

Feline Infectious Peritonitis Viruses Arise by Mutation from Endemic Feline Enteric Coronaviruses

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Received June 11, 1997; returned to author for revision July 2, 1997; accepted January 14, 1998

Feline infectious peritonitis virus (FIPV) strains from six cats and three different geographic areas were compared genetically with feline enteric coronavirus (FECV) isolates obtained from cats inhabiting the same environments. Sequence comparisons were made from 1.2- to 8.9-kb segments on the 3' end of the genome. FECV/FIPV pairs from the same catteries or shelters were 97.3–99.5% related but were genetically distinct from FIPV and FECV strains obtained from cats living in geographically distinct environments. The high genetic similarity between FECVs and FIPVs from the same environment strongly suggested a common ancestry. Based on the presence of deletion mutations in the FIPVs and not in the FECVs, it was concluded that FIPVs evolved as mutants of FECVs. The mutations are deletions in the FIPVs and not insertions in the FECVs since similar sequences are present in other strains that have segregated earlier from a common ancestor. Therefore, the order of descent is from FECV to FIPV. Mutations unique to FIPVs were found in open reading frames (ORFs) 3c in 4 of 6 isolates and/or 7b in 3 of 6 isolates. When the study was extended to include 7 additional FIPV isolates, 11/13 of the FIPVs sequenced were found to have mutated 3c ORFs. © 1998 Academic Press

INTRODUCTION

Feline infectious peritonitis (FIP) is a highly fatal infection of cats. The disease was first recognized in the 1950s (Holzworth, 1963) and is currently the leading infectious cause of mortality in young cats from pedigree catteries and shelters. The coronavirus etiology of this infection was first reported by Ward (1970).

Seroepidemiologic studies of FIP were first conducted using FIPV-infected tissues as antigen substrates in an indirect immunofluorescent antibody assay (Pedersen, 1976). Virtually all normal cats in households experiencing FIP deaths were found to be seropositive, suggesting that FIP was an infrequent disease manifestation of a common infection. However, when FIPV was isolated from naturally infected animals and experimentally transmitted to laboratory cats, it caused fatal FIP rather than inapparent disease (Pedersen *et al.*, 1981). To further complicate the picture, an antigenically indistinguishable feline enteric coronavirus (FECV) was also isolated from catteries where FIP occurred (Pedersen *et al.*, 1981). The FECV-induced antibodies strongly cross-reacted with FIPV but caused only an inapparent or mild transient

enteritis. It was postulated, therefore, that FIPV might be a relatively common and highly pathogenic mutant of the more ubiquitous FECV (Pedersen *et al.*, 1984) (Pedersen and Floyd, 1985). The validity of this mutational theory was questioned when so-called prototypic tissue culture-adapted strains of FIPV (strain WSU 79-1146) and FECV (strain WSU 79-1683) were genetically compared. The latter was found to have a major deletion in the 7b gene (Vennema *et al.*, 1992b), opposite to what would have been expected if FIPV had mutated from FECV. The mutational origin of FIPV from FECV was given new credence following genomic comparisons of 1.2-kb fragments obtained from nine additional FECV and FIPV isolates (Herrewegh *et al.*, 1995). All of these isolates had intact 7b genes, indicating that the 7b gene deletion seen in FECV 79-1683 was associated with cell culture adaptation. Furthermore, while geographically disparate isolates were genetically different, FECV UCD and FIPV UCD3 isolated from cats originating from the same facility were found to be highly related, thus supporting a common origin for FECVs and FIPVs.

The aim of this study was to accumulate direct evidence for FECV to FIPV mutation and to identify regions within the FIPV genome responsible for its unique pathogenicity. In order to do this, FECV/FIPV pairs were isolated from feces and tissues of diseased and normal cats inhabiting the same catteries or shelters. Genetic differences between virus strains in such pairs would be more likely associated with the FIP-inducing phenotype than with geographic segregation.

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TABLE 1

Comparison of Feline and Canine Coronavirus Nucleotide Sequences

Strain	Length (kb) compared	Genes compared	Percentage sequence identity	
			FECV UCD	FECV RM
FECV UCD	8.9	2, 3, 4, 5, 6, 7	—	89.7
FIPV UCD1	2.2	3, 4, 5	94.5	92.0
FIPV UCD2	1.7	3, 4, 5(p)	91.3	91.8
FIPV UCD3	8.9	2, 3, 4, 5, 6, 7	98.7	89.4
FIPV UCD4	2.3	3, 4, 5	91.5	91.8
FIPV UCD5	2.3	3, 4, 5	91.9	93.1
FIPV UCD6	2.4	3, 4, 5	84.1	83.8
FIPV UCD8	8.9	2, 3, 4, 5, 6, 7	89.5	98.7
FIPV UCD9	4.5	3, 4, 5, 6, 7	91.5	99.5
FIPV UCD10	4.5	3, 4, 5, 6, 7	91.5	99.4
FIPV TN406	1.7	3, 4, 5(p)	92.8	92.4
FIPV 79-1146	8.9	2, 3, 4, 5, 6, 7	77.4	76.6
CCV Insavc	8.9	2, 3, 4, 5, 6, 7	72.1	70.1

Genetic comparison of 1.2–8.9 kb in the 3' one-third of the genome strongly suggests that FIPVs evolve as simple mutations from FECVs endemic in the same environment. Although the precise genetic defect has not been identified, deletions or nonsense mutations within the 3c open reading frame (ORF), and less often point mutations in the 7b ORF, are present in FIPVs but not in their FECV counterparts.

RESULTS

Comparison of FECV and FIPV strains

Nucleotide sequence comparisons spanning a region of 2.3 to 8.9 kb were made between eight different FIPV strains, one previously sequenced strain of canine coronavirus [CCV (CCV Insavc) (Horsburgh *et al.*, 1992)], and two strains of FECV (RM and UCD) (Table 1). The two FECV isolates shared 89.7% nucleotide sequence identity in the 3' one-third of their genomes comprising all their genes except for the replicase gene (Fig. 1). FIPV UCD8, UCD9, and UCD10 were closely related to FECV RM (98.7–99.5%), while FIPV UCD3 was strongly related to FECV UCD (98.7%). The genetic relationships between the FECVs and other FIPV isolates and CCV Insavc were significantly more distant (70.1 to 93.1%). The sequence identities among FIPVs from different geographic areas were in the same range as those between them and the FECVs.

The degree of relatedness mirrored the origins of the various viruses. FECV RM was isolated from the same group of cats as FIPV UCD8, FIPV UCD9 and UCD10 were obtained from cats infected with FECV RM, while FECV UCD and FIPV UCD3 originated from the same cattery. FIPV UCD1, UCD2, UCD4, UCD5, UCD6, TN406,

and 79-1146 were all from catteries geographically distinct from where FECV RM and UCD were first found. The serotype II isolates FIPV 79-1146 and UCD6 are more distantly related to the FECVs than the serotype I FIPVs. Nucleotide sequence comparisons demonstrated that closely related pairs of different biotypes exist and that feline coronaviruses show a relatively high degree of genetic diversity. The pairwise comparisons form the basis for phylogenetic analysis, which clearly supports the evolutionary relationships.

The origin of FIPV strains from FECVs

In order to demonstrate that FIPVs originated from FECVs, mutations within the major structural genes were determined. FIPV UCD3 had a major in-frame deletion of 123 nucleotides in the S gene compared to FECV UCD and other strains. This was confirmed by sequencing several overlapping PCR fragments. Deletions of one and two single codons were found in the S genes of FIPV UCD3 and UCD8, compared to their respective FECVs, FECV UCD and RM (Fig. 2). Furthermore, FIPV UCD3 had a deletion of 100 nucleotides in the 3c gene (see below). The occurrence of deletion mutations in FIPVs and not FECVs of each pair confirmed the hypothesis that FIPVs arose from FECVs. The deleted sequences are still present in other strains. Based on phylogenetic analysis these strains diverged from a common ancestor before the epide-

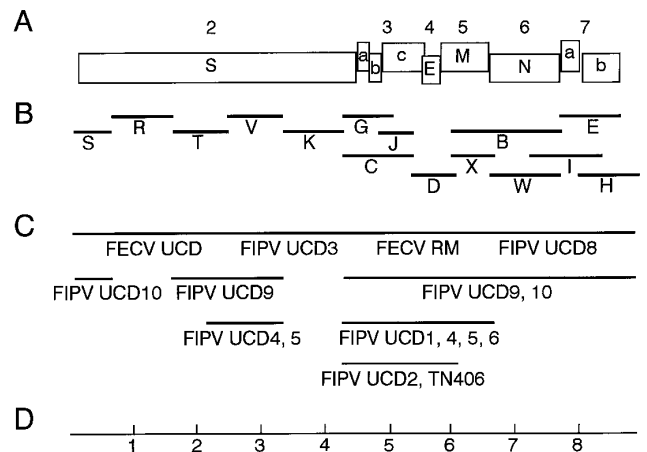


FIG. 1. Genomic organization and sequencing strategy of feline coronaviruses. (A) Genomic organization of the 3' one-third of a representative feline coronavirus. Open reading frames (ORFs) are represented by boxes. The genes are indicated with a number (3 and 7) or with an acronym corresponding to the encoded protein: S for spike, E for envelope, M for membrane, and N for nucleocapsid protein gene, respectively. Gene 3 and gene 7 contain 3 (a, b, and c) and 2 (a, and b) ORFs, respectively. (B) cDNA fragment amplification map. PCR-amplified and cloned cDNA fragments are indicated by a thick line, each coded by a capital letter. Corresponding primers that were used to generate the fragments are listed in Table 3. (C) Extent of sequence information obtained for the listed feline coronavirus strains. (D) Scale representing kilobases.

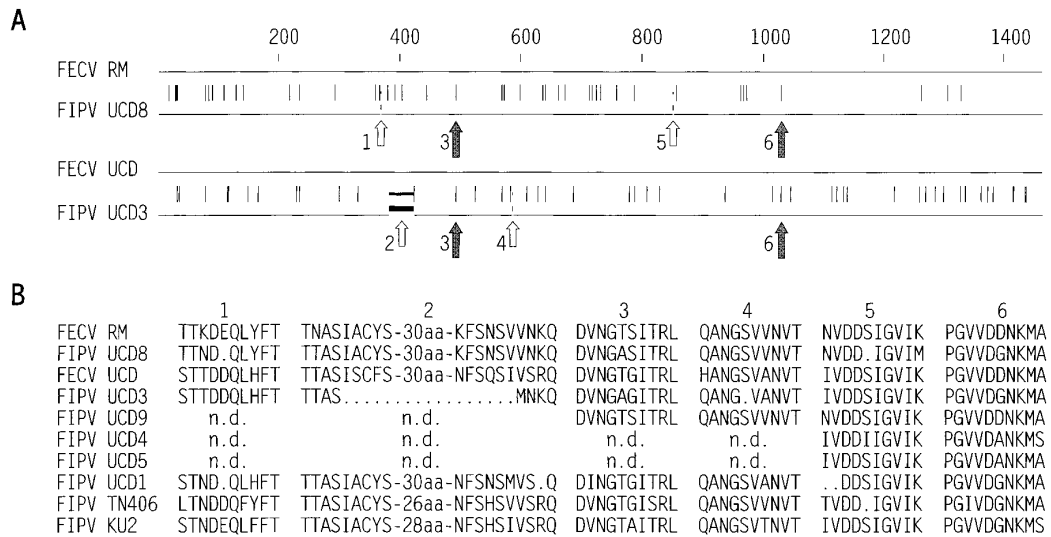


FIG. 2. Deletions and point mutations in FIPV spike protein genes. (A) Graphical representation of the alignment of the spike protein amino acid sequences of FECV RM/FIPV UCD8 and FECV UCD/FIPV UCD3 using the program GAPSHOW. Mismatches are indicated by a vertical line, and deletions by an interrupted vertical line. Mutations listed in B are numbered from left to right and indicated by open arrows for deletions and filled arrows for amino acid changes occurring in both pairs. (B) Multiple sequence alignments surrounding numbered mutations in A. Mutations 1, 2, 4, and 5 are deletion mutations in either one of the FIPV strains. Mutations 3 and 6 are identical changes occurring in both pairs. Note that mutations 3 and 4 (and 2 also) involve potential N-linked glycosylation sites. Amino acids are listed in single-letter code.

miologically related FIPV/FECV isolates segregated. Therefore, the mutations are deletions in the FIPV genomes and not insertions in the FECV genomes.

The search for biotype-specific genotypic markers in the 3' one-third of the genomes of the FIPVs UCD8 and UCD3 compared to the FECVs RM and UCD, respectively, resulted in the identification of only two consistent amino acid changes, both located in the S gene (mutations 3 and 6, Fig. 2). The same changes were not found in the S gene of FIPV UCD9, which indicated that there was not one single consistent point mutation in the major structural protein genes in the 3' one-third of the genome that could be directly linked to transition to the FIPV biotype. Mutation 3 (Fig. 2) was a threonine to alanine substitution which abolished a potential N-glycosylation site in FIPV UCD3 and UCD8. In FIPV strains UCD1, TN406, and KU2, this glycosylation site was conserved (Motokawa *et al.*, 1996). Mutation 6 was an aspartic acid to glycine substitution. The glycine residue was also present in FIPV strains UCD1, TN406, and KU2 (Motokawa *et al.*, 1996). In FIPV UCD4 and UCD5 an alanine residue was found in this position (Fig. 2B). The region in which mutation 6 can be found is highly conserved among coronaviruses, in particular of serogroup 1, but also to some extent in mouse hepatitis virus (MHV) and infectious bronchitis virus (IBV), representing the other two serogroups. In most cases the central part of this region contains two acidic residues, one of which is mutated in all FIPV strains, except for FIPV UCD9.

Potential genetic basis of the FIPV biotype

Two consistent mutations were identified in the 3' end of the ORF 7b of all three FIPVs derived from FECV RM and FIPV UCD8, UCD9 and UCD10, compared to FECV RM. Two nucleotide differences resulted in two amino acid changes. The C-terminal sequence –NQHYKTEL of FECV RM was changed to –NQHHRTTEL in all three FIPV strains (Fig. 3). However, the most significant genetic differences between corresponding FECVs and FIPVs were found in ORF 3c. FIPV UCD3 was found to have a deletion of 100 nucleotides in ORF 3c, while FIPV UCD9 had two deletions of 35 and 25 nucleotides, compared to FECV UCD and FECV RM, respectively (Fig. 4). FIPV UCD8 and UCD10, both derived from FECV RM, did not have deletions in ORF 3c. Seven additional FIPV isolates also showed mutations in ORF 3c (Fig. 4C). FIPV UCD1 had a deletion of 25 nucleotides. FIPV TN406 had a deletion of 62 nucleotides covering the 3' end of the 3b gene and the 5' end of the 3c gene. The deletion resulted in readthrough of the ORFs 3b and 3c. FIPV UCD2 had a small deletion of 4 nucleotides. In addition this strain had mutations that affected the intergenic sequence upstream involved in mRNA synthesis of the 3c gene. The intergenic sequence was also mutated in both type II feline coronaviruses FIPV 79-1146 and FIPV UCD6. FIPV UCD4, UCD5, UCD6, and 79-1146 had nonsense mutations, resulting in early termination. Animal-passaged FIPV UCD4 also exhibited several overlapping deletion mutations in ORF 3c. Remarkably, the smallest of the deletions in FIPV UCD4 was identical to the 35-nucleo-

tion deletion in FIPV UCD9. The 25-nucleotide deletion in FIPV UCD1 was the same as the deletion found in FIPV UCD9 and overlapped with the 100-nucleotide deletion in FIPV UCD3. All deletions resulted in a shift to a different reading frame that soon terminated, resulting in severe truncation of the 3c ORF.

Genetic comparisons of FECVs and FIPVs in multiple cat households

Two littermate kittens from a shelter (cats 6-51 and 6-53) developed FIP during the study period. RNA was isolated from affected tissues of these FIP-affected cats, as well as from feces of a healthy littermate (cat 6-49). The tissue- and feces-derived viral RNAs were partially sequenced and the sequences of pairs FECV 6-49/FIPV 6-51 and FECV 6-49/FIPV 6-53 were compared. The genetic homology between this FECV and the FIPVs was 98.7 and 97.3% and was significantly higher than that between unrelated feline coronavi-

FECV RM	M I V L L L V C V F L A N G I K A T T V Q H D L H E H P V L
FIPV UCD8
FIPV UCD9
FIPV UCD10
FECV RM	T W D L L Q H F I G H T L Y I T T H Q V L A L P L G S R V E
FIPV UCD8
FIPV UCD9
FIPV UCD10
FECV RM	C E S V E G F N C T W P G F Q N P A H D H I D F Y F D L S N
FIPV UCD8
FIPV UCD9
FIPV UCD10
FECV RM	P F Y S F V D N F Y I V S E G N Q R I N L R L V G A V P K Q
FIPV UCD8 S
FIPV UCD9
FIPV UCD10
FECV RM	K R L N V G C Y N S F A V D L P F G T Q I Y H D R D F Q H P
FIPV UCD8
FIPV UCD9 Y
FIPV UCD10 L
FECV RM	V N G R H L E C T H R V Y F V K Y C P H N L H G Y C F N E K
FIPV UCD8
FIPV UCD9
FIPV UCD10
FECV RM	L K V Y N L T Q L R S K K V F D R I N Q H Y K T E L
FIPV UCD8 H R
FIPV UCD9 H R
FIPV UCD10 H R

FIG. 3. Multiple sequence alignment of 7b protein amino acid sequences of FECV RM and its three derived FIPV strains. The FIPV strains all have three amino acid differences with FECV RM, two of these differences are the same in each case, and involve two residues near the carboxy terminus of the protein.

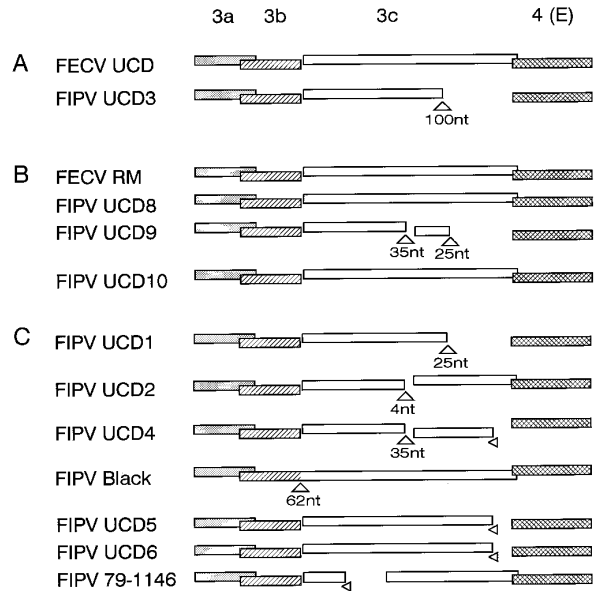


FIG. 4. Deletion and nonsense mutations in ORF 3c of FIPV strains. (A) FIPV UCD3 has a deletion of 100 nucleotides in ORF 3c compared to its predecessor FECV UCD. (B) Of the three FIPV strains derived from FECV RM, two have an intact ORF 3c. FIPV UCD9 has deletions of 35 and 25 nucleotides. (C) Additional FIPV strains without known FECV predecessor contain nonsense and/or deletion mutations. FIPV UCD1 and UCD4 contain deletions of 25 and 35 nucleotides, respectively. FIPV TN406 has a deletion of 62 nucleotides covering the 3' end of the 3b gene and the 5' end of the 3c gene. The deletion resulted in readthrough of ORFs 3b and 3c. FIPV UCD2 has a small deletion of 4 nucleotides. FIPV UCD4, UCD5, UCD6 and 79-1146 all have nonsense mutations. Note that the 35-nucleotide deletions in UCD9 and UCD4 are in the same position. The same is true for the 25-nucleotide deletions in FIPV UCD9 and UCD1 which overlapped with the 100-nucleotide deletion in UCD3. The nonsense mutations in FIPV UCD5 and UCD6 are the same. Deletions are indicated with large triangles pointing up; nonsense mutations are indicated with small triangles pointing left.

ruses (Table 2). The 7b ORFs were intact and colinear in all of three strains. However, there were specific mutations in the 3c ORFs of the FIPVs but not in the FECV (Fig. 5). Deletions were present in the 3c ORFs of FIPV 6-51 and 6-53. Both deletions resulted in a shift to a different reading frame like those of other FIPV strains (see above). Remarkably, the deletions in FIPV 6-51 and 6-53 were distinct, demonstrating that these two FIPVs were independently derived from FECV and not spread horizontally. These FIPVs, from two diseased siblings, were both related to a fecal isolate from a healthy littermate (cat 6-49).

DISCUSSION

Feline coronaviruses have been classified into two serotypes, I and II, and two biotypes, FIPV and FECV (Pedersen, 1995; Pedersen *et al.*, 1984). The predominant serotype I has a distinct spike protein, while the less common serotype II has a CCV-like spike protein

TABLE 2

Percentage Nucleotide Sequence Difference between Selected FECV and FIPV Strains

	FECV		
	UCD	RM	6-49
FIPV			
UCD3	0.9	8.3	7.9
UCD8	8.7	0.9	8.2
UCD9	8.1	0.4	7.9
UCD10	8.0	0.4	7.9
6-51	7.8	7.7	1.3
6-53	6.9	7.1	2.7

(Vennema *et al.*, 1995). All of the various FIPVs/FECVs studied herein, except for FIPV UCD6 and 79-1146, were serotype I feline coronaviruses. FIPV UCD6 and FIPV 79-1146 belonged to serotype II based on the close genetic relationship of their spike proteins to CCV (Vennema, manuscript in preparation).

Sequence comparisons demonstrated that FECVs and FIPVs from the same group of cats were very closely related, while significant genetic variation existed between FECVs and FIPVs that were from different geographic areas. This indicated that feline coronaviruses manifest a high degree of genetic drift, independent of their serotype and biotype.

Sequence comparisons demonstrated that FIPVs originate as simple and relatively frequent mutations from FECVs endemic in the same environments. The most compelling evidence to support this notion is the close relation between FECV RM and its three derivatives FIPV UCD 8, 9, and 10. FIPV UCD8 occurred in the same closed group of cats into which FECV RM had been inadvertently introduced and FIPV UCD9 and UCD10 were obtained directly from cats experimentally infected with FECV RM. The origin of FIPVs as mutations of FECV is also supported by epidemiologic studies: FECV infection is rampant in multiple cat households, while FIP is sporadic (Foley *et al.*, 1997). The conclusion from the combined epidemiologic and molecular data could be that FIPV, unlike FECV, is not transmitted from cat to cat but emerges as mutant of FECV within the cat in which it causes disease.

The potential of a FECV to undergo minor mutations that radically change its biotype is also consistent with what is known about coronaviruses in general. Their high mutability has led to a large number of strains of each species, with each strain differing in cell tropism and disease potential (Compton *et al.*, 1993). As an example, transmissible gastroenteritis virus (TGEV) of swine, a virus closely related to FECV, spontaneously mutated in the 1980s, giving rise to the porcine respiratory coronavirus (PRCV), which has displaced TGEV in many areas (Laude *et al.*, 1993). The TGEV to PRCV

mutation involves deletions in the S gene and point mutations and/or deletions in gene 3 (Rasschaert *et al.*, 1990) (Wesley *et al.*, 1991). Even though PRCV is a relatively new virus, numerous different strains of PRCV have evolved in a decade or less (Vaughn *et al.*, 1994). Most, if not all, PRCV strains appear to be independently derived from TGEV. Recombinations between various strains of coronaviruses have also been documented. New pathogenic variants of MHV have arisen from recombination *in vitro* and *in vivo* between MHV 2 and MHV A59 (Keck *et al.*, 1988a,b). New strains of IBV have apparently developed from recombination between field and attenuated live vaccine viruses (Kusters *et al.*, 1990). Recombinations have also occurred between different species of coronaviruses. Serotype II feline coronaviruses have apparently resulted from recombination in the field between serotype I feline coronaviruses and canine coronaviruses (Vennema, manuscript in preparation).

Extensive sequence analysis and comparison did not point out a single mutation within a single gene that was consistently associated with the transition from FECV to FIPV. All but 1 of the FIPV strains sequenced here and earlier had a specific mutation in the spike gene compared to the spike genes of two FECV strains. All 3 FIPV strains derived from FECV RM had two consistent differences compared to FECV RM in the carboxy-terminal part of the 7b protein gene. This part is known to play a role in the secretion of the 7b protein (Vennema *et al.*, 1992a). The effect of the particular amino acid sequences in FECV RM and its derived FIPVs on secretion of the 7b protein are currently under investigation. Differences affecting the genomic organization in FIPVs compared to FECVs were noted in the 3c ORF. A total of 13 different FIPV strains were ultimately analyzed and the 3c ORF was mutated from wild type in all but 2, FIPV UCD8 and UCD10 (both related to FECV RM).

The 3c ORF is the last of three ORFs in the gene 3 region of FECVs and closely related CCV. The ORF 3c contains a nonsense mutation in CCV Insavc (Horsburgh *et al.*, 1992), in FIPV 79-1146, and in CCV K378 (Vennema and Rossen, unpublished data), but is intact in most, but not all, TGEV and PRCV strains (Vaughn *et al.*, 1994). In

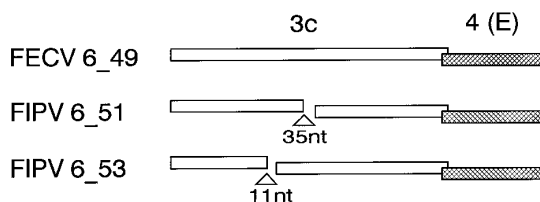


FIG. 5. Deletion mutations in ORF 3c from FIPV strains in a shelter. Independent deletion mutations in 3c ORFs of FIPV 6-51 and 6-53 compared to FECV 6-49 from three littermates. Note that the 35-nucleotide deletion in FIPV 6-51 is in the same position as the 35-nucleotide deletions in FIPV UCD4 and UCD9.

TGEV and PRCV the middle ORF is missing; here the ORF corresponding to ORF 3c of feline and canine coronaviruses is designated ORF 3b. No function has yet been associated with this ORF and no protein encoded by this ORF has been demonstrated in infected cells. *In vitro* translation studies of mRNA from TGEV-infected cells demonstrated a 27-kDa primary translation product (Jacobs *et al.*, 1986).

The ORF 7b is present in feline and canine but not in porcine coronaviruses (Vennema *et al.*, 1992b; de Groot *et al.*, 1988). The 7b protein of FIPV 79-1146 is a secreted nonstructural protein which readily induces an immune response during natural and experimental infections (Vennema *et al.*, 1992a). In the tissue culture-adapted serotype II strain, FECV 79-1683 ORF 7b is mutated by deletion, while the same gene in the highly virulent FIPV 79-1146 strain is intact (Vennema *et al.*, 1992b). However, the non-tissue culture-adapted strain FECV UCD had an intact ORF 7b (Herrewegh *et al.*, 1995). The same was true for the additional FECV strains sequenced for the present study. Sequencing of attenuated derivatives of virulent FIPV strains showed a good correlation between deletions in the 7b gene and attenuation of virulence (Herrewegh *et al.*, 1995). This latter observation indicates that the 7b protein is important for virulence. If this is true than this virulence associated with an intact 7b gene must be suppressed somehow in the non-tissue culture-adapted FECVs. In view of the data presented here it could be speculated that the intact 3c gene present in these FECVs is involved in this suppression.

What is the frequency of FIP-inducing mutations during an outbreak of FECV? Only a half-dozen cases of FIP occurred among several thousand specific-pathogen-free cats that were inadvertently infected with FECV-RM (Hickman *et al.*, 1995). The incidence of FIP, however, was 10% among a small group of chronically FIV-infected cats that were experimentally infected with the same strain of FECV (Poland *et al.*, 1996). The incidence of FIP among large pedigreed catteries and shelters averages 5% and greater (Pedersen, 1995; Postorino-Reeves, 1995; Foley *et al.*, 1997). Assuming that most cats that are experimentally infected with FIPV will ultimately die from FIP, it can be concluded that the FIP incidence rate is an approximation of the actual FECV to FIPV mutation rate. The actual rate of mutation may be affected by at least three different factors: (1) the level of FECV replication (the greater the more chance for mutations to occur) (Poland *et al.*, 1996), (2) the acquired or inherited resistance of the particular breed, bloodline, or individual cat to the mutant virus (Foley and Pedersen, 1995; Poland *et al.*, 1996), and (3) the strain of FECV and the ease with which it can be mutated.

MATERIALS AND METHODS

Virus strains

Six pairs of FECV and FIPV, each originating from the same cattery or shelter, were obtained from naturally or experimentally infected cats. The first pair, FECV UCD/FIPV UCD3, was recognized as being genetically related by (Herrewegh *et al.*, 1995). FECV UCD (Pedersen *et al.*, 1981) and FIPV UCD3 (Pedersen and Floyd, 1985) originated from the same experimental cattery but at different times. The second pair, FECV RM/FIPV UCD8 was isolated from a single closed specific-pathogen-free cat population that had been inadvertently infected with FECV and subsequently suffered sporadic cases of FIP (Hickman *et al.*, 1995). The isolates were obtained an estimated 2 years after the initial introduction of FECV into the colony. Two new strains, designated FIPV UCD9 and UCD10, occurred when chronically feline immunodeficiency virus (FIV)-infected cats were experimentally infected with FECV RM, at 8 and 10 weeks after inoculation, respectively (Poland *et al.*, 1996). This resulted in a third (FECV RM/FIPV UCD9) and fourth (FECV RM/FIPV UCD10) pair. Pairs 5 and 6 were isolated at the same time from littermate kittens in a shelter in Davis, California, and designated FECV 6-49/FIPV 6-51 and FECV 6-49/FIPV 6-53.

Unpaired FIPV isolates used in the study included FIPV UCD1 (Pedersen *et al.*, 1981), FIPV UCD4 and UCD2 (Pedersen and Floyd, 1985), and FIPV TN406 (Black, 1980). FIPV UCD5 and UCD6 were isolated from FIP-affected cats originating from two different pedigreed Persian catteries in northern California; the viruses (in ascitic fluid) were passaged one time in specific-pathogen-free cats to confirm their virulence and saved in the form of ascites and cell-free omental extracts.

Among these various FECVs/FIPVs, only FIPV UCD1, UCD2, UCD3, UCD4, and TN406 could be propagated in tissue culture. Tissue culture-adapted strains used in this study were passaged no more than five times in cell culture after being taken from affected animals. Animal-propagated strains were passaged one to three times in experimental cats and used in the form of either tissue homogenates (FIPV biotypes) or fecal extracts (FECV biotypes).

cDNA synthesis, PCR amplification, cloning, and sequence analysis

A map of the 3' one-third of a representative FECV/FIPV genome is given in Fig. 1A. This portion of the genome, approximately 8.9 kb, contains all the structural protein genes [spike (S), envelope (E), membrane (M), and nucleocapsid (N)] and two presumably nonstructural genes, 3 and 7, containing three (a,b,c) and two (a,b) open reading frames (ORFs), respectively. Figure 1B demonstrates the overlapping PCR fragments, designated by letter, that were used to

TABLE 3
Primers Used for RT-PCR of Feline Coronavirus Genes

Code	Sequence 5'→3'	Orientation	Fragment
D00	GGAAGGGTAAGATACTCATTAG	+	S
RD600	CTCTGGTTGAATACACACTG	-	S
D600	CTTAATTTTGGAGATGGAGG	+	R
RD1400	TAGGCTATGGTCCAAAAGCC	-	R
D1380	CTATTAGCTCAGTTGAGCAGT	+	T (UCD3)
D14	GTCTACCACCTATTAAGAGTGT	+	T
R24	CTCATCGTTAATCACAGCTGC	-	T
D1900	GAACCCAGCTGTTGCAGGTT	+	V (UCD4,5)
D24	TGCCATGTGATCTAACAGCAC	+	V
R34	GCCTCCTATTAAGAGGCAG	-	V
D34	GGCATAATGGTTTTACCTGGTG	+	K
R45	CCGATTAGTAGCCACACATAC	-	K
D38	CTTCCAGCTATTAGTAGTTC	+	K seq.
223	AATGGCCTTGGTATGTGTGG	+	C,G
M32	CCTGAGAAAAGGCTGCATTGT	-	C,J
R54	CTTTTAATACTAGCACTAACAAAC	-	C seq.
264	GTACAGCGATGCTGAACTCTGG	-	G
D53	GTGATTGCTAACACACACCAC	+	J
240	ACATGGCAGAGCTGCACCGTTT	+	D
212	TAATGCCATACACGAACCAGCT	-	D
197	TCTTGCTAACTGGAACCTCAGCTGG	+	B,X
R70	TGACGCGTTGTCCCTGTGTG	-	X
D69	ACTCAACAGAAGCACGTA CTG	+	W
195	CAGCATGGAGAAAAACGAGCATGCG	-	B,W
S75	AGGTCTGGTTCACAGTC	+	B,W seq.
D76	CTCAATCTAGAGGAAGACACC	+	I
177	CACTTACAATATAGAAATTATCTAC	-	I
178	GATGACACACAGGTTGAG	+	E
R205	GTTTTAAACATCGGGTTGCC	-	E
C202	GGGTTTTCTGCTATACATTG	+	H
211	CACTAGATCCAGACGTTAGCTC	-	H

sequence this portion of the genome. The coronavirus strains that were included in this study, and the PCR fragments sequenced for each, are presented schematically in Fig. 1C. The entire 3' one-third of the genome of two independent FECV isolates, FECV UCD and FECV RM, was sequenced, as well as identical segments of their respective FIPVs, FIPV UCD3 and FIPV UCD8. FIPV UCD9 and UCD10, both derived from FECV RM, were sequenced for 4.5 kb, downstream of the S gene and starting with fragment C. Several fragments of the S gene were also sequenced for these two strains. Sequences of a 2.5-kb segment covering gene 3 (ORFs 3a-3c), the E gene, and the M gene were determined for the remainder of the FIPV strains.

Genomic RNA was isolated from the materials mentioned above using published methods (Chomczynski and Sacchi, 1987). cDNA synthesis was performed with M-MLV reverse transcriptase (RT) and followed by polymerase chain reaction (PCR) amplification with *Taq* DNA polymerase. All enzymes were used according to the manufacturer's instructions (Promega Corp., Madison, WI). Both reactions were primed with specific primers (Table 3). Primers were derived from the sequence of

FIPV 79-1146 (de Groot *et al.*, 1988) (Vennema *et al.*, 1991). Primers derived from the spike gene of FIPV 79-1146 did not work with type I FECVs/FIPVs. A set of overlapping fragments of the spike gene of FIPV UCD3 was obtained as described previously (Vennema *et al.*, 1995). This sequence was used to design primers for the spike genes of other type I FECVs/FIPVs. PCR fragments were cloned using TA-cloning kits (Invitrogen or Novagen) according to the manufacturer's instructions. Plasmid DNA was sequenced using the Sequenase 2.0 kit (U.S. Biochemicals) with [³²P]dATP (Amersham) and standard or reverse M13 sequence primers, PCR primers, or internal primers. Sequences from FECV/FIPV pairs 5 and 6 were obtained by direct automated sequencing of PCR products. Sequence data were analyzed using the University of Wisconsin Genetics Computer Group sequence analysis software package (Devereux *et al.*, 1984) (Program Manual for the Wisconsin Package, Version 8, 1994).

ACKNOWLEDGMENTS

We are grateful for Solvay Animal Health, Inc. (Mendota Heights, MN) for their financial support of the Center for Companion Animal Health,

School of Veterinary Medicine, University of California (Davis, CA) and in turn to numerous individual donors. We also acknowledge Jeff Carlson, Steve Ramirez, and Tami Citron for their excellent technical support.

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