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Rapid Antigenic-Type Replacement and DNA Sequence Evolution of Canine Parvovirus

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Analysis of canine parvovirus (CPV) isolates with a panel of monoclonal antibodies showed that after 1986, most viruses isolated from dogs in many parts of the United States differed antigenically from the viruses isolated prior to that date. The new antigenic type (designated CPV type 2b) has largely replaced the previous antigenic type (CPV type 2a) among virus isolates from the United States. This represents the second occurrence of a new antigenic type of this DNA virus since its emergence in 1978, as the original CPV type (CPV type 2) had previously been replaced between 1979 and 1981 by the CPV type 2a strain. DNA sequence comparisons showed that CPV types 2b and 2a differed by as few as two nonsynonymous (amino acid-changing) nucleotide substitutions in the VP-1 and VP-2 capsid protein genes. One mutation, resulting in an Asn-Asp difference at residue 426 in the VP-2 sequence, was shown by comparison with a neutralization-escape mutant selected with a non-CPV type 2b-reactive monoclonal antibody to determine the antigenic change. The mutation selected by that monoclonal antibody, a His-Tyr difference in VP-2 amino acid 222, was immediately adjacent to residue 426 in the three-dimensional structure of the CPV capsid. The CPV type 2b isolates are phylogenetically closely related to the CPV type 2a isolates and are probably derived from a common ancestor. Phylogenetic analysis showed a progressive evolution away from the original CPV type. This pattern of viral evolution appears most similar to that seen in some influenza A viruses.

Canine parvovirus (CPV) is a recently emerged pathogen of dogs which was first observed in 1978. The original 1978 strain is here designated CPV type 2 (CPV-2) to distinguish it from subsequent variant strains. No evidence for the existence of a CPV-like parvovirus in dogs prior to 1974 has been reported. During 1978, CPV-2 became globally distributed (for a review, see reference 36), and CPV-like viruses are now endemic in most populations of domestic and wild canids. We have previously shown that around 1979, a variant CPV strain (designated CPV type 2a [CPV-2a]) became widespread (36, 37). That strain differed from CPV-2 in that it lost at least one epitope recognized by monoclonal antibodies (MAbs) and gained a new specific epitope. By 1981, the CPV-2a strain was the virus most frequently isolated from domestic dogs with clinical disease in the United States, Japan, Denmark, and Australia, and CPV-2 was rarely seen after that time (36, 43).

Variation is seen among many different viruses, and in some cases viruses may evolve over time to give rise to new genetic or antigenic types, while among other viruses the types or subtypes are relatively stable (for examples, see references 2, 15, 20, 22, 23, 26, 35, 44, 45, and 50). The sequential evolution of new antigenic types (antigenic drift) appears uncommon but has been studied extensively as a feature of the epidemiology and evolution of influenza virus isolates (1, 11–13, 16–18, 40). Genetic variation is common among viruses, probably because of their large effective population sizes, their rapid replication, and their selection by the host. The rapid evolution of RNA viruses is reported to be due, at least in part, to the error-prone nature of their RNA-dependent RNA polymerases (11, 46).

Parvoviruses contain about 5,000 bases of single-stranded DNA and encode two structural (VP-1 and VP-2) and one or two nonstructural (NS-1 and NS-2) protein genes (7). VP-1 and VP-2 are translated from differently spliced products of the RNA transcribed from the p40 promoter, and the two proteins have most of their sequences in common, the gene being referred to here as the VP-1/VP-2 gene. The three-dimensional molecular structure of CPV has been determined by X-ray crystallography, and it shows that the capsid comprises a total of 60 copies of the VP-1 and VP-2 proteins (48). The surface features of the capsid include a prominent region surrounding the threefold axis of symmetry (the "threefold spike"), a circular canyon around the fivefold axis of symmetry, and a depression or dimple on the twofold axis of symmetry. Defined antigenic epitopes are affected by residues on the threefold spike (30, 33, 48).

In this study, we have examined many recent isolates of CPV and have shown that a further antigenic variant (designated CPV type 2b [CPV-2b]) emerged around 1984 and that the CPV-2b antigenic type subsequently replaced CPV-2a as the cause of CPV disease in many regions of the United States. The molecular basis of that antigenic variation was determined, and the molecular evolutionary trends of the CPVs were examined.

MATERIALS AND METHODS

Cells and viruses. Cells of the feline NLFK line, a derivative of the Crandell feline kidney cell line (8), were grown in a 50% mixture of McCoy's 5A and Leibovitz L15 media with

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TABLE 1. Identification numbers, sources, and origins of virus isolates

ID[a]	Accession no.[b]	ID	Accession no.	ID	Accession no.
a	780916 (IL[c])[d]	37	C84189070 (TX)[g]	105	80-1496 (WA)[e]
b	80929 (IL)[d]	38	C84173015 (TX)[g]	106	87-1154 (CA)[e]
c	800725 (WI)[d]	39	C8417607 (TX)[g]	108	A88-4/1 (TX)[d]
d	790320 (NY)[d]	40	223639-4 (PA)[f]	126	HBY9 (MI)[d]
e	A6A7 (AL)[d]	43	79668 (PA)[f]	127	AIBY9 (MI)[d]
9	790232 (IL)[d]	44	85702 (NJ)[f]	129	07960 (NJ)[d]
10	AV 29 (IL)[d]	45	85881 (NY)[f]	130	900215 (CA)[d]
12	780939 (NM)[d]	46	89458 (IL)[f]	131	900217 (IL)[d]
13	811102 (PA)[d]	47	95871 (PA)[f]	132	891335 (OH)[d]
14	MIBH (NY)[d]	48	674494 (NY)[d]	133	19680 (GA)[h]
15	840704 (IL)[d]	59	CRCK-3 (TX)[g]	159	88-273 (ID)[e]
16	78-15 (WA)[e]	60	C602373 (TX)[g]	160	88-396 (OR)[e]
17	78-16 (WA)[e]	61	85-0802 (MA)[d]	161	88-532 (AK)[e]
18	79-600 (WA)[e]	63	8002003 (KN)[f]	163	88-1910 (WA)[e]
19	79-1009 (WA)[e]	69	MA[d]	164	88-2401 (WA)[e]
20	79-1529 (OR)[e]	70	851219 (NY)[d]	165	88-3627 (OR)[e]
21	80-22 (WA)[e]	72	"Fargo" (NK)[f]	166	88-3807 (ID)[e]
22	80-139 (WA)[e]	73	741-4 (NY)[f]	167	88-4062 (ID)[e]
23	81-6323#7 (WA)[e]	74	Kansas "A" (KN)[f]	168	88-4305 (ID)[e]
24	81-6323#6 (WA)[e]	89	79-1369 (WA)[e]	169	88-4491 (ID)[e]
25	82-1315#2 (WA)[e]	90	79-1417 (WA)[e]	170	88-4589 (WA)[e]
26	82-2424 (WA)[e]	91	79-1702 (WA)[e]	171	88-5701 (WA)[e]
27	181392 (MD)[f]	92	80-1525 (WA)[e]	172	88-6037 (ID)[e]
29	179137 (CA)[f]	93	80-1541 (WA)[e]	173	90-880 (WA)[e]
30	181654 (NY)[f]	97	80-1669 (WA)[e]	174	90-3575 (WA)[e]
31	179088 (VA)[f]	98	80-1788 (WA)[e]	177	90-6301 (AK)[e]
32	179566 (NJ)[f]	100	81-8390 (WA)[e]	180	90-7034 (WA)[e]
33	177242 (CA)[f]	101	81-8934 (WA)[e]	182	90-7937 (NV)[e]
34	C84157190 (TX)[g]	102	81-9665 (OR)[e]	183	90-8747 (ID)[e]
35	C84173055 (TX)[g]	103	81-10790 (WA)[e]	184	90-9525 (WA)[e]
36	C84189105 (TX)[g]	104	81-9690 (WA)[e]		

[a] ID, identification number used in this study and in some previous studies (32, 34, 37).
[b] Original accession number from diagnostic laboratory or code number.
[c] State abbreviations: AK, Alaska; AL, Alabama; CA, California; GA, Georgia; ID, Idaho; IL, Illinois; KN, Kansas; MA, Massachusetts; MD, Maryland; MI, Michigan; NJ, New Jersey; NM, New Mexico; NV, Nevada; NY, New York; OH, Ohio; OR, Oregon; PA, Pennsylvania; TX, Texas; VA, Virginia; WA, Washington; NK, not known.
[d] Submitted to James A. Institute, New York State College of Veterinary Medicine.
[e] Submitted to Washington Animal Disease Diagnostic Laboratory.
[f] Submitted to New York State Veterinary Diagnostic Laboratory (obtained from E. J. Dubovi).
[g] Submitted to Texas Veterinary Diagnostic Laboratory (obtained from R. A. Crandell).
[h] Submitted to University of Georgia, Veterinary Diagnostic and Investigational Laboratory (obtained from A. R. Purcell).

5% fetal bovine serum. For virus propagation, cells were seeded at a density of $2 \times 10^4/cm^2$ 24 h prior to inoculation. Feline primary fetal heart lung cells were grown in Eagle's minimal essential medium with 10% fetal bovine serum.

The sources and the identification numbers of the virus isolates and the isolates' origins within the United States are listed in Table 1. Field strains of CPV were obtained from veterinary diagnostic laboratories or from canine feces or tissue samples submitted directly to our laboratories. Viruses were antigenically typed directly from clinical specimens if sufficient hemagglutination (HA) titer was present; otherwise, viruses were propagated during from one to five passages in NLFK or feline fetal heart lung cells prior to testing. Prototype strains of CPV-2 (CPV-d) and feline panleukopenia virus (FPV) (FPV-b) were isolated from infectious plasmid clones of those viruses after transfection into NLFK cells, and the viruses were then passaged two to three times to prepare stocks, as described previously (29). Antigenic types of all viruses listed in Table 1 are shown in Fig. 1.

MAb typing and analysis. A panel of 23 MAbs prepared against either CPV-b, FPV-c, or CPV-39 (32, 34, 37) was used for antigenic typing (see Table 2). Antibodies were prepared as tissue culture supernatant fluids of hybridoma cell lines grown in Dulbecco's minimal essential medium with nonessential amino acids and 20% fetal bovine serum. Antigenic typing was performed by using the HA inhibition (HI) assay, with 8 HA units of virus antigen in barbital-acetate-buffered saline (pH 6.2) and 0.5% rhesus macaque erythrocytes (29, 32). Twofold dilutions of 25 μl of the hybridoma culture supernatant were prepared and incubated with 25 μl of antigen for 1 h at room temperature, and then 50 μl of 0.5% erythrocytes was added and the plates were incubated at 4°C for at least 8 h. HI titers were read as the last antibody dilution inhibiting HA by >50%.

Escape mutant selection and analysis. A CPV-d stock from plasmid-derived virus was prepared in NLFK cell cultures and then was concentrated 40× by ultracentrifugation at 100,000 × *g* for 3 h. Virus was incubated with MAb I for 1 h at 37°C, inoculated onto NLFK cells in a 75-cm^2 flask, and cultured for two blind passages in the presence of MAb I. As the derivation of CPV from single plaques is difficult, DNA was cloned by preparing a recombinant infectious plasmid from the mutant virus DNA to ensure that a single viral sequence was analyzed. The replicative-form DNA of the MAb I-selected virus recovered by a modified Hirt extraction procedure (33) was purified by electroelution. The *Pfl*MI (nucleotide [nt] 2814, 54.5 map units [m.u.])-to-*Eco*RV (nt

	Year of Virus Isolation												
	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990
CPV-type 2	a, b, 12 16, 17	d, 9, 10 18, 19, 20 89, 90, 91	c, 21, 22 59, 60, 63	103, 104				48			165, 167		
CPV-type 2a		e	92, 93, 97 98, 105	13, 23, 24 43, 44, 45 46, 47, 100 101, 102	25, 26	14, 27, 29 30, 31, 32 33	15, 34, 35 37, 38, 40	61, 69, 70	72, 73, 74		164, 171		177, 130
CPV-type 2b							36, 39			106	108, 159, 160 161, 163, 166 168, 169, 170 172	129, 132	126, 127, 131 133, 173, 174 180, 182, 183 184

FIG. 1. Antigenic types of CPV isolates collected from 1978 through 1990 from clinically ill dogs in the United States. The numbers or letters are our catalogue designations and refer to the virus isolates described in Table 1. Viruses were typed by the panel of MAbs shown in Table 2, and each virus showed the same pattern with the panel of MAbs as the prototype virus of that type (CPV-d, CPV-15, and CPV-39).

4011, 77.6 m.u.) fragment was isolated and used to replace the same sequence from the plasmid clone of CPV-d (29). The recombinant plasmid was purified by CsCl gradient centrifugation and transfected into NLFK cells, and the antigenic type of the virus reisolated was determined by MAb typing.

DNA sequencing and analysis. Replicative-form DNA was isolated from cultures of CPV-39 and CPV-133. The 21.3-to-77.6-m.u. and 74.4-to-95.4-m.u. sequences were cloned into M13 vectors mp18 and mp19 (51) after digestion with *Eco*RI and *Eco*RV or *Hae*III. Sequences from both strands were determined by using a series of synthetic oligonucleotide primers complementary to various positions in the CPV sequences (29, 30, 41, 47) (Fig. 2B).

The *Pfl*MI-*Eco*RV region of the recombinant plasmid containing that region derived from the MAb I-selected mutant was sequenced in both directions by using some of the same primers after denaturing the plasmid with alkali (14, 47).

Phylogenetic analysis of viral sequences. Phylogenetic analysis of the complete VP-1/VP-2 sequences was performed by the method of Fitch (10). The most parsimonious tree of the sequences was obtained by comparison of the variable positions among the reported sequences of CPV-2 isolates CPV-d (29, 30), a 1978 Belgium isolate of CPV L78-778 (5, 31), and CPV-Norden (38) (isolated in 1978) (21); CPV-2a strains CPV-15 and CPV-31 (30, 37); and the CPV-39 and CPV-133 sequences (Fig. 2A). A published sequence of the VP-1/VP-2 gene of a 1978 CPV-2 isolate (39) contained a number of sequencing errors which made its analysis uncertain (30), and it was not included in this study. The changes unique to that sequence would place that virus further out on the CPV-2 branch of the phylogeny and would not alter the phylogenies of the other viruses (30). The rate of sequence variation of the VP-1/VP-2 gene was estimated after the plotting of the number of variant positions, determined from the total branch lengths measured from the root node of the CPVs.

Nucleotide sequence accession number. Sequences have been deposited in Genbank, and accession numbers are M74849 and M74852.

RESULTS

The HI titers of various antigenic types of virus with the panel of 23 MAbs are shown in Table 2, along with the reactivities of the MAb I-selected virus and an FPV isolate. This shows that CPV-2b retains both the CPV-specific and the CPV-2a-specific epitopes (recognized by MAb 7 or 14 and by MAb 1D1 or 7D6, respectively) but loses its reactiv-

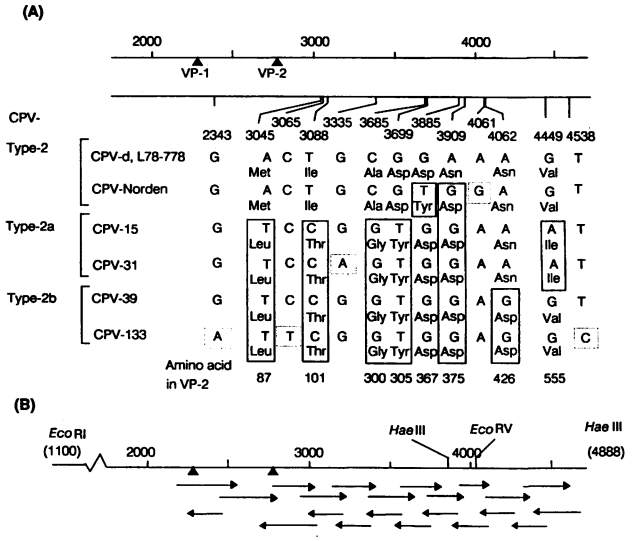

FIG. 2. (A) Variant sequences in the VP-1/VP-2 genes of CPV-2, CPV-2a, and CPV-2b isolates. Sequences of CPV-d, CPV-Norden, Belgium isolate L78-778, CPV-15, and CPV-31 have been previously reported (29–31, 38), and CPV-39 (1984 isolate) and CPV-133 (1990 isolate) were sequenced as shown here. Only positions which varied are shown. Variant positions are boxed; coding changes are boxed by a solid line. Nucleotides are from the complete CPV sequence (29); amino acids are residues in the VP-2 sequence. (B) Strategy used in the sequencing of CPV-39 and CPV-133. Clones were prepared from each virus by using the *Eco*RI, *Eco*RV, or *Hae*III site to insert DNA sequences into M13 vectors. A series of synthetic oligonucleotide primers complementary to each of the DNA strands was used for sequencing.

TABLE 2. MAb reactivities of various virus isolates as determined by HI assay

Antibody ID	HI titer[a] for:				
	CPV-d	CPV-15	CPV-39	Selected CPV-MAb I	FPV-b
7	4,096	512	2,048	4,096	2
14	256	256	512	4	2
13	256	8	64	512	8
D	4,096	32	32	4,096	4,096
E	512	64	64	256	512
J	128	2	4	256	4,096
6	512	256	512	256	512
12	256	512	1,024	256	1,024
B	64	64	<2	<2	128
I	64	128	<2	<2	128
1	4,096	4,096	4,096	4,096	4,096
8	4,096	4,096	4,096	4,096	4,096
16	512	256	512	512	1,024
15	1,024	1,024	2,048	1,024	4,096
F	4,096	4,096	4,096	4,096	4,096
1D1	<2	1,024	4,096	<2	2
7D6	2	512	512	<2	<2
2A9	256	256	512	256	32
2E12	32	32	64	4	32
3G6	128	128	512	128	256
4A12	64	64	4,096	32	512
G	4	8	8	4	512
H	2	2	<2	2	512

[a] HI titers are given as reciprocals of last antibody dilutions inhibiting HA. Numbers in boxes are titers which differ by >10-fold from those of at least one other prototype virus.

ity with MAbs B and I. All isolates showed the same antigenic types as one of the prototype viruses described in this paper, and no intermediate or alternate antigenic types were observed.

Testing 92 isolates collected during each year from 1978 through 1990 showed that the prevalence of viruses with the various antigenic types changed over time (Fig. 1). Along with the change from CPV-2 to CPV-2a between 1979 and 1981, a change to the CPV-2b antigenic type was observed among the viruses collected after 1984. The CPV-2b antigenic type was first seen among virus isolates from Texas in 1984; by 1988, CPV-2b was the common antigenic type isolated from dogs in many parts of the United States (Fig. 1). The trend for isolating particular strains of CPV in specific time intervals was evaluated using the χ^2 statistical test, with the probability of isolating a particular CPV strain in a specific period being estimated by polychotomous logistical regression analysis. The model was specified as follows: $P(\mathrm{CPV2}_i) = 1/[1 + \exp^{-\int(\beta_i\ \mathrm{Xi})}]$, where $P(\mathrm{CPV2}_i)$ is the probability of a CPV isolate being either CPV-2a or CPV-2b in a specific period (Xi) (1978 to 1980, 1981 to 1986, 1987 to 1990) and β is the change in the probability of isolating a specific CPV strain in a particular period (X). Results in Table 3 confirm the significance of the trend for strain replacement, as it was much less likely that CPV-2 would be isolated during recent years (1981 to 1990) than previously (1978 to 1980), and it was more likely that CPV-2a would be isolated during the period from 1981 to 1986 than during any other period.

Sequence differences in the VP-1/VP-2 genes of CPV-39 (isolated in 1984 in Texas) and CPV-133 (isolated in 1990 in Georgia) are shown and compared with sequences of CPV-2 and CPV-2a isolates in Fig. 2A. CPV-39 differs from the CPV-2a isolates in a minimum of two nonsynonymous nucleotide substitutions in the VP-1/VP-2 gene, while CPV-133 differs from those isolates in three further synonymous (non-amino acid-changing) nucleotide substitutions. The two CPV-2b-specific coding changes resulted in differences in amino acids 426 and 555 in the VP-2 sequence. The substitution of amino acid 555 represented a reversion to or retention of the sequence of CPV-2, and only the difference at amino acid 426 represented a replacement unique to CPV-2b.

TABLE 3. Results of polychotomous logistical regression analysis of virus strain distribution, comparing likelihood of isolating CPV-2a and CPV-2b to that of isolating CPV-2 in three periods examined

Outcome or period	Regression coefficient	SE	Probability of isolation of:		
			CPV-2	CPV-2a	CPV-2b
Outcome					
CPV-2	0				
CPV-2a	3.52	0.62			
CPV-2b	1.37	0.50			
Period					
1978–1980	0		0.80	0.17	0.03
1981–1986	−5.36	0.78	0.02	0.86	0.12
1987–1990	−2.56	0.62	0.07	0.13	0.79

An antigenic variant of CPV-d was selected with MAb I, and the *Pfl*MI-*Eco*RV region recovered from the replicative-form DNA of that virus was used to replace the homologous sequence in the CPV-d plasmid clone. The virus reisolated did not react with MAbs B and I (which did not react with CPV-2b strains), and it had also lost reactivity to CPV-specific MAb 14 (Table 2). The only sequence difference in the MAb-selected virus was a C-T substitution at nt 3450 in the complete CPV sequence (29), giving a His-Tyr substitution at VP-2 amino acid 222.

Examination of the molecular structure of CPV-2 showed that most (six of eight) of the amino acid differences among CPV-2, CPV-2a, and CPV-2b sequences are exposed on the surface of the capsid (48) (Fig. 3). The surface structure of the virus at the threefold axis of symmetry is shown in Fig. 3A and B and indicates that the variable positions are located in three regions on the surface of the virus capsid. Figure 3C shows that residue 87 of one VP-2 subunit is close (0.8 to 1.0 nm) to residues 300 and 305 of the neighboring VP-2 molecule and that those differences may therefore all affect one epitope. Figures 3B and D show that residue 426 is located at the top of the threefold spike, the three symmetrically arranged 426 residues being only 2.5 nm apart in the structure. Residue 375 is located on the side of the threefold spike opposite residues 87, 300, and 305 (Fig. 3C).

The phylogenetic relationships among the three CPV-2, two CPV-2a, and two CPV-2b VP-1/VP-2 sequences from Fig. 2A are shown as a "one-trunk" tree in Fig. 4. The root of the tree was derived from the common node of the FPV–mink enteritis virus (MEV)–raccoon parvovirus (RPV) sequences, as previously determined (30). The phylogeny predicts three ancestral viruses (designated A, B, and C in Fig. 4), each of which differs by only one or two nucleotides from the viral sequences determined.

The CPV phylogeny has a total branch length of 13 nucleotide changes among the seven viruses, the most distantly related viruses (CPV-Norden and CPV-133) dif-

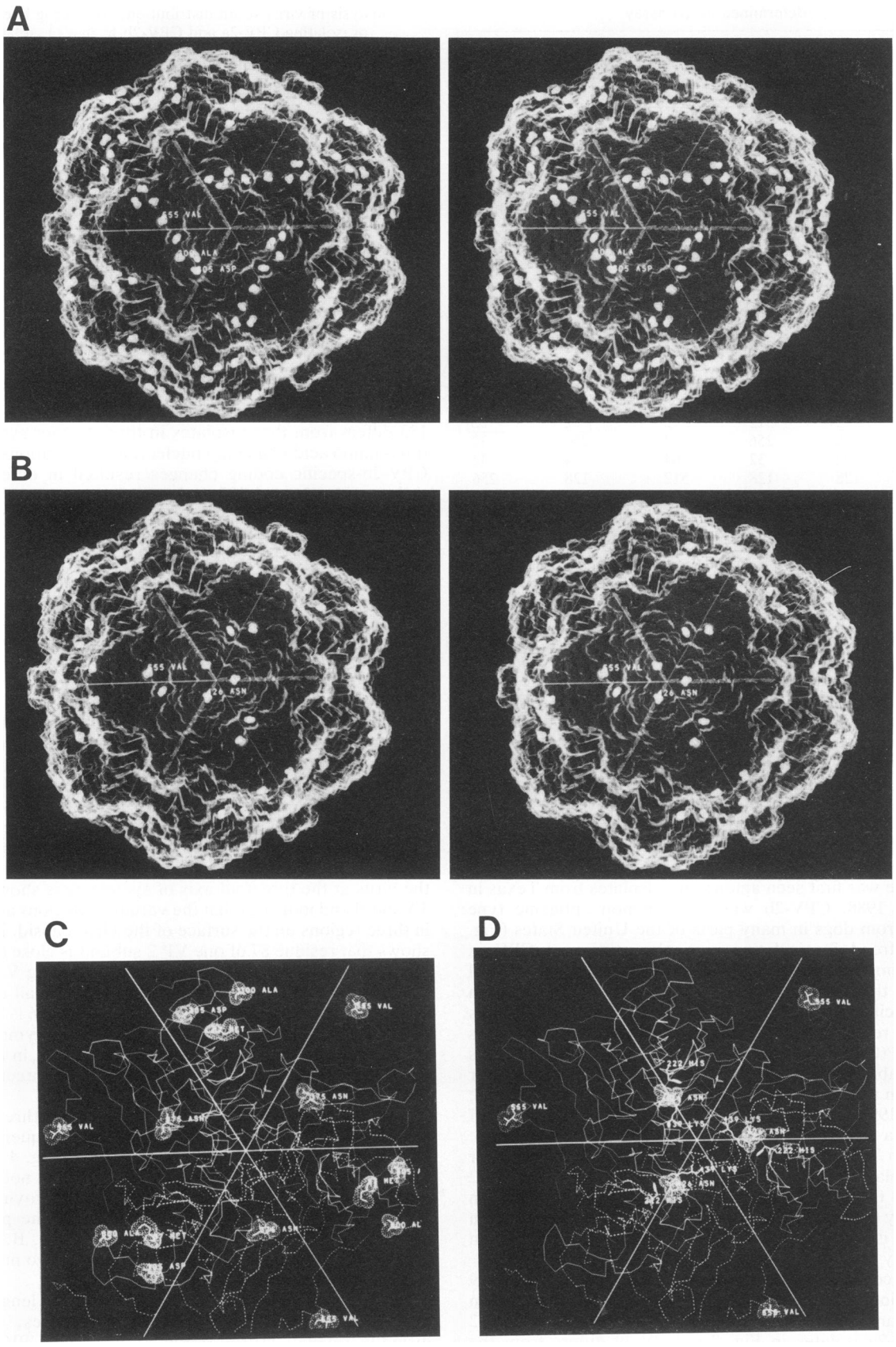
A
B
C
D

FIG. 3. (A) Stereo view of the CPV-2 capsid shown by grid-mesh surfacing, indicating differences between CPV-2 and CPV-2a which are exposed on the surface of the particles. The view is along the threefold axis. The triangular outline depicts the theoretical crystallographic asymmetric unit. Variant residues are represented by dotted spheres (van der Waals radius of each atom). Residues 87 (Met-Leu), 300 (Ala-Gly), and 305 (Gly-Thr) are located on the flanks of the threefold spike, while residue 555 (Val-Ile) is within the dimple, adjacent to the other three changes (see the closer view [C]). (B) Same structure as in panel A but showing the location of residues which differed between CPV-2a and CPV-2b strains. Residue 426 (Asn-Asp) is on the peak of the threefold spike. Residue 555 (Ile-Val) reverts to the CPV-2 sequence (see closer view [D]). (C) View of the differences between CPV-2 and CPV-2a, viewed down the threefold axis. The C-alpha tracings of the threefold related proteins are shown as solid, dotted, and dashed lines, with the altered residues represented by dotted spheres (van der Waals radius of each atom). Residue 375 (Asn-Asp) is not exposed directly on the surface, but its mutation affects the pH dependence of HA. The residue is located underneath residues 320 and 321 of a threefold related protein. (D) Similar to panel C but showing the position of residue 426 (Asn-Asp) (van der Waals radius of the atoms), which differs between CPV-2a and CPV-2b. Adjacent residues 222 (His, mutated to Tyr in the MAb I escape mutant virus) and 439 (Lys) are shown as line tracings.

fering by 10 nucleotides in the VP-1/VP-2 gene (0.44%). All six nucleotide changes which separate the distinct antigenic types of virus within the phylogeny were nonsynonymous changes, while only two of seven of the differences among viruses of the same antigenic type were nonsynonymous differences. Plotting the number of changes against time leads to an estimate of the rate of retained nucleotide sequence substitution of 0.382 (standard error = 0.064) nt per year in the VP-1/VP-2 gene over the 12 years sampled, equivalent to 1.69×10^{-4}/nt/year (Fig. 5).

DISCUSSION

We demonstrated here the continuing evolution of the antigenic type of CPV. After 1983, an antigenically variant virus which differed from both CPV-2 and CPV-2a emerged, and we designate that new antigenic type CPV-2b to distinguish it from the previous types. By 1988, CPV-2b was the predominant virus type (Fig. 1). CPV-2b was characterized by the loss of a neutralizing epitope recognized by two MAbs (Table 2), and it also differed from CPV-2a in two coding sequence changes in the VP-1/VP-2 genes. Although many of the isolates were from Washington state, the same variation occurred in other distant areas of the United States (Table 1), and we have also observed the CPV-2b antigenic type for a CPV isolate collected in France in 1984 (28). Analysis of the data in Fig. 1 indicated that there was a high probability that this represents antigenic drift with strain replacement and was not an artifact of the sampling of variants (Table 3).

The successive replacement of CPV-2 by CPV-2a and then CPV-2b was likely due to selection to escape canine immunity, as the changes in neutralizing epitopes were the markers used to define the strains. However, we cannot rule out the possibility that some of the advantage of the new virus strains is due to factors other than immune selection. We have shown that surface residues in the VP-1/VP-2 proteins are important in determining the host ranges of these viruses (29, 30, 33), and it is possible that some of the surface changes also represent a further adaptation of CPV for optimal replication in and spread between dogs. For example, VP-2 residues 87, 300, and 305, which differ between CPV-2 and the later virus types, are all very close in the

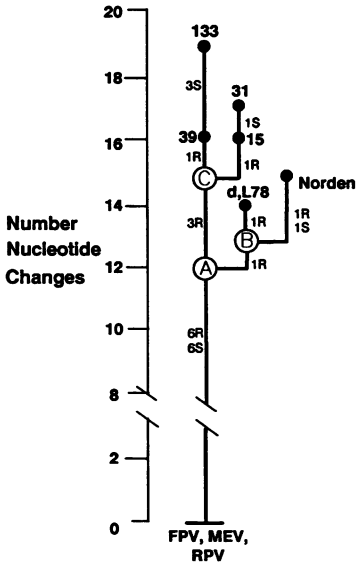

FIG. 4. One-trunk phylogenetic tree of the CPVs, derived from the sequences of the VP-1/VP-2 genes shown in Fig. 2A. The most parsimonious tree was calculated by the method of Fitch (10). Viruses are indicated by their isolate numbers (see Table 1) except for the Norden isolate (38) and the L78-778 isolate from Belgium (L78). The basal node of the CPV isolates was derived from the FPV, MEV, and RPV sequences, as previously reported (30). Branch lengths are proportional to the number of changes, and the number and type of sequence changes along each branch of the tree are indicated (R, replacement change; S, silent change). A, B, and C indicate hypothetical ancestral viruses that have sequences in common with two or more of the viruses.

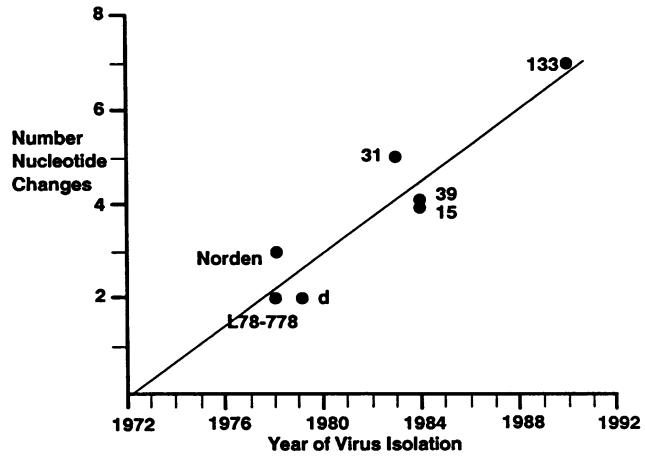

FIG. 5. Plot of the number of nucleotide substitutions in the VP-1/VP-2 gene versus time of isolation of various CPV isolates. The number of changes was calculated from the hypothetical common ancestor of the CPVs (virus A in Fig. 4). The linear regression was calculated as the best fit to the data.

structure and may affect a single epitope. The new CPV-2a- and CPV-2b-specific epitope is also most likely determined by one or more of those changes, as VP-2 residue 101 was not exposed on the surface of the capsid and the other two changes either were present in FPV (residue 375) (30) or were not present in CPV-2b (residue 555). The epitope which is affected by those changes is also affected in a mutant virus (CPV 102/10) (33). The mutations in that case were Ala-to-Asp and Thr-to-Ile changes of amino acids 300 and 301, respectively, and also resulted in the virus losing the host range for canine cells. The sequence difference at residue 375 in CPV-2a and CPV-2b was a reversion to or retention of an FPV sequence (30). That residue, along with a change in residue 323 in FPV, affects the pH dependence and also possibly the temperature dependence of HA of the viruses (29, 43). The CPV-2b epitope altered by the sequence at residue 426 is close in the structure to residue 93, which is clearly involved in determining a CPV-specific epitope and the canine host range of CPV (6, 29, 30). Indeed, the MAb I-selected virus isolated here lost reactivity with CPV-specific MAb 14 in addition to MAbs A and I (Table 2).

DNA sequence analysis of CPV-39 and CPV-133 showed that the CPV-2b strains differed by only two amino acids in their VP-1/VP-2 genes from the CPV-2a strains, which differ in only five or six amino acids from CPV-2 isolates (Fig. 2A). That the Asn-Asp change at amino acid 426 determined the altered epitope recognized by MAbs B and I was shown by comparison with the escape mutant derived with MAb I. That virus showed a His-Tyr change at amino acid 222 in the VP-2 sequence, immediately adjacent to residue 426 in the structure of the virus (Fig. 3D). As the only other CPV-2b-specific change (residue 555) was a reversion to the CPV-2 sequence and was 4.8 to 5.2 nm distant from the residue 426 molecules in the threefold spike, it is unlikely to be involved in the altered epitope.

The evolutionary relationships among the virus strains revealed by phylogenetic analysis emphasizes the likely origin of CPV from one member of the FPV–MEV–RPV group (30) and shows that subsequent evolution is away from that virus group (Fig. 4). Although only seven virus sequences were analyzed here, the phylogeny suggests that CPV-2 and CPV-2a occupy evolutionary side branches which have become uncommon and may be becoming extinct. Whether the CPV-2b isolates are on the main trunk of the tree or are simply the currently dominant side branch will be revealed only by future studies. It is likely that these VP-1/VP-2 sequences are being positively selected, since the six differences on the trunk and main branches of the phylogeny are nonsynonymous changes, five of which are either surface exposed or within one residue of the surface, while most sequence differences among viruses of the same antigenic type are synonymous. The type of variation defined here for the CPV isolates appears similar to the antigenic and genetic variations of influenza A virus in humans and in horses (1, 11, 17), where a progressive evolution of viral strains over time is apparently selected by immune pressure from the host (11).

Assuming a constant rate of change, extrapolation from the known sequences indicates that the common ancestor of CPV-2 and CPV-2a (virus A in Fig. 4) was most likely present during the early 1970s (Fig. 5). The first reported evidence of CPV antibodies was from canine sera collected in 1974 in Greece (19) and in 1976 in the Netherlands (42). Analysis of further sequences is in progress to more precisely define the ancestry of the CPVs and their likely origins from the FPV–MEV–RPV group.

Variation of other parvoviruses has not been extensively studied. Little antigenic variation among FPVs has been described (24, 32). Antigenic variation of one or more epitopes has been described for MEV isolates from the United States and Scandinavia (32, 35), although those different antigenic types appeared to coexist in the mink populations. Genetic variation of the human B19 parvovirus has been revealed by restriction enzyme mapping of viral DNA. However, no consistent patterns of variation were seen in viruses from Europe and the United Kingdom over a period of 10 years (25). Viruses from two outbreaks in Japan were shown to be caused by distinct strains (49), although the significance of that variation was not defined.

Virus variations and evolution have been revealed in studies of many different viruses. These variations include a variation of sequences from point sources during epidemic spread, variations within endemic virus populations (1, 9, 13, 15, 20, 25, 44, 47), and evolution with sequential replacement of strains by new antigenic types over time (3, 16). The variation rate of the CPV VP-1/VP-2 gene over the 12 years after its first isolation ($\sim 1.69 \times 10^{-4}$ /nt/year) is 10- to 100-fold lower than has been reported for the influenza virus HA gene (11, 13, 17, 40), for human or simian immunodeficiency virus sequences (2, 15, 44, 50), or for recently emerged picornaviruses (22, 23), although it is perhaps similar to variation rates described for an alphavirus epidemic (4). This rate may be comparable to, or higher than, those observed for baculoviruses (9) or hepadnaviruses (27). The mutation rate of CPV has not been reported, but since parvovirus DNA is replicated by host cell DNA polymerases (7), which have low error rates, it appears that a high polymerase error rate is not required for this type of virus variation and antigenic drift.

This observation of rapid antigenic replacement and sequence evolution is unusual among DNA viruses. In future studies we will more closely define (i) the phylogenetic relationships among the viruses, (ii) their rates of change, and (iii) their possible ancestors among other carnivore parvoviruses in order to reveal the mechanisms by which these viruses vary and the selective pressures which resulted in their gaining the host range for dogs during the 1970s.

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