

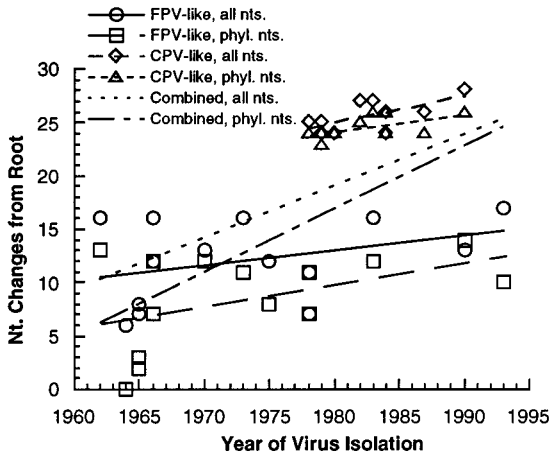

FIG. 5. Numbers of nucleotide sequence differences in the VP1 genes of the viruses versus time of virus isolation. Data for each virus include the total nucleotide sequence variation for the virus from the root node of the phylogeny shown in Fig. 2A (all nts.) or the variation to the nearest common node in the phylogeny (phyl. nts.). Linear regressions of the data are shown for all virus sequences combined or for FPV-like or CPV-like viruses separately.

within internal branches of the CPVs were mostly coding changes. Most phylogenetically informative sequences in common between BFPV or FPV-377 and CPV were silent changes, and so the amino acid sequences of those viruses were not as similar to the CPV sequence. There were minor differences in the relationships of the amino acid sequences of the viruses from cats, mink, and raccoons compared with the nucleotide sequences, but again the overall relationships were the same.

Viruses from dogs and Asiatic raccoon dogs formed a single clade (which we term CPV). The CPV isolates clearly subdivided between the CPV type 2 and CPV type 2a or CPV type 2b isolates, as previously described (39). Viruses from cats, mink, raccoons, or an Arctic fox did not subdivide according to the hosts from which they were isolated (Fig. 2). Two recently isolated viruses from Europe (BFPV and FPV-377) were most closely related to CPV in nucleotide sequence (Fig. 2A).

All of the phylogenetically informative amino acid sequence differences between these viruses which could be located in the CPV structure were on or close to the outer or inner surfaces of the virus capsid (Fig. 3).

PPV was the closest outgroup virus (Fig. 4), and roots of phylogenies obtained from analysis of nucleotide sequences were positioned by using PPV as an outgroup. The divergence of CPV from the PPV sequence (36%) was much greater than that seen among the feline-subgroup viruses (1.3%), but the root was consistently located among the earliest FPV or MEV isolates (Fig. 2A). That root was used to allow estimation of the rates of sequence substitution over the period which these viruses represent (Fig. 5). There was considerable variation in the sequences of FPV and MEV isolates, but the rates of change observed were similar between canine isolates and those from other species.

NS1 gene sequences. The single most parsimonious phylogeny of the eight NS1 gene DNA sequences analyzed showed a topology similar to that of the capsid protein genes (Fig. 6). The CPV sequences again formed a distinct clade, and FPV-Carlson and MEV-Abashiri differed from FPV-193 and FPV-b. The CPV-Carlson sequence contained only 53.5% of the NS1 gene, but inspection of the phylogenetically informative sequences available for that sequence confirms the topology shown (Fig. 1B). There were 23 transitions, 8 transversions, and 18 synonymous and 13 nonsynonymous differences among those sequences.

Correlations of functions with sequence variation. Functional differences of the capsid protein between CPV and FPV include CPV- and FPV-specific antigenic epitopes, canine host range in tissue cultured cells, and in vivo canine and feline host range. The genetic mapping of those functions has been previously described (1, 5, 17, 39, 60). The known associations with the phylogenetically informative differences in sequence are shown in Table 2.

The NS1 protein is important for DNA replication and gene regulation; it contains single-stranded DNA binding, DNA helicase, and ATPase activities, and it is also bound covalently to the 5′ end of viral DNAs (55, 70). The coding changes observed are not known to be associated with differences in

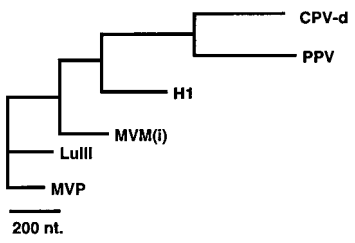

FIG. 4. Phylogeny of the VP1 genes of minute virus of mice [MVM (i)], LuIII, H1, PPV, and CPV-d, indicating the relationships between the various viruses. The single tree was derived by branch-and-bound analysis.

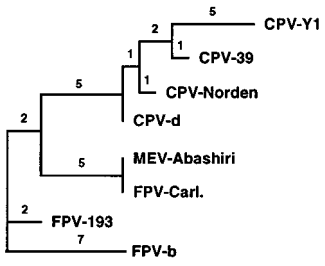

FIG. 6. The single unrooted phylogenetic tree derived from the NS1 gene sequences of eight virus isolates. All were complete gene sequences except that for FPV-Carlson (FPV-Carl.), which contained only the 1,118 3′ nt of the gene.

TABLE 2. Known associations with the phylogenetically informative coding differences in the VP1/VP2 sequences

Amino acid difference (VP2)	Comparison	Associated function
80	FPV vs CPV	Antigenicity, feline host range
87	CPV type 2 vs CPV type 2a/2b	Antigenicity
93	FPV vs CPV	Antigenicity, canine host range
101	CPV type 2 vs CPV type 2a/2b	Unknown
103	FPV vs CPV	Viability in presence of changes of residues 93 and 323
300	CPV type 2 vs CPV type 2a/2b	Antigenicity
305	CPV type 2 vs CPV type 2a/2b	Antigenicity
323	FPV vs CPV	Antigenicity, canine host range, pH of hemagglutination
375	CPV type 2 vs CPV type 2a/2b	pH of hemagglutination
426	CPV type 2/2a vs CPV type 2b	Antigenicity
564	FPV vs CPV	Feline host range (?)
568	FPV vs CPV	Feline host range (?)

functions, although the Tyr-544→Phe and Glu-545→Gly differences might affect the functions of the protein (55).

Canine host range in vivo. Both the recombinant (vBI410) and site-directed mutant (vBI319) viruses derived from FPV replicated in dogs to titers similar to those seen for CPV (Fig. 7). Replication in the ileum and mesenteric lymph nodes is a characteristic of CPV host range in dogs (61), and both recombinant viruses replicated to high titers in those tissues. Virus-infected cells were also observed after immunoperoxidase staining in the intestine (Fig. 8) and other tissues (not shown). Viral DNA was demonstrated by dot blot Southern hybridization analysis, and viral DNAs from the tissues isolated by PCR amplification were cloned and sequenced, confirming that the correct mutations were present in the virus replicating in each dog (results not shown).

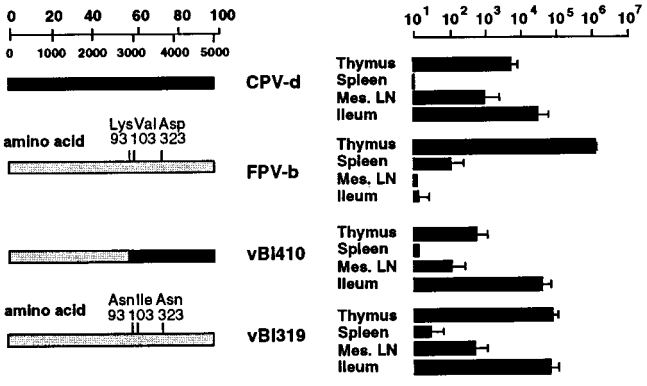

FIG. 7. Replication of CPV-d, FPV-d, and mutant viruses recombinant between those isolates or prepared by site-directed mutagenesis. The shading indicates the sources of sequences in each recombinant or prepared as a site-directed mutant (5), and the identities of residues 93, 103, and 323 (numbered in the VP2 sequence) are indicated for the mutants. Dogs were inoculated by the intramuscular route, and then tissues were assayed for viral PFU 4.5 days later. Mes. LN, mesenteric lymph nodes.

DISCUSSION

This study provides a new understanding of the sequence evolution and host range variation of these small, simple DNA viruses. Only low levels of sequence variation were observed, with 86 variant nucleotide positions being observed in the 2,256-base VP1/VP2 gene (3.8%), after examination of 53,239 bases in 24 virus sequences. The most distantly related viruses differed from each other by 1.3% in sequence. Phylogenetic analysis was complicated by the low amounts of phylogenetically informative variation within many of the sequences (Fig. 1), and only low bootstrap confidence levels were obtained for the positions of several of the older FPV and MEV isolates (Fig. 2A). However, the phylogenies obtained clearly confirmed that all CPV strains evolved from a single common ancestor and also showed that the strains most similar to CPV were BFPV and FPV-377, recent European isolates from a fox and a cat, respectively. Retrospective serological studies have suggested that CPV first emerged in dogs in Europe in the mid-1970s prior to spreading around the globe in 1978 (reviewed in references 35 and 52), and the closer association of CPV with those viruses would tend to support a Eurasian origin of the canine virus.

The nonsynonymous/synonymous difference ratio in the VP1/VP2 gene (1.1:1 for all differences and 1.7:1 for phylogenetically informative differences) was much higher than observed for many other viruses (9, 11–13, 20, 21, 45, 56, 68), indicating that there has been selection for amino acid substitutions. This finding also indicates that a molecular clock of neutral sequence variation is unlikely to be present for these genes (11). Amino acid substitutions were not randomly distributed within the VP1/VP2 gene sequence, and the localization of virtually all of the phylogenetically informative sequence differences on the outer or inner surface of the VP2 molecule (Fig. 3) was probably due to selection for functions of the capsid, such as virus infection processes, receptor binding, the formation of antigenic sites, or because those regions were structurally less constrained.

The role of residues 93, 103, and 323 in determining the canine host range in vivo was confirmed (Fig. 7 and 8), showing that the ability to replicate in the mesenteric lymph nodes or the ileum of the dog represented the same host range that has previously been defined for replication in canine cells in tissue culture (5, 17, 61). How those changes affect host range is still unclear, although it appears that in tissue cultured cells, they control the ability of the virus to undergo the changes required during the early stages of the infectious process.

The relationships of two newly identified viruses from other groups of carnivores was revealed. RD isolates collected in 1980 and 1987 in Finland were CPV type 2 isolates, while BFPV was related to the more recent FPV-like viruses (67) (Fig. 2). Sequences in BFPV which were in common with the CPV-like viruses were synonymous changes, and in the amino acid sequence phylogeny, BFPV was positioned close to MEV-b (Fig. 2B).

To look for any progressive evolution, we sought an outgroup which would identify those viruses which were closer to the ancestral sequences. The closest virus outside the feline virus subgroup was PPV. The scatter in the data shown in Fig. 5 indicates that the older FPV-like isolates were quite diverse and may have been separated for various amounts of time or in some cases were subjected to extensive tissue culture passage. However, when PPV was used as an outgroup, earlier isolates tended to be positioned closer to the root of the phylogeny (Fig. 2A). Using this root to estimate the temporal rate of variation of the sequences gave rates of 1×10^{-4} to $2 \times$

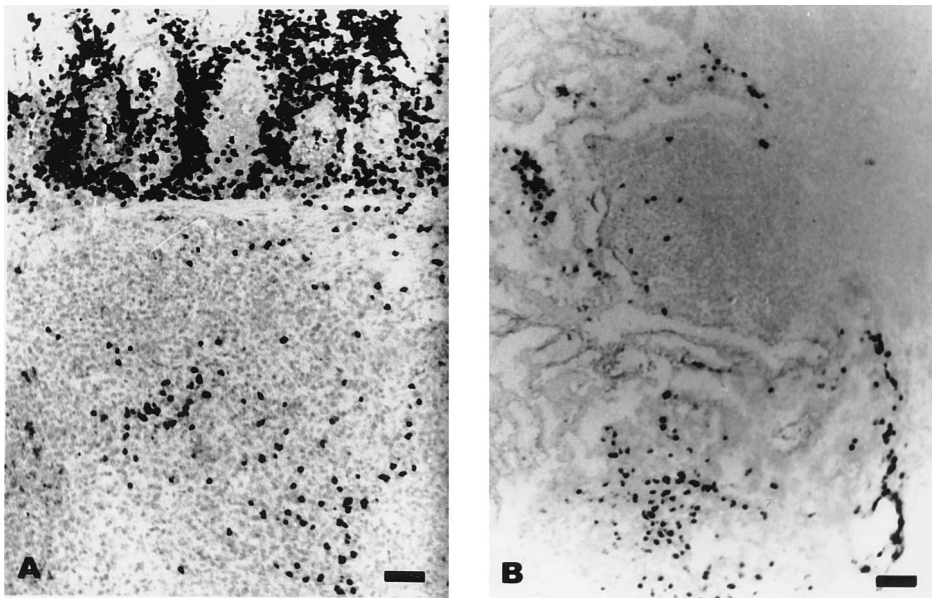

FIG. 8. Replication of mutant viruses vBI410 (A) and vBI319 (B) in the canine ileum, as shown by immunohistochemistry with an anti-CPV monoclonal antibody. Viral antigen was demonstrated in cells of the Peyer's patches and in the gut epithelium. Bars represent 100 μm.

10^{-4}/nt/year for the CPV and FPV-like viruses separately, which gave the closest fit to the data (Fig. 5). If all sequences were combined, then a rate of about 4×10^{-4}/nt/year was determined. The rates were similar whether total nucleotide sequence changes or only those changes to the nearest node to each sequence were considered. The rate previously determined for CPV on the basis of divergence over 11 years from the sequence of the well-defined CPV common ancestor was about 1.69×10^{-4}/nt/year (39), and so it appears that this rate may be similar for the newly emerged viruses as well as those that have long been in equilibrium with their hosts.

Because of the scatter in the data, those rates are clearly only approximate. They are about 10-fold lower than those observed for many RNA viruses or retroviruses (12, 18, 22, 45, 50, 59, 68, 69) but would be many orders of magnitude higher than rates estimated for other DNA viruses (15, 29, 34, 46, 49). This difference most likely reflects selection for variation, as is also suggested by the generally high nonsynonymous/synonymous and transversion/transition ratios. Although parvoviruses are replicated by host cell DNA polymerases, the rate of mutation is unknown; however, it is likely to be higher than those seen for many double-stranded DNA or persistently infecting viruses. It is possible that the spontaneous mutation rate of this virus is higher as a consequence of the small genome size, as has been reported for other organisms or bacteriophage (10). In addition, infection by these parvoviruses results in acute disease, with virus being shed in the feces (at titers of up to 10^9 50% tissue culture infective doses per g) for only 4 to 5 days (4), indicating that the virus undergoes repeated animal-animal infection. The virion is viable in the environment for weeks or months and is probably widely dispersed by transport on inanimate objects. During cell-cell spread within an animal or while in the environment, the encapsidated single-stranded DNA genome would be subject to modification such as depurination, or cytosine and adenine deamination (28), which would not be corrected during the initial stages of replication.

There was no clear distinction between viruses isolated from different parts of the globe (Fig. 2), and there appear to be few barriers to their ready spread around the world. CPV type 2 spread around the globe within a few months of emerging as an epidemic virus in 1978 (reviewed in references 35, 37, and 52). Subsequently, the different CPV antigenic types each became globally distributed within a few years of their first recognition, and CPV type 2a and CPV type 2b now infect dogs in various proportions in the United States, Europe, and Japan (31, 42, 48, 62). Examination of human B19 virus isolates by using restriction enzyme analysis or limited sequence comparisons revealed variation among isolates collected in Japan at various times during the 1980s, suggesting that there were distinct strains circulating (64, 65). It is not possible to directly compare the data for B19 with those obtained here. As B19 replicates only in bone marrow cells and is assumed to be transmitted by respiratory routes, the epidemiology of that virus in humans may be quite distinct from that seen for these carnivore parvoviruses.

Several of the viruses differ in antigenic epitopes. Antigenic sites have been mapped by analysis of escape mutants into at least two regions of the virus capsid (57), while peptide mapping indicates that there are linear epitopes in several other regions of the structure (27). Many of the variant residues were within the two major antigenic determinants on the threefold spike. Replacement of the CPV type 2 by CPV type 2a and CPV type 2b may have had a component of antigenic selection, but as those viruses also differ in host range, the selection was probably complex.

Although the viruses have been named according to the animals from which they were isolated, the phylogenetic analysis showed that the cat, mink, raccoon, and fox isolates were not readily distinguished (Fig. 2), indicating that interspecies transmission of those viruses occurs and confirming results of studies of experimental infections (2, 43). The host ranges of the viruses from dogs appear to be complex; it appears that the feline host ranges of those viruses in vivo vary and that CPV type 2 isolates do not replicate in cats, while CPV type 2a and CPV type 2b strains replicate efficiently (31, 61, 62).

These results indicate that the evolution of these DNA viruses shows many of the features described for many epidemic viruses, despite the prediction of relatively low rates of nucle-

otide substitution in the DNA genomes. Since viruses in this group show many antigenic, host range, and other functional differences, it would appear that the extremely high rates of substitution during replication of RNA viruses or retroviruses are not required for viruses to undergo rapid evolution.

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