Canine parvovirus (CPV) emerged in 1978 as the cause of new enteric and myocardial diseases in dogs. The new virus spread globally in a pandemic of disease during 1978 and has since remained endemic in dogs throughout the world (27, 43). The 1978 strain of CPV (termed CPV type 2) was a new virus infecting dogs since there is no serological or other evidence for infection of dogs by a related virus prior to the mid-1970s (27). Phylogenetic analysis shows that all CPV isolates were descended from a single ancestor which emerged during the mid-1970s, which was closely related to the long-known feline panleukopenia virus (FPV) which infects cats, mink, and raccoons but not dogs or cultured dog cells (43). FPV and CPV isolates differ by as little as 0.5% in DNA sequence, and the characteristic properties of CPV type 2 are controlled by a small number of changes in the capsid surface. Two differences between FPV and CPV changed VP2 residues 93 from Lys to Asn and 323 from Asp to Asn, and those changes alone could introduce the canine host range, a CPV-specific antigenic epitope, and a difference in the pH dependence of hemagglutination into FPV (9, 14). Despite the close relationship to FPV, CPV type 2 isolates did not replicate in cats (42, 44), and this host range was determined at least in part by VP2 residues 80, 564, and 568 which are in close proximity in the capsid structure (41). Other mutations in the same structural region of CPV type 2 were selected by passage in cat cells (VP2 residue 300 from Ala to Asp), and these reduced the infection of canine cells, as did closely adjacent changes in in vitro prepared mutants (VP2 residue 299 Gly to Glu) (18, 26).

Host range-controlling residues are located on a raised region of the capsid that surrounds the threefold axis (the threefold spike) (9, 46). VP2 residues 93 and 323 are found near the top of that structure, whereas residues 299 and 300, and changes controlling feline host range, are all on a ridge on the side (the shoulder) (18, 26).

During 1979 a CPV variant (CPV type 2a) emerged that spread worldwide within 1 year and replaced the CPV type 2 strain. CPV type 2a contained five substitutions in the capsid sequence compared to CPV type 2, including changes of VP2 residues 87 from Met to Leu, 300 from Ala to Gly, and 305 from Asp to Tyr (16, 29, 42). CPV type 2a isolates were antigenically variant from CPV type 2 and also infected and caused disease in cats (29, 30, 42). An antigenic variant of CPV type 2a (CPV type 2b) was recognized in 1984, and it differed in an antigenic epitope as a result of the substitution of VP2 residue 426 from Asn to Asp (29).

CPV and FPV are autonomous paroviruses with single-stranded DNA genomes of ca. 5,120 bases. The genomes encode two genes which each form two proteins by alternative mRNA splicing (10, 49). The 28-nm-diameter nonenveloped capsid is assembled from 60 copies of a combination of the overlapping capsid proteins VP1 and VP2 (46). The three sites on the capsid that can affect canine host range on the threefold spike are separated from each other by 25 to 30 Å, and all affect the folding or flexibility of loops within the capsid structure, suggesting roles in virus-receptor interactions or in capsid uncoating (1, 18, 36).

CPV type 2 and FPV capsids bind the human or feline transferrin receptors (TfRs) and use those receptors to infect normally resistant Chinese hamster ovary (CHO) cells (25).
The capsids normally enter cells by clathrin-mediated endocytosis, colocalize with transferrin (Tf) in perinuclear endosomes, and then slowly leave the endosome and enter the cytoplasm prior to the DNA gaining access to the nucleus for replication (24, 47, 48, 51).

Here we show that CPV infection of dog cells was associated with its specific ability to bind the canine TfR and that resistance of canine cells to FPV could be overcome by expression of the canine Tf among those of the feline and human TfRs, and differences were mapped onto the human TfR structure (7, 17). The intact cDNA of the canine Tf gene was prepared and cloned into the vector pcDNA3.1(−) (Invitrogen, Carlsbad, Calif.) for expression.

Receptor expression from plasmids. TRbV or C2Th cells seeded at 2 × 10⁴ per cm² in 25-cm² flasks were transfected with 5 μg of plasmid cloning into the feline or canine Tf cDNAs. The DNA was mixed with 15 μl of Lipofectamine (Invitrogen) and added to the cells according to the manufacturer’s directions. Cells were incubated at 37°C for 2 to 4 days, and then binding and uptake of capsids and Tf was determined by microscopy and flow cytometry. For fluorescence microscopy, cells were incubated at 37°C for 1 h with labeled Tf or with labeled or unlabeled virus capsids at 37°C for 1 h, and then they were washed and fixed with 4% PFA in PBS. For antibody staining, the cells were permeabilized with PBS containing 0.1% Triton X-100 and 0.5% bovine serum albumin, incubated with Cy2-labeled MBA8 to detect virus, and then examined with a UV microscope.

For flow cytometry the cells were removed from the plastic with 1 mM EDTA in Hanks buffered saline without Ca²⁺ and Mg²⁺, fixed with 4% PFA, and stained with Cy2-labeled MBA8 as described above. Cells were then analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.).

The role of sialic acid in virus binding was examined by neuraminidase treatment of cells. CRFK cells were incubated for 1 h at 37°C with 0.5 U of neuraminidase (Clostridium perfringens, type X; Sigma)/ml and then incubated for 1 h with either fluorescein isothiocyanate-labeled peanut agglutinin or with 10 μg of capsids/ml, and virus binding was assayed by flow cytometry as described above.

Infection assays. Cells transfected with plasmids expressing canine or feline TfR or with the empty vector were incubated for 4 days. Cells were inoculated with 10 TCID₅₀ of either CPV type 2, CPV type 2b, FPV, or CPV type 2-G299E per cell for 1 h at 37°C and then incubated for 24 or 48 h at 37°C. The cells were then incubated with serum-free medium for 30 min, followed by the addition of Texas Red-labeled canine Tf for 60 min at 37°C. After a washing step and fixation with 4% PFA, the infected cells were stained with Cy2-labeled anti-CPV and then examined with a UV microscope.

RESULTS

Expressing the canine TfR in C2Th cells made them susceptible to infection by both FPV and the CPV type 2-G299E host range mutant (26) (Fig. 1). The percentage of transfected cells infected was determined by comparison with the proportion of cells transfected in parallel with a plasmid expressing green fluorescent protein. A total of 6 to 9% of the cells expressing C2Th cells became infected by either FPV or CPV type 2-G299E (Fig. 1). A total of 10 to 20% of the C2Th cells were infected by wild-type CPV type 2 that could potentially infect all of the cells, a finding which is typical for these viruses in susceptible nonsynchronized cells, since cellular S phase is needed for virus DNA replication.

The canine TfR was required for infection of C2Th cells by CPV type 2. When we injected an antibody against the TfR cytoplasmic tail into these cells before virus inoculation, there
FIG. 1. Expression of the feline TfR in canine C2Th cells makes them susceptible to FPV and CPV type 2-G299E infection. C2Th cells were transfected with a plasmid expressing the feline TfR or with the empty plasmid vector, incubated for 4 days, and then inoculated with 10 TCID50 per cell of CPV type 2, FPV, or CPV type 2-G299E. After 24 h more incubation, the cells were fixed and infection was detected with an antibody against the NS1 protein. Bars show one standard deviation of the data from three separate experiments.

was an 80 to 100% reduction in virus infection, whereas no significant effect was seen after injection of a control IgG (Fig. 2).

There was only a partial correlation between binding of the viruses to canine and feline cells at 37°C and their ability to infect these cells. All viruses tested bound and were taken up into feline CRFK cells efficiently (Fig. 3). However, between 5- and 20-fold more capsids of the wild-type CPV type 2 and CPV type 2–R377K became cell associated than did those of FPV or CPV type 2b (Fig. 3A). When incubated with canine C2Th cells, CPV type 2 and CPV type 2–R377K bound and were taken up at high levels, CPV type 2b capsids was taken up at 10- to 20-fold-lower levels, and FPV capsids showed no detectable cell association (Fig. 3A). Although these viruses can bind sialic acids on erythrocytes and cells at lower pHs and temperatures (4, 36, 39), this does not appear to be a factor in the binding to the cells examined here. The CPV type 2–R377K does not bind sialic acid, but it bound feline cells to levels similar to those of the wild-type CPV type 2 (Fig. 3A). In addition, pretreatment of cells with neuraminidase prior to incubation with CPV type 2 capsids did not reduce virus binding (Fig. 3C), although increased binding of peanut agglutinin to the galactosyl(β1,3)-N-acetylgalactosamine exposed on the treated cells indicated that the sialic acids had been removed (Fig. 3D).

The increased binding of CPV type 2 to the host cells was affected by mutations replacing residues within the shoulder region of the capsid. CPV type 2–G299E showed binding similar to that of CPV type 2b (Fig. 3B). Capsids of CPV type 2 containing changes derived from the CPV type 2a and type 2b sequence (D305Y or A300G/D305Y/N375D) bound to both cell types at levels intermediate between those seen for CPV type 2 and CPV type 2b capsids, indicating a partial role for the 305Y change (Fig. 3B).

To directly determine the role of the canine TfR in specific viral binding and infection, we prepared the canine TfR cDNA by reverse transcription-PCR and expressed that receptor in CHO-derived TRVb cells (19). Cells were then incubated with FPV, CPV type 2, CPV type 2–G299E, or CPV type 2b capsids for 1 h at 37°C. All four viruses bound and were taken up into TRVb cells expressing the feline TfR, whereas only CPV type 2 or CPV type 2b capsids bound efficiently to cells expressing the canine TfR (Fig. 4). No binding above background was seen to cells transfected with the control plasmid (results not shown). The susceptibility of TRVb cells expressing the feline or canine TfRs to virus infection generally paralleled the natural host ranges of these viruses (Fig. 5). Cells expressing the feline TfR were highly susceptible to FPV, CPV type 2, or CPV type 2b. TRVb cells expressing the canine TfR completely resisted infection by FPV, whereas 0.5 to 1% of these cells were infected by CPV type 2 and 7 to 10% were infected by CPV type 2b (Fig. 5A). When the TRVb cells expressing the canine TfR were inoculated with a 9:1 TCID50 ratio of CPV type 2 and CPV type 2b and the cells were incubated for 5 days, the CPV type 2b sequence became dominant in the culture, as seen in the DNA sequence profile (Fig. 5B).

The translated sequences of the feline and canine TfRs differed by 13%, and both differed from the human TfR sequence by ca. 22% (Fig. 6). Differences between the feline and canine TfR sequences were found in all three domains defined for the human TfR structure, and most were of surface-exposed residues (Fig. 7A) (7, 17). Although a detailed model of the docking of the virus and TfRs is not yet possible, it is clear...
that changes of VP2 residues 93 or 299 on the top or the shoulder of the threefold spike of the capsid can affect binding to the canine TfR (Fig. 7C and D).

**DISCUSSION**

The emergence of CPV as a new pathogen of dogs presents a unique opportunity for understanding the adaptation of a virus to a new host, since we can examine the ancestral viruses, as well as the variants derived during the process of host adaptation. The viral controls of host range involve only a small number of changes on or near the viral capsid surface, and here we show that some of those changes control a critical molecular interaction with the host cell: the specific binding by CPV capsids to the canine TfR. In addition, a second step in the adaptation of CPV to dogs lead to the natural global replacement of CPV type 2 by the CPV type 2a variant, and the CPV type 2b strain later emerged as a variant of the CPV type 2a through an additional point mutation. We show that a CPV type 2b isolate was more efficient in its use of the canine TfR for infection of TRVb cells and, by comparison with CPV type 2, show that it bound canine and feline cells to low levels, perhaps indicating that it had lost the ability to bind an additional receptor on the host cells.

The host range for canine cells was controlled by virus-specific binding to the canine TfR. FPV did not bind to the canine cells at 37°C (Fig. 3), indicating that the block to FPV infection of canine cells involved the absence of a functional surface receptor for that virus. However, the expression of the canine TfR in these cells made them susceptible to virus infection (Fig. 1). This presents a simpler model of host range control than has been proposed in previous studies, in which...
radioactively labeled capsids of the closely related mink enteritis virus or of CPV host range mutants bound to canine cells when incubated at 4°C; these studies had suggested that the infection was blocked at a stage after cell entry (15, 26). The lack of binding and uptake of FPV in dog cells at 37°C seen here suggests that at 4°C labeled capsids can associate with cells that they cannot infect. That the canine TfR is a major determinant of the infection of dog cells by CPV was confirmed by the block to infection seen after microinjection of Cf2Th cells with an antibody against the TfR cytoplasmic tail (Fig. 2).

Expression of the feline and canine TfRs in TRVb cells yielded cells that acted as surrogates for the original host cells; these cells expressing the feline TfR bound and were infected by all of the viruses that infected feline cells, whereas those expressing the canine TfR bound and were infected by CPV type 2 and CPV type 2b but not by FPV (Fig. 4 and 5). An interesting finding was that the two natural variants of CPV differed in their ability to efficiently infect the canine TfR-expressing TRVb cells, with CPV type 2 infecting the cells only to very low levels, whereas the CPV type 2b isolate infected the cells at levels similar to those seen for TRVb cells expressing the feline TfR (Fig. 5). The CPV type 2 strain was the cause of the original pandemic in 1978, while the CPV type 2a later spread worldwide and replaced CPV type 2. This suggests that several biological differences can explain the rapid rise and dominance of the CPV type 2a strain, since those viruses differ antigenically, in their ability to infect cats, and in their ability to use the canine TfR as an efficient receptor when it is present alone on cells.

Sequences in the shoulder region of the CPV type 2 capsid control increased binding to feline and canine TfRs. CPV type 2 capsids bound to 5- to 20-fold-higher levels on feline and canine cells than did either CPV type 2b or FPV capsids (Fig. 3A). That additional binding of CPV type 2 was partially or completely reduced by mutations that introduced changes adjacent to VP2 residue 300 in the shoulder region of the capsid. The CPV type 2b-derived change of 305 D-Y reduced binding by about fivefold, whereas the host range mutant CPV type 2-G299E was reduced in binding to feline cells to levels similar

![Figure 4](image-url)  
**FIG. 4.** Virus binding to TRVb cells expressing feline or canine TfRs. TRVb cells were transfected with plasmids expressing the feline or canine TfR and then incubated for 4 days. The cells were incubated at 37°C for 1 h with Cy5-labeled canine Tf and with 10 μg of FPV, CPV type 2, CPV type 2b, or CPV type 2-G299E capsids/ml and then washed, suspended by EDTA treatment, fixed, and permeabilized. Capsids were detected with Cy2-labeled antibody. Cell associated Tf is shown on the y axis, and virus capsids are shown on the x axis.

![Figure 5](image-url)  
**FIG. 5.** (A) Virus susceptibility of TRVb cells expressing feline or canine TfRs. Cells transfected with plasmids expressing the feline or canine TfRs were inoculated with CPV type 2, FPV, or CPV type 2b and then incubated for 24 h before incubation for 30 min with Texas Red-labeled canine Tf. After fixation and permeabilization virus infection detected by staining for the viral NS1 protein. The bars represent one standard deviation of the mean of the percentage of Tf-binding cells that became infected in six separate experiments. (B) The replacement of CPV type 2 by CPV type 2a when the two viruses were grown together in TRVb cells expressing the canine TfR for 5 days. The inoculum contained nine times more TCID_{50} of CPV type 2 than CPV type 2b, which was reflected in the double sequencing profile at position 3046. Samples of virus were collected from the culture inoculum and from the culture at days 2 and 5 after inoculation. The viral DNA was amplified by PCR and sequenced, and the profiles of sequences from nucleotides 3043 to 3049 are shown.
to those seen for CPV type 2b (Fig. 3 and 7C). FPV bound to fivefold-lower levels to feline cells compared to CPV type 2, and these viruses differ in residues 80, 564, and 568, which are also within the structure of the shoulder region (Fig. 7C and D). The region of the receptor that determines the specific virus binding of the canine TfR has not yet been defined, and feline-canine variant sequences were found throughout the receptor structure (Fig. 7A). However, the large size of the receptor relative to the capsid suggests that the interacting surface would be near the apical domain of the TfR structure (Fig. 7B).

One of the mysteries of the evolution of CPV was the inability of CPV type 2 to infect cats despite its efficient infection of feline cells in culture (44), and the subsequent reacquisition of feline host range by CPV type 2a (16, 42). Recombinants between CPV type 2 and FPV showed that cat infection required VP2 residue 80, along with residues 564 and/or 568, to be the FPV sequence (41). It is therefore possible that the same changes that control the high binding levels of CPV type 2 to cat and dog cells also affect the in vivo replication of that virus in cats through an unknown mechanism.

Host range and changes in receptor usage in the evolution of CPV. These data suggest a model whereby CPV type 2 emerged as a variant of an FPV-like virus through acquisition of up to six changes that gave it the ability to bind the canine TfR and to efficiently infect both canine cells and dogs. That initial group of mutations also resulted in increased virus binding to feline cells compared to FPV or to the later CPV type 2a/b strain of virus. That additional binding activity of the CPV type 2 is not due to sialic acid, a potential ligand for the virus (4, 39), since the CPV type 2 R377K nonhemagglutinating mutant bound feline cell, as well as the wild-type virus, and neuraminidase pretreatment of the cells did not reduce the binding. Potential explanations for this result would be that CPV type 2 capsids bind both the TfR and a second receptor on the feline or canine cells or, alternatively, that those viruses bind only to the canine or feline TfRs, but they bind with higher affinity or avidity when the receptors are expressed on the natural host cells. That increased binding of CPV type 2 to the canine cells presumably allows the efficient infection to occur. The use of multiple receptors by viruses or variation in receptor usage is seen with other viruses (8, 13, 23, 37, 38, 52).
Subsequent changes in CPV type 2a (which were also retained in CPV type 2b) allowed the virus to use the canine TfR more efficiently for infection, and these viruses therefore no longer needed the additional or coreceptor binding. A number of possible mechanisms remain to be investigated. It is possible that CPV type 2a/b capsids have a higher affinity or avidity of binding to the canine TfR, which permits the more efficient use for cell binding and infection, or that the changes in the CPV type 2a/b capsid structure may facilitate later uncoating or membrane penetration steps in infection with the canine TfR. Many different viruses undergo receptor-induced changes in viral structural proteins that are required for successful infection of cells either directly or through second steps involving low-pH exposure (21, 22, 33, 35, 45).

Host range or tissue tropism controlled by capsid sequences has been defined for several different paroviruses. The tissue specificities of the fibrotropic and lymphotropic strains of minute virus of mice (MVM(p) and MVM(i), respectively) are also determined by a small number of changes in the surface of the capsid in a position similar to VP2 residue 323 in CPV which controls the host range for dogs (2, 3). The infection of mink by the Aleutian mink disease parovirus is also controlled by a small group of residues that are probably on the surface of the capsid (20).

Host range switching by viruses. Natural host range switches by viruses are rare events, but when they occur the results can be severe since the viruses may then spread widely through immunologically naive and nonadapted host populations. Examining the ancestors and descendents of CPV allows us to define the important cellular and viral determinants involved. It is clear that the emergence of CPV was a multistep process, where small numbers of mutations in the capsid protein gene...
allowed it to efficiently infect and spread within a new host order. Even the partially adapted CPV type 2 was very successful, spreading worldwide in only a few months. The subsequent replacement of that strain by CPV type 2a was also completed within 1 or 2 years in the face of immunity to the original virus.

These results show that under the right circumstances even a genetically stable DNA virus can gain the mutations required to adapt to a new host. This type of multistep adaptation is seen in the emergence of a new influenza viruses in humans or other animals (31, 50) and possibly is seen in the adaptation from other primates of human immunodeficiency virus type 1 to humans (11, 34). Although each system has distinct features, they all show that specific sets of mutations need to occur for the virus to become a successful pathogen of the new host, that they are likely selected in a specific order, and that they are favored where viable intermediate viruses can be selected. Although these types of host range jumps are likely to remain very rare events, understanding the mechanisms involved may allow us to anticipate and perhaps prevent the emergence of new viruses in the future.

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