Feline Coronavirus Type II Strains 79-1683 and 79-1146 Originate from a Double Recombination between Feline Coronavirus Type I and Canine Coronavirus

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Recent evidence suggests that the type II feline coronavirus (FCoV) strains 79-1146 and 79-1683 have arisen from a homologous RNA recombination event between FCoV type I and canine coronavirus (CCV). In both cases, the template switch apparently took place between the S and M genes, giving rise to recombinant viruses which encode a CCV-like S protein and the M, N, 7a, and 7b proteins of FCoV type I (K. Motowaka, T. Hohdatsu, H. Hashimoto, and H. Koyama, Microbiol. Immunol. 40:425–433, 1996; H. Vennema, A. Poland, K. Floyd Hawkins, and N. C. Pedersen, Feline Pract. 23:40–44, 1995). In the present study, we have looked for additional FCoV-CCV recombination sites. Four regions in the *pol* gene were selected for comparative sequence analysis of the type II FCoV strains 79-1683 and 79-1146, the type I FCoV strains TN406 and UCD1, the CCV strain K378, and the TGEV strain Purdue. Our data show that the type II FCoVs have arisen from double recombination events: additional crossover sites were mapped in the ORF1ab frameshifting region of strain 79-1683 and in the 5′ half of ORF1b of strain 79-1146.

Coronaviruses, pathogens of mammals and birds, are enveloped RNA viruses with a nonsegmented, positive-stranded genome that is 27 to 32 kb in length (for a review, see reference 41). The 5′ two-thirds of the genome is occupied by open reading frames (ORFs) 1a and 1b, the expression of which yields large polyproteins from which the viral replicase is derived. Downstream of ORF1b, there are 8 to 10 smaller ORFs which are expressed through subgenomic mRNAs. These code for the structural proteins S, E, M, and N and for a number of presumptive nonstructural proteins (reviewed in reference 32) (for a schematic representation of a coronavirus genome, see Fig. 1).

One of the most intriguing aspects of coronavirus replication is the occurrence of high-frequency homologous RNA recombination (30, 33; for reviews, see references 28 and 29). This process is thought to be mediated by a "copy-choice" mechanism (7, 24, 33). In this model, the RNA-dependent RNA polymerase together with the nascent cRNA product dissociates from the original template, reassociates with another template at the identical or nearly identical position, and subsequently recommences RNA synthesis. Template switching occurs randomly (1, 44) and, in the case of mouse hepatitis virus, at an estimated frequency of approximately 1% per 1,300 nucleotides, i.e., 25% for the entire 32-kb genome (2). Homologous recombination not only allows the rapid exchange of beneficial mutations but also may serve as a correction mechanism counteracting Muller's ratchet (6, 28). Recombination of coronavirus genomes has been observed in tissue culture (30, 33), in experimentally infected animals (23), and in embryonated eggs (26). In the case of infectious bronchitis virus, there is evidence for homologous recombination occurring in

the field (22, 27, 47). Recent findings suggest that homologous RNA recombination may also be an important factor in the evolution of feline coronaviruses (FCoVs) (16, 37, 46).

Together with porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCV), and human coronavirus (HCV) 229E, FCoVs form a separate cluster within the genus *Coronavirus*. They are commonly associated with mild enteric infections but are also associated with feline infectious peritonitis, a fatal immune-mediated disease of both domestic and wild felidae (for a review, see reference 8). Two FCoV serotypes exist; they can be distinguished by an in vitro virus neutralization assay using either type-specific feline sera (39) or monoclonal antisera directed against the S protein (18–20). The prevalence of these serotypes has not been studied exten-
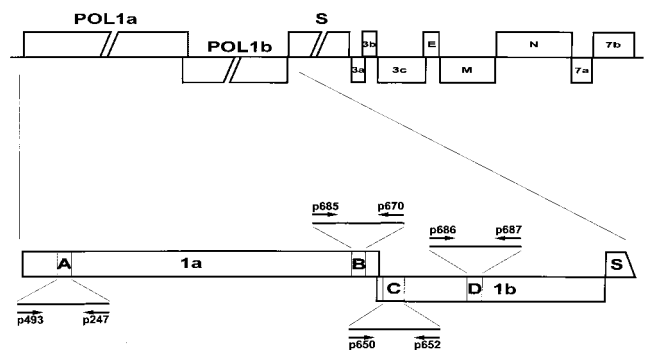
FIG. 1. Genomic organization of FCoV and an outline of the strategies used for the amplification of specific regions in the polymerase genes. (Upper panel) Schematic representation of the genome of FCoV strain 79-1146 with the various genes represented by boxes. The genes for the polymerase (POL1a, POL1b), the structural proteins S, M, E, and N, and the presumptive nonstructural proteins 3a, 3b, 3c, 7a, and 7b are indicated. (Lower panel) Schematic presentation of ORF1a and ORF1b, indicating the locations of regions A through D. The positions and polarities of the oligonucleotide primers used for RT-PCR amplification and sequence analysis are indicated.

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FIG. 2. Comparative sequence analysis of the M gene. An alignment is shown of the nucleotide sequences of the FCoV type I strains UCD1 and TN406 (37), the FCoV type II strains 79-1683 (15, 37) and 79-1146 (45), the CCV strain Insavc-1 (21), and the TGEV strain Purdue (40). Alignments were performed by using the PILEUP program (University of Wisconsin) (17). Only the nucleotides differing from the consensus sequence are depicted. Those nucleotides of the 79-1683 sequence which are identical to those of the CCV and TGEV sequences or to residues of at least two other FCoV strains are boxed. Template switching between the CCV and FCoV type I genomes apparently occurred in the region formed by nt 327 through 376 (underlined).

sively but according to a recent sero-survey, type II FCoVs may account for 20 to 30% of the feline infectious peritonitis cases in Japan (18).

Interestingly, serologic studies suggested that the type II FCoVs are more closely related to CCV than to type I strains; i.e., immunodominant neutralization epitopes shared by the S proteins of CCV and FCoV type II are absent in the S protein of FCoV type I. Puzzlingly, however, comparative sequence analysis of the ORF7a-ORF7b region (Fig. 1) revealed that in the 3′-most 1.5 kb of the genome, type I and II FCoVs are

more closely related to each other than they are to CCV (16). These seemingly conflicting observations could be reconciled upon genetic comparison of the region between ORF1b and ORF7a. Whereas the type I and II viruses are also closely related in the genes encoding M and N proteins, the S genes are markedly different (36, 37, 46). In fact, the S proteins of the type II FCoV strains 79-1146 and 79-1683 bear a much greater amino acid sequence identity to those of CCV (91%) and TGEV (81%) than to the S proteins of the type I strains TN406, UCD1, and Ku2 (46%) (36, 49). The data strongly

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Region A
              1
79-1683                              w   c        t          c              g  t      c  c
79-1146          a                       c        t          t              a    c    c  c
TN406              g                      ag                  c              a      c   c  t
UCD1                                     a                    t              g    c    c  t
CCV        aac        t gac  c  c     a        t  a           g  t      a  g  t      t  t  g    t  c
TGEV     g  aaca    g  c gac  c     a        t  a           g  g      a  a        t  t  g    tt  c
Consensus AACCTGTGTTTGTAGTTTCATCTAATAGTGTTAAGGATCTCGTTGACAAGTGTGT-AAAATTCTTGTGAA-GCCTTTGATGT-TT-ACACAGACAATTAC

79-1683              g        g  t    t                        c  c                   g
79-1146    c          a        g                               c  t        t                        t
TN406                g        a                                c  c                             c
UCD1                 a        g  t    t                        t  c             c    a
CCV          t   a  t  a  c  a                 g        c  t  g  c  t  t          a
TGEV       a  t    a  t    c  a                 g        c  t  g  c  t  t          a                  c
Consensus AATTGCAGGTGT-GAGGCTAA-TGCTTTGTGCTTGGTTCTAAATACTTGCTTTTTAA-AA-GCACTTGTCAAGCTTGTCAGTGTTAAAATACTTGGCAAG

                                    233
79-1683           g                  c
79-1146           a
TN406             g                        t  n
UCD1           g  g    c      a
CCV        ag  a  g      c  a                t
TGEV       ag  a  g        at                t
Consensus AGACA-AAAGGTCTTGATAGTGCATTCTTTGCC


Region B
              1
TN406                    c                                                              c
UCD1                                          g                                 t    t
79-1146                  c              g                    g                       c              c
79-1683                               g                                                            c
CCV          c  t    c      ta               a  g      g  c      t  a  c ttt a      a  tc  c  t  a
TGEV         c  t    c      ta               a  g        c  g      g  a  a  tc      a  tc    c  t  a
Consensus AACCACAAAGTTGCAGAATAATGAAATCTTGCCAGGTAAACTTAAAGAGAAAGCTGTTAAAGCATCTGCTACAATTGATGGTGATGCTTATGGTAG-GGT

TN406                    t    t
UCD1                     g            t      c                                                   c
79-1146                  g
79-1683           c      t
CCV        g  t      ag      a    c  a        g              c        a    t          c                t      a
TGEV       g  t      a      a    a  a        g              c        a    t              g              t      a
Consensus AAAGCGCTTATGGCTTCTGAA-GTGGTAAGAGCTTTATATATGCATTTATAGCATCAGACAGCAACCTTAAGTATGTTAAATGGGAGAGCAACAATGATG

TN406                    g                  a              g  t      c              a      g
UCD1       t             a                                a  t      cc      c      g  t  a
79-1146                  g                  a              g  t      t              a      g
79-1683                  g                    a            g cc      c              a      g
CCV        c             a            t  c          c      a  c  g  t      c  c  c  gca      a
TGEV       t             a            t  c        c  c  c            a  c  g  t          c  g at  a
Consensus TAATACCTATTGAACTTGA-GCTCCATTGCGCTTTTATGTTGATGGTGTTAATGGTCCTGA-GT-AAATA-TTGTATTTTGTGAA-AGCTT-AACACTCT

                       330
TN406
UCD1                          cc
79-1146
79-1683
CCV                c  c      c
TGEV               c
Consensus TAGACGTGGTGCTGTTCTTGGTTATATCGG
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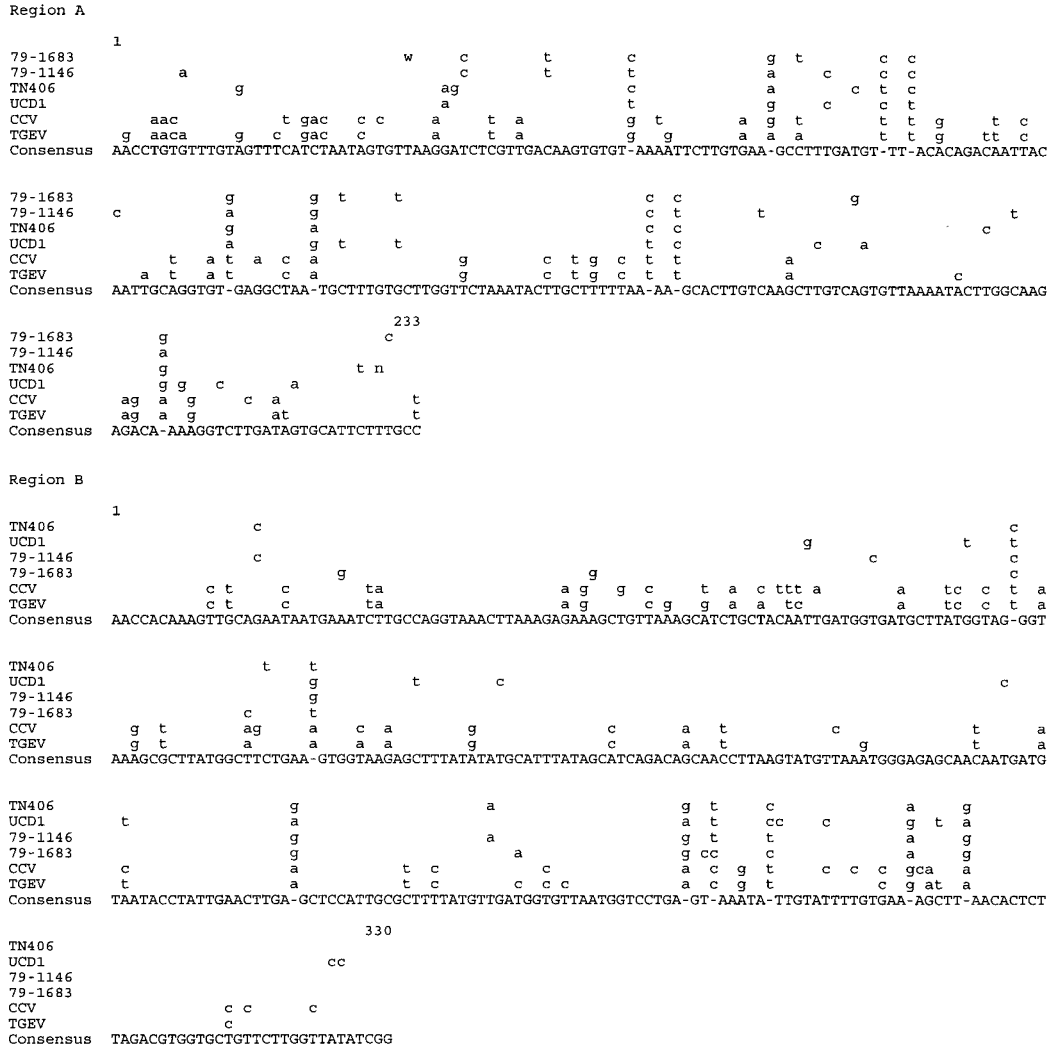
FIG. 3. Comparative sequence analysis of genomic regions A through D. Alignments were made by using the PILEUP program (University of Wisconsin), which scores identity between every possible pair (17). Alignment of the nucleotide sequences of regions A through D, as determined for the FCoV type I strains UCD1 and TN406, the FCoV type II strains 79-1683 and 79-1146, the CCV strain K378, and the TGEV strain Purdue (11), is shown. Only those nucleotides differing from the consensus sequence are depicted. n, r, and w represent nucleotides A/C/G/T, A/G, and A/T, respectively.

suggest that the type II FCoV strains have arisen from a homologous RNA recombination event between CCV and a type I FCoV (16, 37, 46). In the case of the FCoV type II strain 79-1146, the template switch has occurred within the E gene (44a). Data published by Motokawa et al. (37) indicated that for FCoV type II strain 79-1683, the template switch also took place between the S and M genes. From comparative sequence analysis of the ORF3a to ORF3c region (15) and a reevaluation of published data (37), it now appears that the crossover occurred in the M gene in the region formed by nucleotides (nt) 327 to 376 (Fig. 2). The fact that the template switch occurred at different sites in strains 79-1146 and 79-1683 suggests that they have arisen from separate recombination events.

One important question remaining was whether the type II strains had originated from single crossovers or from multiple template switches. Here, we have investigated the presence of additional switch sites in the *pol* genes. To this end, four regions in ORF1a and ORF1b, designated A through D (Fig. 1), were chosen for comparative sequence analysis of the type II FCoV strains 79-1146 and 79-1683, the type I FCoV strains Black and UCD1, the CCV strain K378, and the TGEV strain

Purdue (11). The FCoV and CCV strains were grown in fcwf-D cells, and viral RNA was isolated from infected cells as described previously (9). Oligonucleotide primers (Table 1) were designed after regions conserved in the *pol* genes of TGEV

TABLE 1. Oligonucleotide primers used for RT-PCR and cycle sequence analysis

Primer	Sequence (5′ to 3′)	Position (nt)[a]	Orientation
p247	CCGTTGTGGTGTCACATTAAC	1895–1875	Antisense
p493	GTAGTAGCATCACTCTGCACTTC	1575–1597	Sense
p650	TGGTGCTGTTGCTGAGCATGA	12574–12594	Sense
p652	ACAACTACTGGTACACCATCAA	13323–13302	Antisense
p670	TCAGTGGTTTACCAGCTTGCA	11920–11899	Antisense
p685	GCCCTTGAGTATAACTTGTGAG	11531–11552	Sense
p686	GAAACTGTGAAAGCTAAGGAGG	15500–15521	Sense
p687	ACACAATGAGATTTACCGCTACC	15972–15950	Antisense

[a] Numerical position on the genome of TGEV strain Purdue as counted from the first nucleotide of the sequence deposited under GenBank accession no. Z34093 (11).
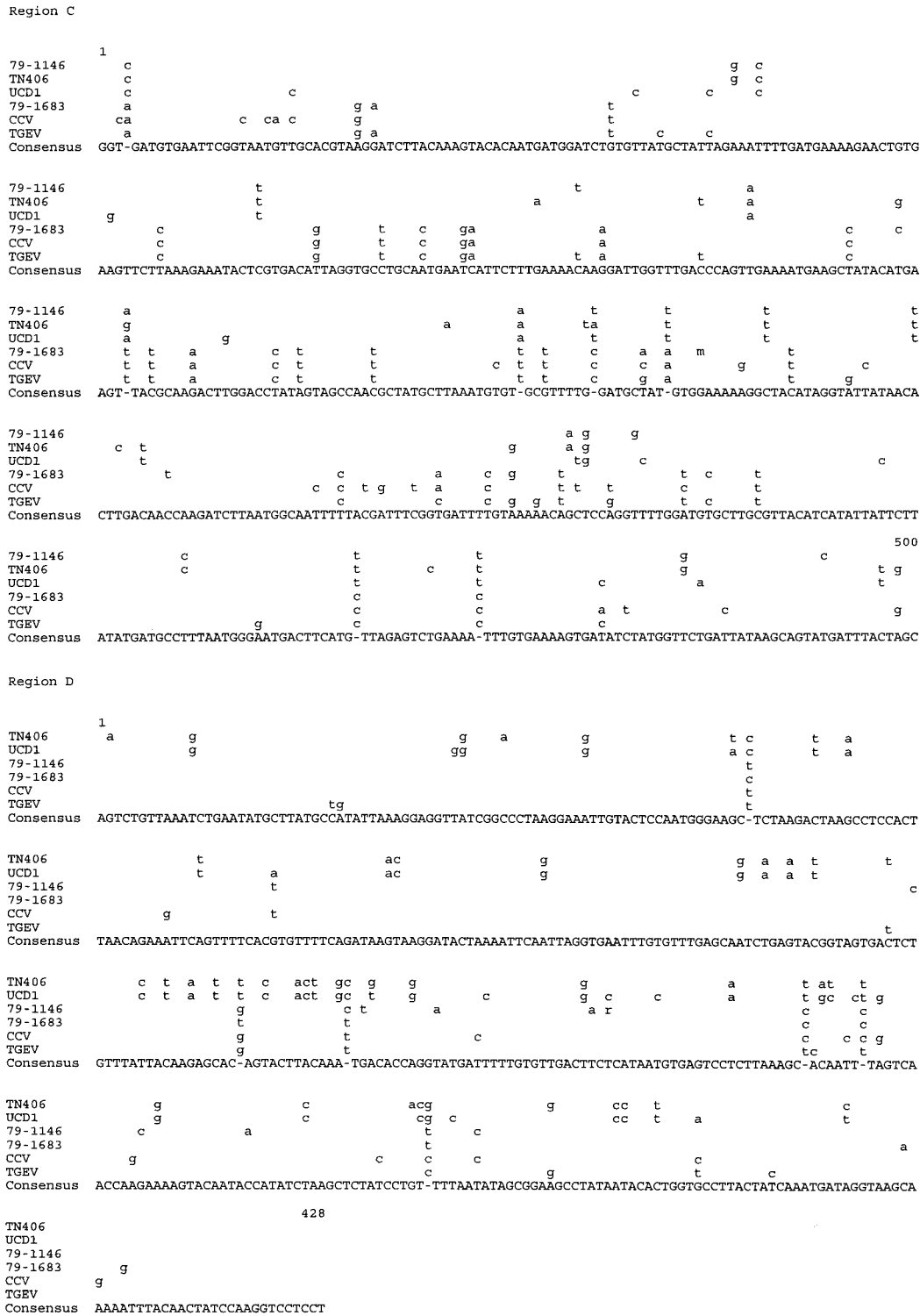
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Region C
           1
79-1146      c                                                              g c
TN406        c                                                              g c
UCD1         c                    c                               c       c   c
79-1683      a                        g a                     t
CCV         ca          c  ca c       g                       t
TGEV         a                        g a                     t    c    c
Consensus GGT-GATGTGAATTCGGTAATGTTGCACGTAAGGATCTTACAAAGTACACAATGATGGATCTGTGTTATGCTATTAGAAATTTTGATGAAAAGAACTGTG

79-1146            t                              t                  a
TN406             t                         a                   t    a                     g
UCD1      g       t                                             t    a
79-1683      c             g     t   c   ga          a                         c    c
CCV          c             g     t   c   ga          a                         c
TGEV         c             g     t   c   ga        t   a        t              c
Consensus AAGTTCTTAAAGAAATACTCGTGACATTAGGTGCCTGCAATGAATCATTCTTTGAAAACAAGGATTGGTTTGACCCAGTTGAAAATGAAGCTATACATGA

79-1146   a                             a     t     t        t                 t
TN406     g                         a    a     ta    t        t                 t t
UCD1      a           g                   a     t     t        t                 t
79-1683   t  t   a      c  t    t         t t   c     a  a  m     t
CCV       t  t   a      c  t    t       c t t   c     c  a        g    c
TGEV      t  t   a      c  t    t         t t   c     g  a        t    g
Consensus AGT-TACGCAAGACTTGGACCTATAGTAGCCAACGCTATGCTTAAATGTGT-GCGTTTTG-GATGCTAT-GTGGAAAAAGGCTACATAGGTATTATAACA

79-1146                                          a g     g
TN406      c  t                              g    a g
UCD1          t                                    tg    c                              c
79-1683        t                 c       a    c g    t       t  c    t
CCV                  c   c  t g  t  a     c       t t t       c     t
TGEV                     c            c     c g g t     g        t c    t
Consensus CTTGACAACCAAGATCTTAATGGCAATTTTTACGATTTCGGTGATTTTGTAAAAACAGCTCCAGGTTTTGGATGTGCTTGCGTTACATCATATTATTCTT

                                                                                     500
79-1146        c                t       t              g                 c
TN406          c                t   c   t              g                   t g
UCD1                            t       t          c        a              t
79-1683                         c       c
CCV                            c       c         a  t      c              g
TGEV           g               c       c          c
Consensus ATATGATGCCTTTAATGGGAATGACTTCATG-TTAGAGTCTGAAAA-TTTGTGAAAAGTGATATCTATGGTTCTGATTATAAGCAGTATGATTTACTAGC

Region D
          1
TN406     a          g                    g  a        g              t c     t  a
UCD1                 g                    gg           g              a c     t  a
79-1146                                                              t
79-1683                                                              c
CCV                                                                  t
TGEV                              tg                                 t
Consensus AGTCTGTTAAATCTGAATATGCTTATGCCATATTAAAGGAGGTTATCGGCCCTAAGGAAATTGTACTCCAATGGGAAGC-TCTAAGACTAAGCCTCCACT

TN406           t              ac                g           g a a t     t
UCD1            t        a      ac                g           g a a t
79-1146                  t                                                            c
79-1683
CCV          g         t
TGEV                                                                                 t
Consensus TAACAGAAATTCAGTTTTCACGTGTTTTCAGATAAGTAAGGATACTAAAATTCAATTAGGTGAATTTGTGTTTGAGCAATCTGAGTACGGTAGTGACTCT

TN406      c t a t t c  act gc g  g             g         a    t at   t
UCD1       c t a t t c  act gc t  g      c      g c    c  a    t gc  ct g
79-1146           g        c t      a          a r          c      c
79-1683           t        t                               c      c
CCV               g        t            c                  c     c c g
TGEV              g        t                               tc      t
Consensus GTTTATTACAAGAGCAC-AGTACTTACAAA-TGACACCAGGTATGATTTTTGTGTTGACTTCTCATAATGTGAGTCCTCTTAAAGC-ACAATT-TAGTCA

TN406        g              c          acg         g       cc  t                 c
UCD1         g              c          cg  c       g       cc  t  a              t
79-1146    c          a                t   c
79-1683                                t                                         a
CCV       g                          c   c  c            c
TGEV                                  c          g        t     c
Consensus ACCAAGAAAAGTACAATACCATATCTAAGCTCTATCCTGT-TTTAATATAGCGGAAGCCTATAATACACTGGTGCCTTACTATCAAATGATAGGTAAGCA

                     428
TN406
UCD1
79-1146
79-1683    g
CCV       g
TGEV
Consensus AAAATTTACAACTATCCAAGGTCCTCCT
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FIG. 3—*Continued.*

strain Purdue (11) and HCV strain 229E (13), and reverse transcriptase PCR (RT-PCR) was performed as described previously (16) (for a schematic outline, see Fig. 1). In all cases, PCR products of the anticipated size were obtained (data not shown). These were purified from agarose gels and subjected to cycle sequence analysis using the AmpliCycle sequencing kit (Perkin Elmer/Roche, Branchburg, N.J.) in accordance with the instructions of the manufacturer. For each region, the nucleotide sequence was determined in both orientations on at least two independently generated RT-PCR products. Computer-assisted nucleotide sequence alignment was performed by using the PILEUP program (Genetics Computer Group
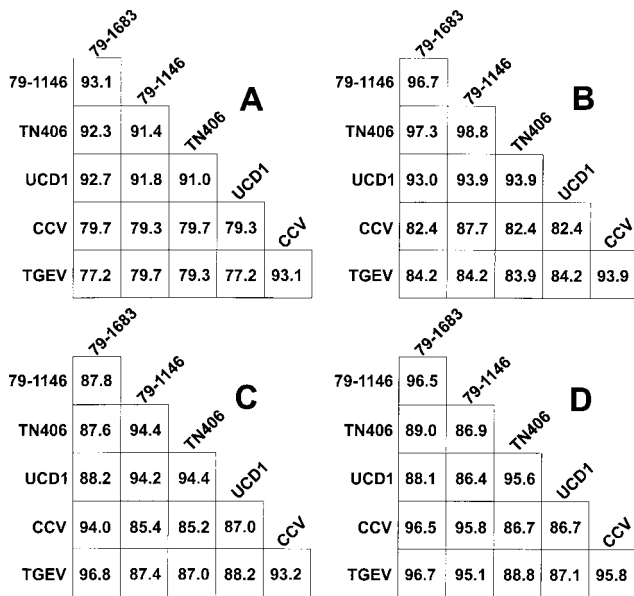
FIG. 4. Percentages of nucleotide sequence identity between polymerase regions A to D of the FCoV type I and II strains, CCV, and TGEV.
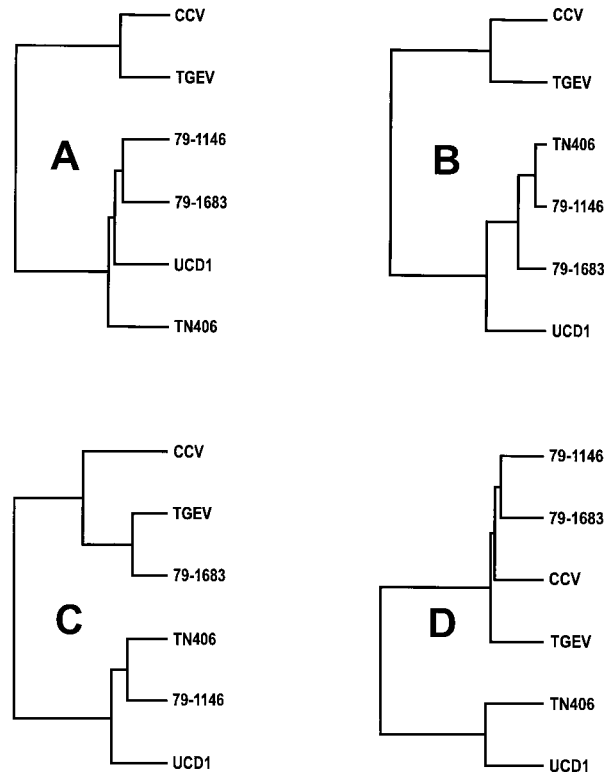

FIG. 5. Dendrograms schematically representing the sequence identity between polymerase regions A to D of FCoV type I and II strains, CCV, and TGEV. The similarity scores as calculated by the PILEUP program were used to create dendrograms by the unweighted pair-group method using arithmetic averages as described by Sneath and Sokal (42). The distances along the vertical axis are proportional to the number of nucleotide differences.

Wisconsin Package) (17), and the similarity scores were used to create dendrograms by the unweighted pair-group method using arithmetic averages as described by Sneath and Sokal (42).

The results are presented in Fig. 3 to 5. In region A, which is located at the 5' end of ORF1a (corresponding to nt 1575 to 1895 of TGEV strain Purdue) (11), the type II FCoV strains 79-1146 and 79-1683 have 91.4 to 92.7% sequence identity with the type I strains Black and UCD1, compared with only 77.2 to 79.7% with CCV and TGEV. In this region, the TGEV and CCV sequences are 93.1% identical (Fig. 3 and 4). Consistently, in a dendrogram schematically displaying the nucleotide sequence identity between the different viruses, the FCoV strains form a separate cluster distinct from that of CCV and TGEV (Fig. 5). A similar situation exists in region B, located at the 3' end of POL1a (corresponding to nt 11531 to 11920 of TGEV strain Purdue). Here, the sequence identity among the FCoV strains ranges between 93.0 and 98.8%, whereas sequence identity with CCV and TGEV varies between 82.4 and 87.7% (Fig. 4). Again, CCV and TGEV cluster, displaying 93.9% sequence identity in this region. In contrast, in region C at the 5' end of ORF1b (corresponding to nt 12574 to 13323 of TGEV strain Purdue), FCoV strain 79-1683 is more closely related to CCV and TGEV, with sequence identities of 94.0 and 96.8%, respectively (Fig. 3 and 4) than to the type I FCoVs. The other type II strain, 79-1146, however, still clusters with the type I FCoV strains. Finally, in region D, located in the middle of ORF1b (corresponding to nt 15500 to 15972 of TGEV strain Purdue), both type II FCoV strains are more closely related to TGEV and CCV, with sequence identities ranging from 95.1 to 96.7%, than to type I FCoVs, with sequence identities of 86.4 to 89.0%. The type I FCoV strains have a sequence identity of 95.6% in region D (Fig. 4).

Our findings provide further evidence that the type II FCoV strains 79-1146 and 79-1683 are recombinant viruses and suggest that they actually originated from double RNA recombination events. In the case of strain 79-1146, an additional template switch apparently occurred in ORF1b, between regions C and D, whereas for strain 79-1683, switching took place

further upstream, in the ORF1ab frameshifting region between sites B and C (Fig. 6). The fact that the template switches in the *pol* gene occurred at different sites again is consistent with strains 79-1146 and 79-1683 having originated independently. FCoV type I and CCV are the presumptive parental viruses (37, 46), but it is not known in which host species the recombination took place. Under experimental conditions, cats can be infected with CCV (3, 34, 35, 43). Whether CCV and FCoV readily cross species barriers in the field remains to be determined.
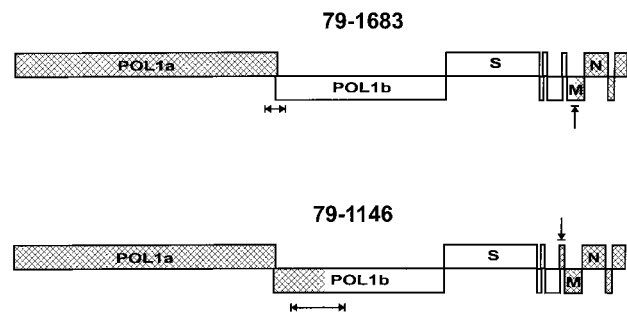
FIG. 6. Recombination patterns of the FCoV strains 79-1146 and 79-1683. The schematic representations of the two virus genomes are as detailed in Fig. 1. Hatched boxes indicate parts of the FCoV type II genome thought to have originated from FCoV type I. Open boxes represent sequences apparently obtained from CCV. The regions where the template switches have taken place are underlined.

The recombination events from which the type II viruses resulted can be explained by two models. In the most simple scenario, the recombination may involve only two parental virus strains, with RNA replication initiating on a type I FCoV template of either negative or positive polarity (31) and then switching of the polymerase-nascent cRNA complex to a CCV template, followed by a switchback. Alternatively, a more complicated scheme can be envisaged; in this scheme, a CCV-FCoV hybrid, arisen from a single template switch, spreads into the cat population and in turn engages in additional recombination events with another type I FCoV strain(s). Recent evidence suggests that FCoV type I viruses cause chronic enteric infections that may last for at least 7 months (14). Conceivably, persistence of FCoV would raise the odds of occurrence of double infections.

In general, a recombinant virus, to emerge and establish itself in the field, needs not just to be viable but to have a selective advantage. Thus, the uptake of CCV sequences by the type II FCoVs may have led to increased viral fitness as compared to the type I FCoV. Which of the acquired genes provided the selective advantage is as yet unknown. Studies of other coronaviruses have shown that the S protein plays a crucial role in eliciting protective immunity (4, 5, 12, 25, 38, 48). Moreover, genetic characterization of FCoVs isolated from persistently infected cats suggested that S is subject to antigenic drift, implying that this protein is a prime target for the immune system during chronic infection. From this work, it also appears that FCoV-infected cats develop resistance against FCoV superinfection, at least by antigenically closely related strains (14). Perhaps the acquisition of a CCV spike by a type I FCoV resulted in an antigenic shift, allowing the recombinant virus to escape host and/or herd immunity. However, it is also possible that the acquired CCV sequences provided a growth advantage. As compared to the type I FCoVs, type II FCoVs replicate far more efficiently in tissue culture cells and produce 50- to 100-fold-higher titers of extracellular virus (39).

It is of equal interest that both type II strains have retained FCoV sequences from the 5′ and 3′ ends of the FCoV type I genome. One interpretation is that these FCoV sequences are required for efficient replication in the cat and that selection against recombinant viruses, arisen from a single template switch, occurs. It is particularly intriguing that both strains 79-1146 and 79-1683 have retained the FCoV type I ORF1a. The POL1a polyprotein gives rise to a number of cleavage products of unknown function (reviewed in reference 10), some of which may be involved in specific virus-host interactions. Further genetic characterization of type II FCoVs and mapping of recombination sites will provide more insight into these issues and increase our understanding of coronavirus pathobiology and evolution.

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