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Multiple Amino Acids in the Capsid Structure of Canine Parvovirus Coordinately Determine the Canine Host Range and Specific Antigenic and Hemagglutination Properties

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Canine parvovirus (CPV) and feline panleukopenia virus (FPV) are over 98% similar in DNA sequence but have specific host range, antigenic, and hemagglutination (HA) properties which were located within the capsid protein gene. In vitro mutagenesis and recombination were used to prepare 16 different recombinant genomic clones, and viruses derived from those clones were analyzed for their in vitro host range, antigenic, and HA properties. The region of CPV from 59 to 91 map units determined the ability to replicate in canine cells. A complex series of interactions was observed among the individual sequence differences between 59 and 73 map units. The canine host range required that VP2 amino acids (aa) 93 and 323 both be the CPV sequence, and those two CPV sequences introduced alone into FPV greatly increased viral replication in canine cells. Changing any one of aa 93, 103, or 323 of CPV to the FPV sequence either greatly decreased replication in canine cells or resulted in an inviable plasmid. The Asn-Lys difference of aa 93 alone was responsible for the CPV-specific epitope recognized by monoclonal antibodies. An FPV-specific epitope was affected by aa 323. Amino acids 323 and 375 together determined the pH dependence of HA. Amino acids involved in the various specific properties were all around the threefold spikes of the viral particle.

Canine parvovirus (CPV) and feline panleukopenia virus (FPV) are classified as host range variants among the feline parvoviruses of the genus *Parvovirus* in the family *Parvoviridae* (49). CPV was first recognized in a number of countries during 1978 (3, 23; reviewed in references 10, 31, 41, and 48). Retrospective serological studies indicated that CPV was a new infectious agent of canids, the first serological evidence of canine infection by CPV being from sera collected in Europe around 1974 or 1976 (reviewed in references 10, 31, 41, and 48). Diseases associated with FPV, mink enteritis virus (MEV), or raccoon parvovirus (RPV) in cats, minks, or raccoons, respectively, have been known for many years.

CPV is closely related to FPV, RPV, and MEV. Those viruses are all >98% similar in DNA sequence within the capsid protein gene (9, 27, 32, 33, 42, 43), and studies with monoclonal antibodies (MAbs) indicate that the viruses are very similar antigenically (36, 38, 56). However, there is a specific epitope on CPV which is not present on the other viruses, and there are also epitopes which can distinguish FPV and MEV isolates from CPV (36, 38, 56). In addition, the CPV isolates differ from FPV and MEV in their pH dependence of hemagglutination (HA), as the CPV isolates hemagglutinate at all pH values between 6.0 and 8.0, while FPV isolates hemagglutinate only below about pH 6.8 (11, 29, 33). The host ranges of CPV and FPV are complex, and there are differences in the results obtained from in vivo versus in vitro analysis (52). CPV isolates replicate in dogs and in cultured canine cells as well as in many cultured feline cells. FPV isolates replicate systemically in cats and in cultured feline cells but not in cultured canine cells (3, 10, 33, 52). However, CPV does not appear to replicate in cats after parenteral inoculation, while FPV replicates in vivo in the canine thymus and possibly bone marrow but not in other tissues (52).

Studies of recombinants between CPV and FPV indicate that sequence differences which affect the canine host range and antigenic type of CPV are located within the VP1 and VP2 genes, between 59 and 73 map units (m.u.) (32, 33, 37). There are eight coding nucleotide differences in the 59 to 100 m.u. region between the prototype strains CPV-d and FPV-b (36, 39). Five of those are phylogenetically conserved among CPV isolates and differ in the sequences of the viruses from other species (32, 33). Earlier analysis of CPV/FPV recombinants indicated that the pH dependence of HA was determined by the region between 59 to 80 m.u. and that it was specifically affected by VP2 residue 375 (32, 33).

Antigenic drift with strain replacement has been described among recent isolates of CPV, with two or three epitopes varying between virus strains (34, 40). However, none of the CPV-specific sequences within the 59 to 73 m.u. region changed during that variation.

The atomic structures of CPV full and empty particles and of FPV empty particles have been solved by X-ray crystallography (1, 53, 54), and the results show that the capsids are assembled from 60 copies of a combination of the VP1 and VP2 proteins. The VP2 molecule contains an eight-stranded β barrel with large elaborated loops between the β strands. Surface features include a cylindrical structure surrounded by a 15-Å (1 Å = 0.1 nm) deep canyon on the fivefold axis of symmetry, a prominent 22-Å high spike at the threefold axis, and a 15-Å deep dimple at the twofold axis (53).

Other studies of parvovirus host range include those of CPV-102/10, an antigenic and host range mutant derived by in vitro passage of CPV in feline cells; in those studies, the differences resulted from sequence changes in the VP2 gene (37). A role for the VP1 or VP2 gene in controlling viral host range has also been described for minute virus of mice

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(MVM), for which the tissue tropism was determined by as few as two sequence differences in VP1 or VP2 (2, 4, 5, 18). Those differences determined the ability of MVM to replicate in fibroblastic cells. That tissue tropism of MVM was due to a block at an early stage of viral infection (5, 50, 51). Studies of in vivo tissue tropism of MVM confirmed that the allotropic determinants for each tissue tropism were located in the viral capsid gene (8). Expression of the viral nonstructural protein NS2 was also found to be essential for the lymphotropic phenotype of MVM (8). Studies on the in vitro host range of porcine parvovirus (PPV) showed that the host specificity was determined intracellularly (30, 44). A PPV mutant which was adapted to canine A72 cells had changes within both the nonstructural protein and capsid protein gene regions which were involved in host range determination (55).

In this study, we used site-directed mutagenesis and recombination mapping of the infectious plasmid clones of CPV and FPV to define the precise roles of each of the specific sequences within the 59 to 73 m.u. region in the determination of in vitro canine host range, the pH dependence of HA, and the CPV- and FPV-specific epitopes of these viruses.

(Some of these data have been presented in abstract form at the Keystone Meeting on the Cell Biology of Virus Entry, Replication and Pathogenesis [39].)

MATERIALS AND METHODS

Cells and viruses. NLFK feline kidney-derived cells (14) and A72 canine fibroma-derived cells (7) were grown in a mixture of 50% McCoy's 5A and 50% Leibovitz L15 media with 5% fetal bovine serum. The A72 cells were used at passage levels between 145 and 160.

CPV-d (CPV type 2) and FPV-b were derived from the infectious plasmid clones of those viruses (pBI265 and pBI292, respectively [32]) after transfection of NLFK cells.

Mutagenesis and recombination. To simplify the genetic analysis, a clone that contained the *Hin*dIII-*Hae*III sequence (m.u. 49 to 75) of the FPV-a isolate (mBI100 in the M13 mp18 vector) (33) was used for mutagenesis. That FPV-a sequence contained only three coding (nucleotides [nt] 3065, 3094, and 3753) differences and one noncoding (nt 3485) difference from the CPV-d sequence within the 59 to 73 m.u. (*Pst*I-*Pvu*II) region analyzed here (33) (Fig. 1A). That clone was mutagenized as depicted in Fig. 1B, using the method described by Kunkel (24). M13 single-stranded DNA substituted with uracil by growth in *Escherichia coli* CJ236 (*dut-1 ung-1*) was used as a template for synthesis of a mutant strand by priming with a synthetic oligonucleotide, and then mutants were selected by growth in the wild-type MV1190 strain.

After recloning into plasmids, various sequence combinations were prepared by recombination at an *Spe*I site (nt 3459) (Fig. 1B and 2). Each sequence was used to reconstruct the infectious virus genome by substitution of the *Pst*I-*Pvu*II fragments (59 to 73 m.u.) into the infectious clones of CPV or FPV (Fig. 2 and 3). Recombinant plasmids were designated pBI, while the virus derived from each plasmid was referred to as vBI. All plasmids were confirmed as being correctly constructed by various digestions with *Hin*dIII, *Hin*fI, *Hin*cII, *Pst*I, *Pvu*II, or *Sty*I followed by agarose gel electrophoresis. The *Pst*I-to-*Pvu*II regions of all clones were sequenced from one strand by the dideoxy method, using Sequenase (United States Biochemical Corp.) (20, 46) with a series of oligonucleotide primers complementary to various VP1 and VP2 gene sequences (32, 33). Characteristic restriction enzyme sites which differed between the recombinants were also examined in some cases in the replicative-form (RF) DNAs recovered from infected cells. The changed nucleotides in viruses vBI280, vBI288, and vBI282 (Fig. 3) were further confirmed by direct sequencing of the polymerase chain reaction (PCR) products derived from viral DNA. Templates were generated by asymmetric PCR, using two primers (CGTTTGAGTT CGTCTG [58.6 m.u.] and CGCAGATGTGTTCCCG [75.5 m.u.]) in a 1:100 molar ratio and using virus in 1 μl of unpurified tissue culture supernatant as the template for PCR. PCR was run for a total 30 cycles, and excess primers were removed by washing in an Amicon concentration system 30 filter. Recombinant pBI410 was prepared from pBI319 (Fig. 3) by replacing the *Spe*I-*Pac*I region (68 to 91 m.u.) with the sequence from CPV-d (pBI265) (Fig. 3), making a genome with the 59 to 91 m.u. region from CPV in an FPV background.

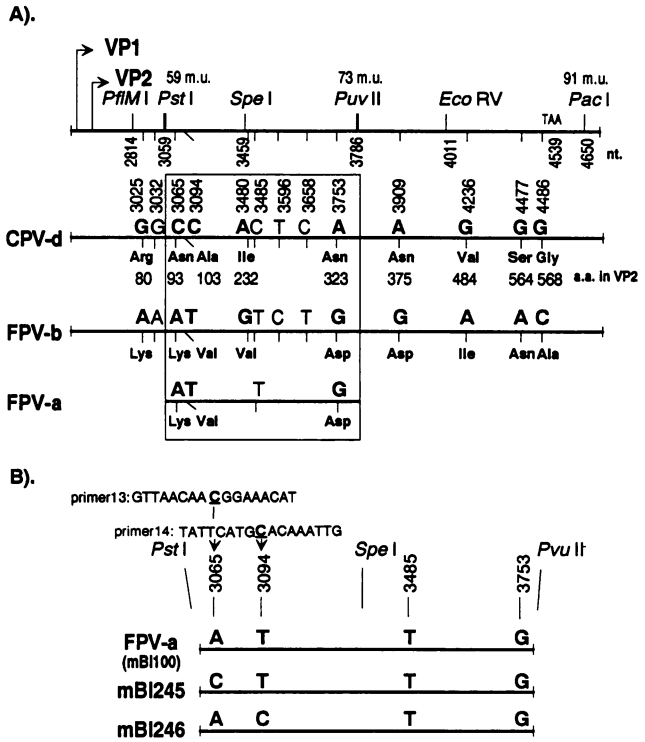

FIG. 1. (A) DNA sequence differences between the VP1 and VP2 genes of CPV-d, FPV-b, and FPV-a in the region studied. All differences between CPV-d and FPV-b in the region between *Pfl*MI and the VP1 or VP2 termination codon (nt 4539) are shown, and the region boxed is that which was analyzed by mutagenesis. The sequence of FPV-a shown was that used for preparation of all mutant viruses. Nucleotide positions and map units are from the complete CPV-d and FPV-b sequences (32). (B) Preparation of specific mutants (mBI245 and mBI246) from the M13 clone of the FPV-a capsid protein gene sequence (mBI100). Oligonucleotide site-directed mutagenesis was used to change nt 3065 and 3094 as shown.

Transfection of cells and titration of viruses. CsCl-banded plasmids were transfected as 5- or 10-μg amounts into the NLFK cell line by using calcium phosphate precipitation (17, 19) or electroporation. Electroporation was performed with 5×10^5 NLFK cells in 0.5 ml of Dulbecco's minimal

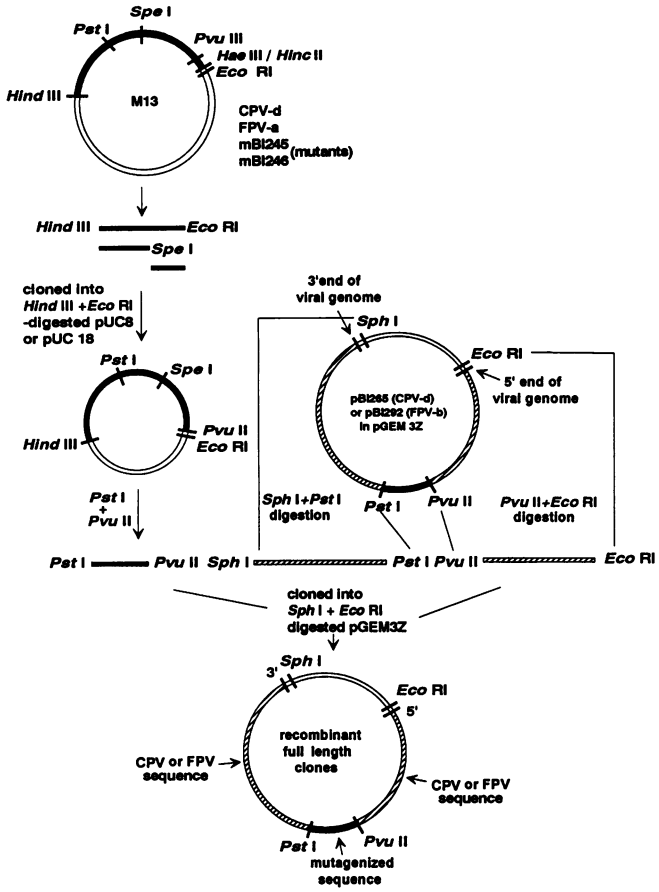

FIG. 2. Strategy used to prepare the recombinant plasmid clones from the mutant M13 phage clones (see Fig. 1). All sequence combinations were inserted between *Pst*I and *Pvu*II sites of CPV-d (pBI265) or FPV-b (pBI292) infectious clones. The individual plasmids were identified as shown in Fig. 3.

essential medium at 500 V/cm for 15.3 ms, after which the cells were cultured in 25-cm^2 flasks in growth medium. To isolate viruses, transfected cells were passaged blind one or two times. Viruses were titrated by an immune staining plaque assay (25, 37).

Analysis of virus properties. (i) Antigenic properties. Viruses were examined for the presence of the CPV- or FPV-specific epitopes by testing with 8 HA units of virus in the HA inhibition (HI) assays with various MAbs (36, 38) (Fig. 3). Some viruses were also tested by enzyme-linked immunosorbent assay (ELISA). Viral antigens were prepared for ELISA from infected NLFK cell cultures. Culture supernatants were clarified at 16,000 × *g* for 10 min, and viruses were pelleted at 140,000 × *g* for 3 h at 4°C. Resulting virus pellets were resuspended in 10 mM Tris-HCl–0.1 mM EDTA buffer (pH 8.0) and partially purified by Sephadex G-200 gel chromatography. Antigens were diluted in carbonate-bicarbonate buffer (pH 9.6) and coated onto plates at room temperature overnight. Antigens were standardized to give equal reactivity with MAb 8 (for mouse immunoglobulin G [IgG]) or MAb F (for rat IgG) (33), as those MAbs react equally well with CPV and FPV antigens. Plates were incubated with 0.5% ovalbumin in phosphate-buffered saline (PBS) (pH 7.2) for 30 min at room temperature, then fourfold dilutions of MAbs in 0.5% ovalbumin–0.05% Tween 20–PBS were added to plates, and the plates were incubated for 1 h. Antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rat IgG and the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and then the A_{405} was determined.

(ii) Host range. The canine host range was examined by infection of A72 cells, using plaque titration, RF DNA recovery, and growth curve analyses, and results were compared with those for parallel control infections of NLFK cells. For RF DNA analysis, cells seeded at a density of 10^4/cm^2 were infected after 24 h with 10-fold dilutions of each virus as shown in Fig. 4 and 5 and then incubated at 37°C for 3 or 4 days. Low-molecular-weight DNA was recovered by using a modified Hirt extraction procedure (21, 28) and electrophoresed in a 1% agarose gel containing 1 μg of ethidium bromide per ml. After Southern blotting, viral DNA was detected with a ^{32}P-labeled CPV DNA probe.

The relative ability of each virus to plaque in A72 or NLFK cells was examined in the immune-staining plaque assay. To compare levels of replication in dog cells, growth curves of certain recombinants were determined and compared with growth of wild-type CPV-d. A72 or NLFK cells seeded at 10^4/cm^2 were inoculated with 0.1 PFU per cell for 1 h at 37°C. Cells were washed twice with medium after removal of the inoculum and then incubated with growth medium. Duplicate cultures were harvested by freezing and thawing after 1 h (day 0) and then on days 1, 2, 4, and 6. Titers were determined by PFU titration in NLFK cells.

(iii) pH dependence of HA. The HA titer of each virus was determined by using 0.5% (vol/vol) rhesus macaque erythrocytes in pH 6.2 or 7.4 sodium barbital-sodium acetate buffer at 4°C (32, 33).

RESULTS

One virus prepared in this study, vBI410, was a recombinant which contained the 59 to 91 m.u. region of CPV in the FPV background (Fig. 3). That virus replicated in A72 cells approximately as well as did the wild-type CPV-d (Fig. 3 to 5), and it had the same antigenic type and pH dependence of HA (Fig. 3; see also 8), confirming that those properties were all contained within the 59 to 91 m.u. region (32, 33). We examined the roles of viral sequences within the 59 to 73 m.u. region, which has been previously shown to influence host range and antigenic properties of the viruses (33), by preparing a series of mutants by site-directed mutagenesis and recombination (Fig. 1 to 3). All amino acid positions refer to location in the VP2 sequence.

Canine host range. Viable recombinant viruses were analyzed for their host ranges in NLFK and A72 cells by using three different assays: plaque formation, RF DNA formation, and in some cases growth curve analysis. The analysis of RF DNA revealed a low level of viral replication in some cases in which no plaques were detected by the plaque assay. This finding most likely indicates that the formation of a detectable plaque required multiple rounds of replication and secondary cell infections, and the lowered replication of some viruses (e.g., vBI286 and vBI279) resulted in foci of infected A72 cells which were too small to be detected by the autoradiographic method used in the plaque assay. The original wild-type CPV-d and those FPV recombinants which had CPV sequences at amino acids (aa) 93 and 323 (vBI319, vBI340, and vBI410) formed visible plaques in A72 cells in the plaque assay (Fig. 3B), and those viruses also showed higher levels of RF DNA replication (Fig. 5A). All viable viruses with the CPV background which had changes

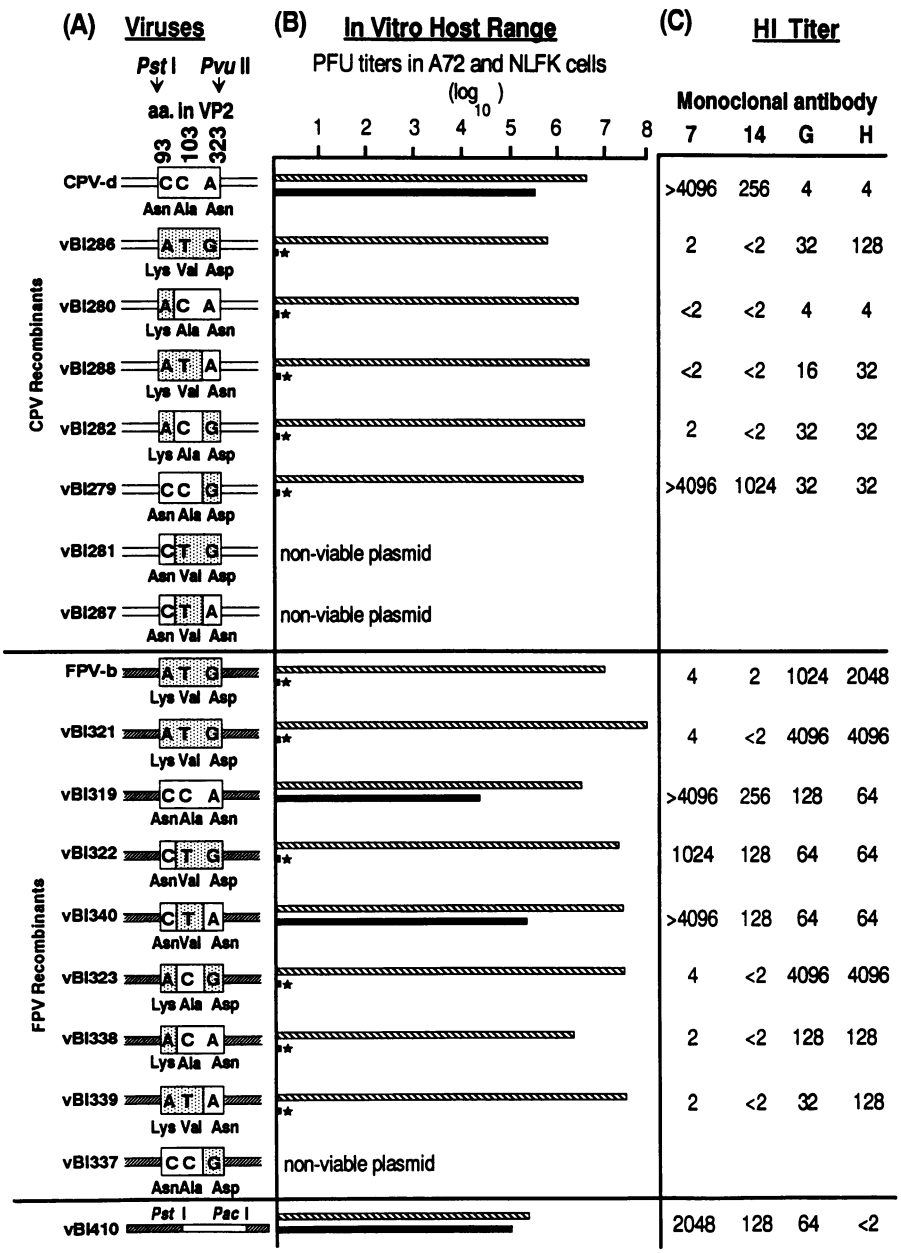

FIG. 3. Analysis of the host range and antigenic properties of different mutants and recombinants between CPV and FPV. (A) The various mutations and amino acid sequence combinations which were introduced into the infectious plasmid clones of CPV-d (CPV recombinants) or FPV-b (FPV recombinants). The viable viruses isolated were analyzed. Virus vBI321 was prepared by replacing the *Pst*I-to-*Pvu*II region of FPV-b with the same region of the FPV-a clone (mBI100). The vBI410 virus was prepared from plasmid clones vBI319 and CPV-d by recombination at the *Spe*I and *Pac*I sites (see Fig. 1). (B) PFU titer per milliliter in NLFK cells (hatched bar) and A72 cells (solid bar) of a stock of each of the viable viruses which was prepared in the nonrestrictive NLFK cells. Virus plaques were assayed by immunostaining of the inoculated cell monolayers 6 days after infection. ⋆, <3 PFU/ml. (C) Antigenic typing of viable viruses. CPV-specific MAbs 7 and 14 and FPV-specific MAbs G and H were titrated in the HI test against 8 HA units of each virus.

of aa 93, 103, or 323 to FPV sequence lost the ability to plaque in A72 cells (Fig. 3).

For efficient replication in A72 cells, CPV Asn-93 and Asn-323 were both required to be present in viruses with an FPV genetic background (vBI319, vBI340, and vBI410) (Fig. 4 and 5). For viruses with a CPV background, the single changes of either Asn-93 to Lys (vBI280) or Asn-323 to Asp (vBI279) greatly reduced viral replication in A72 cells (Fig. 4). When Asn-93 and Ala-103 both were replaced by the FPV sequences (vBI286 and vBI288), viral DNA replication in A72 cells was essentially abolished (Fig. 4).

Although recombinants vBI319 and vBI340 replicated in A72 cells, both the RF DNA production (Fig. 5A) and the relative growth (Fig. 6B) of those viruses were lower than for CPV-d. Since the recombinant which contained the entire 59 to 91 m.u. region of CPV (vBI410) (Fig. 3) replicated approximately as well as did CPV-d in both of those assays (Fig. 5B and 6A), this finding suggests that additional se-

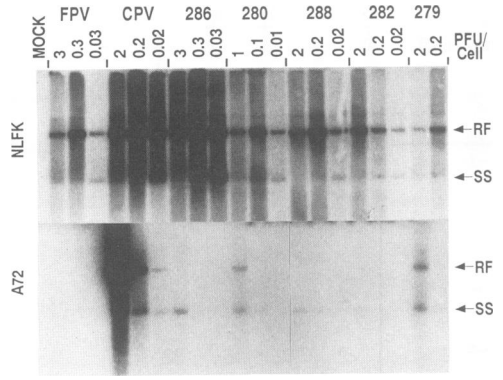

FIG. 4. Host ranges of viable mutant viruses with a CPV background defined by examining DNA replication in NLFK and A72 cells. The specific sequence combination in each virus is shown in Fig. 3. Low-molecular-weight DNA was recovered 3 days after inoculation of feline (NLFK) or canine (A72) cells with dilutions of virus stocks prepared in NLFK cells. Each virus was inoculated at two or three 10-fold dilutions, giving the multiplicities of infection shown above the lanes. One culture of each cell line was mock inoculated. Half of the DNA recovered from a 9-cm^2 culture was electrophoresed in a 1% agarose gel in the presence of 1 μg of ethidium bromide per ml, Southern blotted to a nylon membrane, then probed with a ^{32}P-labeled CPV DNA fragment. RF, viral monomer RF DNA; SS, viral single-stranded DNA.

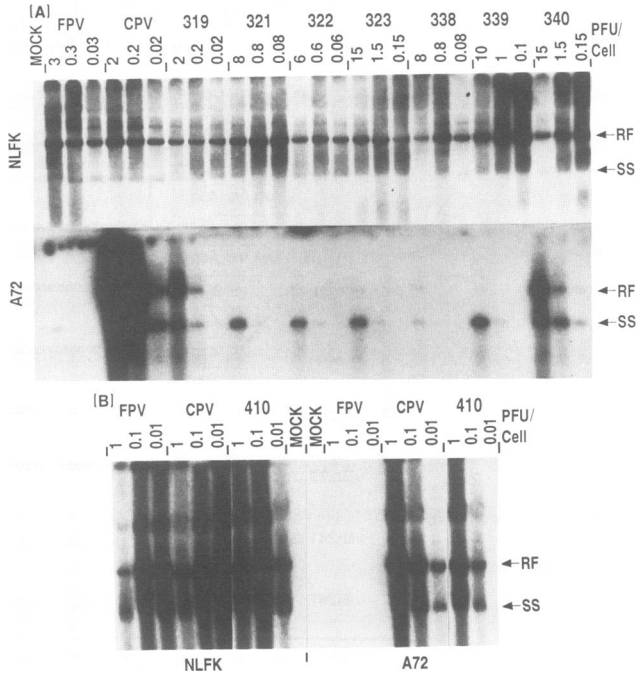

FIG. 5. Host ranges of viable mutant viruses with an FPV background defined by examining DNA replication in NLFK and A72 cells. The specific sequence combination in each virus is shown in Fig. 3. Low-molecular-weight DNA was recovered 4 days after inoculation of feline (NLFK) or canine (A72) cells with dilutions of virus stocks prepared in NLFK cells. Each virus was inoculated at three 10-fold dilutions, giving the multiplicities of infection shown above the lanes. One culture of each cell line was mock inoculated. Half of the DNA recovered from a 9-cm^2 culture was electrophoresed in a 1% agarose gel in the presence of 1 μg of ethidium bromide per ml, Southern blotted to a nylon membrane, then probed with a viral DNA fragment. (A) Analysis of CPV-d, FPV-b, and mutant viruses with an FPV background; (B) analysis of CPV-d, FPV-b, and vBI410 with the 59 to 91 m.u. region of CPV in an FPV background. RF, viral monomer RF DNA; SS, viral single-stranded DNA.

quences between 73 and 91 m.u. are also required for the full expression of the canine host range as seen in A72 cells. Three of the plasmid clones (pBI281, pBI287, and pBI337) were inviable, and no virus could be recovered after transfection or electroporation into either NLFK or A72 cells. The 59 to 73 m.u. region of each of those plasmids which had been subjected to mutagenesis was confirmed by sequencing from one strand, and the *Pfl*MI (nt 2814)-to-*Eco*RV (nt 4011) region (Fig. 1A) of each plasmid was recloned along with the remaining sequences from CPV-d (pBI265) or FPV-b (pBI292). Those plasmids remained inviable. This finding suggests that combinations of the CPV sequence at residue 93 (Asn) and the FPV sequence at residue 103 (Val) in the CPV background (vBI281 and vBI287), or combinations of CPV sequences at residues 93 (Asn) and 103 (Ala) along with FPV sequence at residue 323 (Asp) in the FPV background (vBI337), resulted in inviable plasmids (Fig. 3).

Antigenic epitope analysis. The sequences affecting the CPV- or FPV-specific epitopes were analyzed by testing viruses with specific MAbs in the HI assay (Fig. 3C) and ELISA (Fig. 7). Both tests clearly revealed the presence or absence of the CPV-specific epitope (Fig. 3C and 7B). The FPV-specific epitopes recognized by MAbs G and H were not as clearly distinguished by either HI or ELISA (Fig. 3C, 7D, and 7E), as previously described (36). However, those FPV-specific MAbs showed both lower ELISA binding and lower HI titers to CPV and to certain mutant viruses (Fig. 3C, 7D, and 7E).

When Asn-93 was introduced alone into the FPV sequence (vBI322), it gave that virus the CPV-specific epitope but had little effect on the binding of the FPV-specific MAbs (Fig. 3C, 7D, and 7E). Conversely, any virus with Lys-93 lost the CPV-specific epitope recognized by MAbs 7 and 14 (Fig. 3C and 7B). The epitope recognized by FPV-specific MAb G was affected by the sequence of residue 323 (Fig. 3C and 7D). However, full expression of the epitope for MAb G may need other residues outside the region studied here to give the same HI or ELISA titers as did wild-type FPV. The effects of multiple residues in determining the epitope recognized by MAb H were also observed (Fig. 3 and 7).

Residues affecting pH dependence of HA. Previous studies indicated that sequences affecting the pH dependence of HA mapped between 59 and 80 m.u. (6, 32, 33). However, as shown in Fig. 8, Asn-323→Asp and Asn-375→Asp both affect the HA dependence on pH in a coordinated fashion. If the virus had Asn-375 (CPV sequence), then it exhibited the CPV-like pH of HA whether residue 323 was the CPV or FPV sequence. However, if aa 375 was the FPV-specific sequence (Asp), then the pH dependence of HA was determined by whether aa 323 was Asn (CPV-like pH dependence) or Asp (FPV-like pH dependence) (Fig. 8).

DISCUSSION

The origin of CPV has been of interest since it emerged in 1978 as the cause of a panzootic outbreak of disease in dogs. Because of the close antigenic and genetic relationship to FPV, it has long been suggested that CPV was derived as a variant of FPV or some other closely related virus. In previous studies, we have shown that various properties

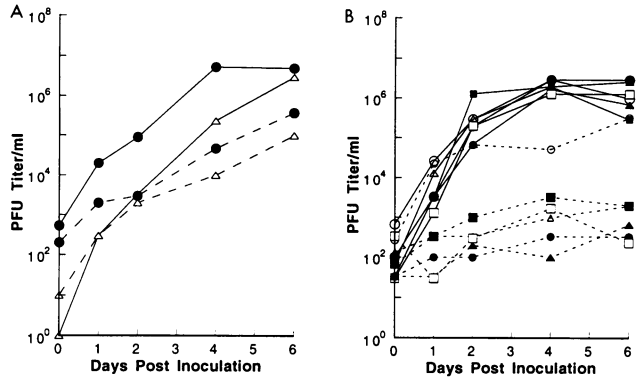

FIG. 6. (A) Replication of vBI410 (△) in A72 and NLFK cells compared with that of CPV-d (●). A72 cells (dashed lines) and NLFK cells (solid lines) were inoculated on day 0 with 0.1 PFU per cell. Virus was collected after freezing and thawing of cultures on day 0, 1, 2, 4, or 6, and the PFU titers were determined in NLFK cells. (B) Comparison of the replication of each of several viruses in NLFK and A72 cells. A72 cells (dashed lines) and NLFK cells (solid lines) were inoculated, and viruses were collected as described for panel A. Results for CPV-d (○), FPV-b (●), vBI279 (△), vBI280 (▲), vBI319 (□), and vBI340 (■) are shown.

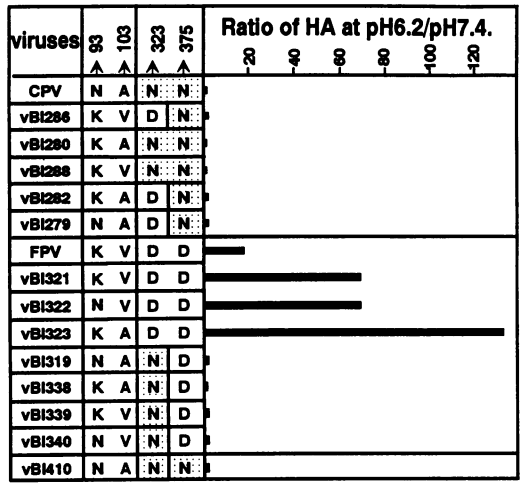

FIG. 8. pH dependence of HA of mutant viruses. The sequences of residues 93, 103, 323, and 375 are shown. Shading of the CPV-encoded Asn (N) residues at aa 323 and 375 emphasizes the correlation between those sequences and the pH dependence phenotype of the virus. The ratios between the HA titers at pH 6.2 and 7.4 are shown.

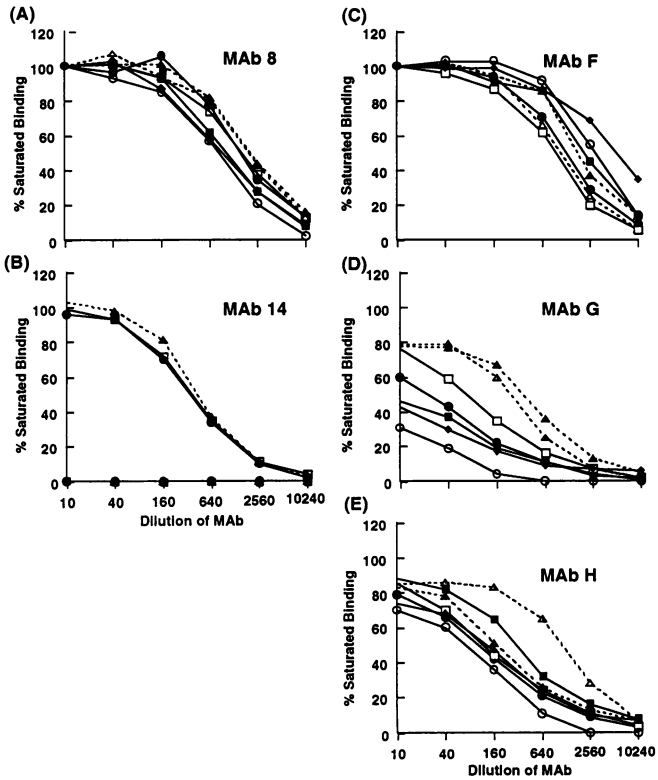

FIG. 7. Antigenic analysis of viruses by ELISA with either a virus type-common mouse (MAb 8) or rat (MAb F) antibody or with antibodies that distinguish between CPV and FPV (MAbs 14, G, and H). Saturated binding of each MAb was normalized against the saturated binding of MAb 8 or F to the same antigen. Viruses are CPV-d (●), FPV-b (△), vBI279 (□), vBI280 (○), vBI282 (◆), vBI288 (■), and vBI322 (▲). ——, viruses with a CPV background; – – –, viruses with an FPV background.

which distinguish CPV and FPV map in the capsid protein gene (32, 33). Here we examined in detail the functional roles of each of the coding sequence differences between 59 and 73 m.u. No function has been associated with the noncoding change of nt 3485 within that region, and that mutation was not examined separately.

The CPV-specific epitope associated with the single sequence Asn-93 has been found on all CPV isolates that we have tested to date (34), and Asn-93 is also observed in all reported CPV sequences (33, 34, 42, 43). The FPV-specific epitopes were examined by HI testing and ELISA (Fig. 3 and 7). The epitope recognized by MAb G was affected by the difference of residue 323 (Asn-Asp), although both the HI and ELISA analyses showed intermediate titers with the mutant viruses. The residues affecting the epitope recognized by MAb H were not clearly defined in these studies.

The ability of CPV to replicate efficiently in A72 canine cells was found to map between 59 and 91 m.u. in the viral genome, i.e., in the right-hand 84% of the VP2 gene and 111 bases of the right-hand noncoding region (Fig. 3, 5B, and 6A). That canine host range was determined by combinations of several amino acids. A combination of the CPV-specific sequences at nt 3065 (Asn-93) and 3753 (Asn-323) introduced into FPV allowed it to replicate in A72 cells (Fig. 3 and 5), albeit to a lower level than did wild-type CPV (Fig. 6). Those differences as well as that of nt 3094 (Ala-103-Val) were all required to be in the CPV sequence to allow efficient replication in canine cells. This finding indicates that of the five phylogenetically informative coding differences in the 59 to 91 m.u. region which differ between CPV and FPV or the related mink and raccoon viruses, at least three are important for determining the efficient replication in canine cells. The roles of the other changes have not yet been clearly defined. However, Asn-375 was found only in some isolates of the original strain of CPV (CPV type 2) (33, 42, 43), and in later CPV types that residue reverted to an Asp (33, 34), suggesting that Asn-375 is not critical to the success of CPV in nature. It is, however, possible that in CPV type 2 that sequence was required and that other sequence changes in

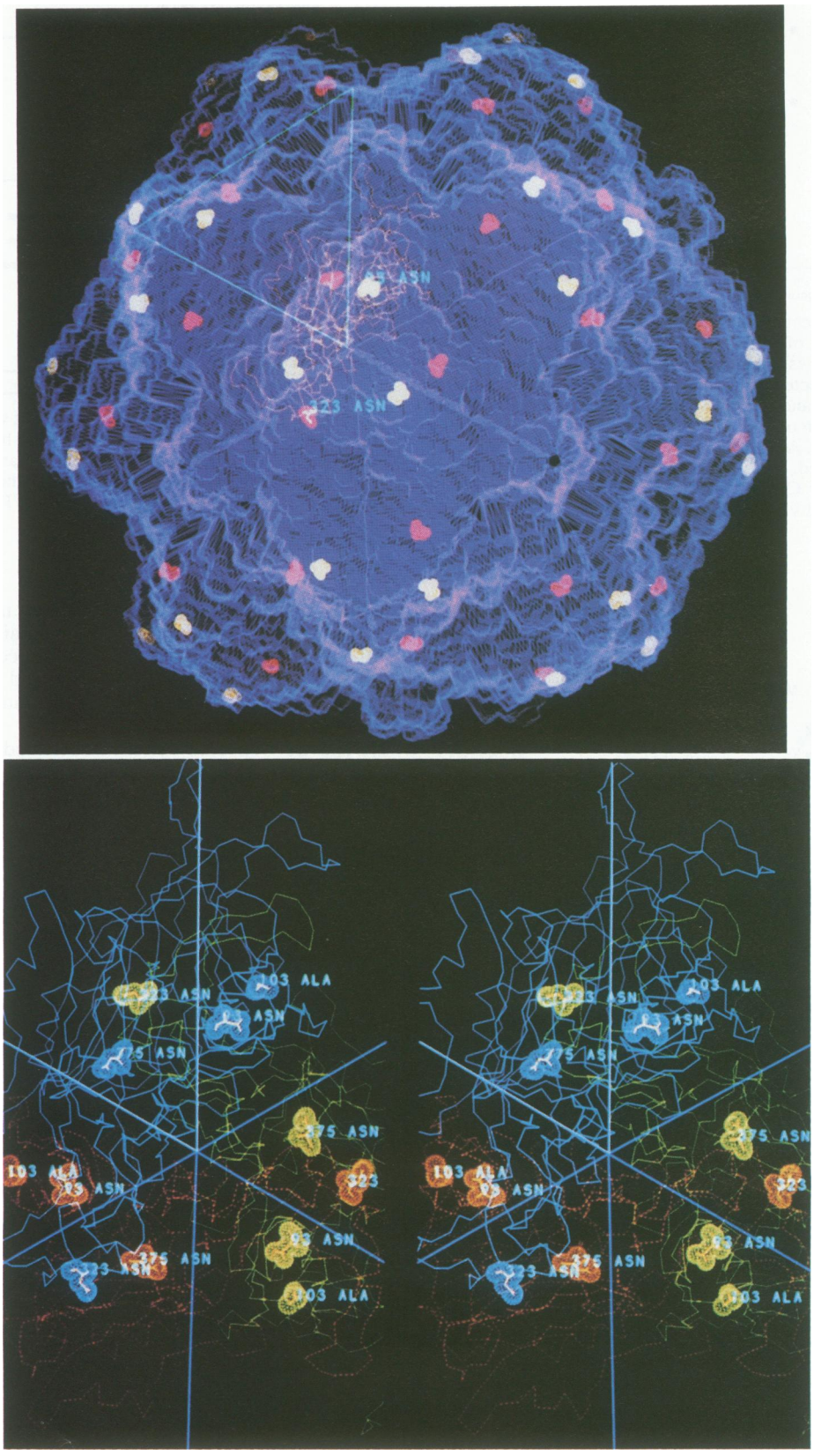
ASN
123 ASN
103 ALA
ASN
375 ASN
323
93 ASN
103 ALA

FIG. 9. Locations of the changes examined within the atomic structure of CPV as determined by X-ray crystallography. (A) Structure of the CPV capsid represented by grid mesh surfacing. The view is along the threefold axis, and the location of one VP2 α-carbon chain is shown in relation to an icosahedral face of the virus, which is outlined by a triangle. VP2 residues 93 and 323 on the surface of the capsid are shown, the different colors indicating the origin of each residue within the asymmetric unit of the virus is from a different, threefold-related VP2 molecule. The surface grid was calculated as originally described by Rossmann and Palmenberg (45). (B) A stereo pair showing the interrelationships of the VP2 molecules around the threefold axis of symmetry of the virus capsid and showing the origins and locations of various residues that were examined in this study. The view is along the threefold axis, and the three VP2 molecules and the variable amino acids within each chain are indicated in red, blue, and yellow. Shading indicates the van der Waals radius of the atoms.

the later CPV types compensated for the change of that residue back to the FPV sequence. Residue 323 is close in sequence alignment and probably also in capsid structure to the two changes in the MVM VP2 sequence (residues 317 and 321) which coordinately determine the ability of that virus to replicate in fibroblasts (5, 18).

The host ranges of these viruses are complex, and the in vitro host ranges assayed here do not necessarily reflect the true animal host ranges. After inoculation in vivo, FPV-b replicated in the thymus and bone marrow cells of dogs but not in the intestine or in the peripheral or mesenteric lymph nodes (52). We have previously shown that a recombinant virus similar to vBI319 but prepared from viral RF DNA replicated to high titers in the canine intestine and mesenteric lymph nodes (33), indicating that the changes examined here most likely also define the ability of the virus to replicate systemically in dogs.

The biological significance of the pH dependence of HA is unknown, but this is a consistent difference between CPV and FPV. We have shown that the HA receptors of rhesus macaque erythrocytes which bind CPV are destroyed by neuraminidase or periodate treatment (6), indicating that the viruses bind to sialic acid on glycoproteins or glycolipids on the erythrocyte surface. The CPV-like ability to hemagglutinate at a range of pH values up to at least 7.4 was affected by combinations of residues 323 and 375, in which the presence of the CPV Asn-375 predominated over the effect of the Asn-323-Asp sequence. However, when residue 375 was Asp, then the sequence of residue 323 determined the pH dependence of the erythrocyte binding. Residues 323 and 375 are in close proximity in the structure (Fig. 9), probably allowing them to influence the same function. Analysis of two MAb-selected escape mutants of CPV which had mutations either of Asp-375 and Asp-302 or of Asp-323 and Arg-224 showed that both viruses had CPV-like HA dependence on pH (12), supporting the results presented here. In addition, a non-HA mutant of CPV had a mutation of residue 377 from Arg to Lys which eliminated all virus binding to erythrocytes (6, 35). Residue 375 reverts to the FPV sequence (Asp) in the CPV type 2a and CPV type 2b virus strains (33, 34). While that change would not have affected the pH dependence of HA of those CPV strains since residue 323 was still Asn (Fig. 8), it may have affected the temperature dependence of HA, which is a marker for the later CPV strains (47). Various pH-induced changes have been reported for other viruses, including picornavirus (23) and polyomavirus (15, 16). Mengovirus had a higher infectivity as a result of a conformational change around the pit structure caused by exposure to a low-pH condition, and that pit structure was shown to be the receptor attachment site (23). A single amino acid change in polyomavirus VP1 affected the viral plaque size and pH dependence of HA (16) and was also involved in determining the tissue tropism of that virus (15). That residue was located on the outside of the viral capsid in a region thought to be involved in receptor attachment (16).

The locations of the residues examined here are shown in the CPV capsid structure in Fig. 9. All of the changes are within the elaborated loops which make up the threefold spikes of the virus capsid. The changes Asn-93→Lys and Asn-323→Asp may both influence interactions between residues in neighboring loops of either the same VP2 molecule or the threefold-related VP2 (Fig. 9). Residue Ala-103→Val may not directly influence the host range, but as it is close to residue 93 (Fig. 9), it may be involved in compensating for the Asn-93 sequence in CPV, allowing virus viability (Fig. 3). The structure of FPV empty particles has recently been determined and has revealed significant differences in the conformations of the loops which make up the threefold spike around these differences in sequence (1). This finding may be similar to the results of studies of poliovirus host range mutants, wherein residues outside the receptor binding canyon were found to affect host and tissue tropism by changing the conformation of loops around the fivefold axis of the viral structure (26, 57).

The differences between CPV and FPV capsids may affect some early stage involving attachment, entry, uncoating, or initiation of DNA replication of virus during infection, as has been observed for the tissue tropisms of MVM and PPV (5, 30, 44, 50). Our studies have shown that the restriction of the replication of FPV in canine cells was at an early stage of infection, prior to DNA replication (Fig. 4 and 5), and the FPV genome was expressed efficiently in A72 cells after DNA transfection (12). It has been suggested that the receptor is not the determinant of tissue tropism of MVM (50) or PPV (30, 44, 55). A lack of receptor appears to be unlikely to be the major block in the replication of FPV in canine cells; for example, in Fig. 4 and 5, the input virion single-stranded DNA was recovered from the washed A72 cells 3 or 4 days after inoculation with high PFU titers, indicating efficient association of the virus with those cells.

Phylogenetic analysis indicates that there are six coding differences in the VP1 or VP2 gene between the root node of the FPV, MEV, and RPV isolates and that of the CPV isolates (33, 34). These studies show that a combination of several specific sequence changes in the VP1 or VP2 gene would have been required to derive a virus with the properties of CPV from FPV or a related virus and that at least three of those changes are required for replication of CPV in canine cells. This finding defines some of the important changes that would have been necessary for the emergence of CPV. In future studies, we are interested in defining the roles of the other sequences not examined here in the various CPV functions and also in examining the sequence differences in the more recent strains of CPV which have since replaced the CPV type 2 strain in nature.

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