Feline infectious peritonitis (FIP) is a lethal immunopathological disease caused by feline coronaviruses (FCoVs). Here, we describe a reverse genetics approach to study FIP by assessing the pathogenicity of recombinant type I and type II chimeric FCoVs. All recombinant FCoVs were successfully rescued after electroporation of full-length in vitro-transcribed RNA into BHK-FCoV-N cells, and their integrity was confirmed by sequencing analysis. Following infection of Felis catus whole-fetus 4 (FCWF-4) cells (25), the recombinant virus recFCoV-ΔStop-7b displayed growth kinetics indistinguishable from those of the recombinant type I FCoV strain Black (recFCoV) (25) and the corresponding isolate with peak titers of 10^6 PFU/ml at 36 h post-infection (p.i.) (Fig. 2A). Similar to what was previously described for FCoVs encoding the type II FCoV strain 79-1146 S protein (24), viruses containing type II sequences (recFCoV-II) induced significantly larger plaques (CPE) on FCWF-4 cells faster than the type I FCoVs (data not shown), gave rise to significantly larger plaques (recFCoV-II, 0.80 mm ± 0.15 mm; recFCoV, 0.28 mm ± 0.07 mm; n = 31; P < 0.001), and reached peak titers in the range of 10^6 PFU/ml within 24 h p.i. We reproducibly observed slightly higher peak titers for the type II FCoV strain 79-1146 isolate than for the recombinant recFCoV-II isolate (P < 0.001; 24 h p.i.), although their genomic sequences have only two silent nucleotide differences (Table 1).

To assess viral pathogenicity, groups of two FCoV-seronegative cats were inoculated with the type I FCoV strain Black (25), the recombinant type I FCoV recFCoV, and the type II FCoV strain 79-1146. The cats were monitored for clinical signs of FIP, and their sera were tested for FCoV antibody titers. The results indicated that the type I FCoV strain Black caused a mild clinical disease, while the type II FCoV strain 79-1146 caused a severe and fatal disease. The recombinant type I FCoV recFCoV was less pathogenic than the type II FCoV strain 79-1146, indicating that the accessory gene 3c is required for FCoV replication in vivo.

Despite these compelling observations, it should be noted that samples are usually from field cases and it is difficult to accurately trace with which FCoVs these animals have been infected. Thus, it remains to be experimentally verified which mutations indeed promote a biotype switch. Ideally, such experimental validation may be done using well-defined prototype viruses. However, FCoV field isolates are hardly, if at all, cultivable, and to date, there are mainly only FIPV isolates available that can be grown in tissue culture (8, 19). Moreover, with very few exceptions, these FIPV isolates are known to lose their pathogenicity through cell culture adaptation (19).

In order to overcome this limitation, recombinant FCoVs generated from cloned full-length cDNA are desirable because they permit the generation of well-defined virus stocks which originate from a cloned sequence after a few passages in cell culture. Based on our recently established reverse genetics system for the type I FCoV strain Black (25), we therefore generated a set of recombinant FCoVs using vaccinia virus-mediated recombination of full-length FCoV cDNA cloned in vaccinia virus (6, 24, 25). The recombinant FCoVs used in this study comprised (i) the type I FCoV strain Black (25), (ii) a type I FCoV Black strain with restored accessory gene 7b, (iii) a chimeric type I/type II FCoV, recFCoV-S-3abcII, encoding the structural gene S and the accessory genes 3a, 3b, and 3c from the highly pathogenic type II FCoV strain 79-1146, (iv), a chimeric type I/type II FCoV, recFCoV-1b-3abcII, possessing a genome organization similar to that of natural type II FCoVs, and (v) the type II FCoV strain 79-1146 (Fig. 1).

All recombinant FCoVs were successfully rescued after electroporation of full-length in vitro-transcribed RNA into BHK-FCoV-N cells (25), and their integrity was confirmed by sequencing analysis. Following infection of Felis catus whole-fetus 4 (FCWF-4) cells (25), the recombinant virus recFCoV-ΔStop-7b displayed growth kinetics indistinguishable from those of the recombinant type I FCoV strain Black (recFCoV) (25) and the corresponding isolate with peak titers of 10^6 PFU/ml at 36 h post-infection (p.i.) (Fig. 2A). Similar to what was previously described for FCoVs encoding the type II FCoV strain 79-1146 S protein (24), viruses containing type II sequences (recFCoV-II) induced significantly larger plaques (CPE) on FCWF-4 cells faster than the type I FCoVs (data not shown), gave rise to significantly larger plaques (recFCoV-II, 0.80 mm ± 0.15 mm; recFCoV, 0.28 mm ± 0.07 mm; n = 31; P < 0.001), and reached peak titers in the range of 10^6 PFU/ml within 24 h p.i. We reproducibly observed slightly higher peak titers for the type II FCoV strain 79-1146 isolate than for the recombinant recFCoV-II isolate (P < 0.001; 24 h p.i.), although their genomic sequences have only two silent nucleotide differences (Table 1).

To assess viral pathogenicity, groups of two FCoV-seronega-
tive, specific-pathogen-free (SPF) cats (Charles Rivers, France) at an age of 4 to 5 months were inoculated intraperitoneally (i.p.) with 10^6 PFU of virus or phosphate-buffered saline (PBS) as a negative control. Cats were monitored daily for clinical signs, and blood and fecal samples were collected weekly over a period of 7 to 8 weeks. All FCoVs established productive infection in cats (Fig. 3), as determined by detection of viral RNA in feces by reverse transcription-PCR (RT-PCR) and induction of FCoV-specific antibody responses, measured in serum samples by endpoint dilution using FCoV-infected CRFK cells. Notably, viral RNA in fecal samples was not detectable by RT-PCR at all time points, a result which is in concordance with a recent report of experimental i.p. FCoV infection (22). None of the type I or chimeric FCoVs induced clinical signs of FIP, suggesting that the type I FCoV strain Black isolate used in our laboratory had already lost pathogenicity through cell culture adaptation. Furthermore, an intact 7b accessory gene carried by recFCoV-ΔStop-7b did not confer a detectable gain of pathogenicity in vivo, suggesting that additional attenuating mutations are elsewhere in the genome. The apathogenic phenotype of the chimeric FCoVs suggests that the type-II-de-
derived sequences (open reading frame [ORF] 1b; genes S, 3a, 3b, and 3c) are not sufficient to increase pathogenicity and further supports that the remaining sequences derived from type I FCoV strain Black (5’ untranslated region [UTR]; ORF 1a; genes M, N, 7a, and 7b; 3’ UTR) may contain attenuating cell culture adaptations.

In sharp contrast, animals infected with the type II FCoV strain 79-1146 and recFCoV-II showed signs of clinical disease that started to develop at 2 to 3 weeks p.i. The animals showed high antibody titers, anorexia, loss of appetite and weight, conjunctivitis, and anemia, which were accompanied by recurring fever typical for FIP (Fig. 3F and G). In both groups, one cat had to be euthanized at 7 weeks p.i. when clinical manifestation of FIP became apparent (cats 11 and 13). In both of these cats, abdominal fluid (ascites) was found, while the liver, the spleen, the kidneys, and the serosa of the peritoneal cavity were covered by pyogranulomas. During the postmortem investigation, characteristic signs of FIP could also be detected in cats 12 and 14. These data demonstrate that the recombinant recFCoV-II derived from cloned cDNA, like the type II FCoV strain 79-1146 isolate, is highly pathogenic and can induce FIP in cats.

By amplifying a set of overlapping FCoV-specific PCR products using RNA isolated from ascites of cat 11 (infected with the type II FCoV strain 79-1146), we successfully determined the full-

TABLE 1 Summary of nucleotide and amino acid differences compared to the published type II FCoV strain 79-1146 sequencea

<table>
<thead>
<tr>
<th>Published sequence</th>
<th>Type II FCoV strain 79-1146b</th>
<th>recFCoV-II</th>
<th>Ascites (cat 11 infected with the type II FCoV strain 79-1146)</th>
<th>Spleen (cat 13 infected with recFCoV-II)</th>
<th>Kidney (cat 13 infected with recFCoV-II)</th>
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a The published type II FCoV strain 79-1146 sequence is in GenBank under accession number NC_002306.
b This sequence corresponds to the type II FCoV strain 79-1146 determined in our laboratory.
c 1a, open reading frame 1a; nsp; nonstructural protein; S, spike protein; 3c, 3c protein; E, envelope protein; N, nucleocapsid protein.
length genome sequence. We noticed nine nucleotide differences, with three nonsynonymous mutations in the S gene, one in nonstructural protein (nsp) 3 of the replicase gene, and one in the E gene (Table 1). Surprisingly, we found that the stop codon in the 3c accessory gene of type II FCoV strain 79-1146 (input virus) was changed to encode a glutamine residue (Table 1). We also determined the full-length genomic FCoV sequence by RT-PCR using RNA isolated from the spleen and kidney of the FIP-diseased cat 13 (infected with recFCoV-II). We detected 2 nonsynonymous nucleotide changes in RNA isolated from the spleen and 7 nonsynonymous and 3 silent changes in RNA from kidney, suggesting that in vivo, recFCoV-II further diversified in the kidneys (Table 1). Importantly, the restoration of the ORF 3c was also present in the viral RNA derived from the recFCoV-II-infected FIP-diseased cat. Moreover, by performing a 3c-specific RT-PCR, we also detected the restoration of ORF 3c in viral RNA in the gut and fecal samples from FIP-diseased cats 11 and 13, which had been infected with the type II FCoV strain 79-1146 and recFCoV-II, respectively (data not shown). Although this finding appears to contradict previous reports (3, 22), it remains to be determined at which phase during the infection in vivo, and in which specific target cells/organs, a functional 3c protein is promoting replication and when it becomes dispensable. It should be noted that previous studies reported mutated 3c genes mainly in the context of natural FCoV infection. This includes a first phase of replication of an FECV in the gut before pathogenic FIPV variants, often containing mutations in the 3c gene, may emerge.

In conclusion, we have generated a set of full-length FCoV cDNA clones that provides a valuable basis to study the molecular pathogenesis of FIP. In the long term, it will be important to extend this reverse genetics approach to pathogenic type I FCoVs. This will greatly help to identify and compare pathogenicity factors of type I and type II FCoVs that critically contribute to the development of FIP. Furthermore, we believe that this approach is exceptionally well suited to generate recombinant FECV that may be used to study early events of enteric FCoV replication and the possible emergence of pathogenic FIPV variants in individual cats. Finally, we expect that FCoV reverse genetics will significantly contribute to furthering our knowledge on the molecular biology and pathogenesis of FIP and, thus, provide the basis for the rational design of efficacious vaccines to prevent FIP.

ACKNOWLEDGMENTS

We thank Svenja Wiese for excellent technical assistance.

This study was supported by the Bundesministerium für Bildung und Forschung of the German Government (Zoonosis Network, Consortium on ecology and pathogenesis of SARS, project code 01K1005A-F; http://www.gesundheitsforschung-bmbf.de/de/1721.php#SARS).
All animal experiments were done in accordance with the Hungarian legislation on animal protection. The protocol was approved by the Fovarosi Allategeszegyőv és Elelmiszer Ellenorzo Allomas, Budapest (assurance number 1082/003/FOV/2006).

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