Genome Organization and Reverse Genetic Analysis of a Type I Feline Coronavirus[▽]

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In this study we report the complete sequence and genome organization of the serotype I feline coronavirus (FCoV) strain Black. Furthermore, a reverse genetic system was established for this FCoV strain by cloning a full-length cDNA copy into vaccinia virus. This clone served as basis for the generation of recombinant FCoV (recFCoV) that was shown to bear the same features in vitro as the parental FCoV. Using this system, accessory 3abc genes in the FCoV genome were replaced by green fluorescent protein (recFCoV-GFP) and *Renilla* luciferase genes (recFCoV-RL). In addition, we showed that feline CD14⁺ blood monocytes and dendritic cells can be easily detected after infection with recFCoV-GFP. Thus, our established reverse genetic system provides a suitable tool to study the molecular biology of serotype I FCoV.

Coronaviruses (CoVs) are enveloped, positive-strand RNA viruses that mainly cause enteric and respiratory diseases in humans and animals. Together with arteriviruses, toroviruses, and roniviruses, they belong to the order *Nidovirales*. CoVs are classified into three groups. This classification was initially based on serological investigations and has received support from recent phylogenetic analyses (19). Feline CoVs (FCoVs) are members of group I and are placed together with the closely related porcine transmissible gastroenteritis virus and canine CoV (CCoV) into the CoV subgroup Ia (18). Members of the more distantly related subgroup Ib CoVs are human CoVs (HCoVs) 229E and NL63 and porcine epidemic diarrhea virus.

FCoVs cause infections in wild and domestic *Felidae* and are widespread, with seropositivity of 20 to 60% in domestic cats and up to 90% in animal shelters or households with multiple cats (25). Infected animals often develop persistent infection and shed the virus over longer periods of time (1, 2, 17, 24, 26). On the basis of serological differences, FCoVs can be separated into type I and type II (29, 30, 43). Eighty to 90% of the naturally occurring infections are caused by type I (2, 31, 34, 38). Type II FCoVs most likely have arisen by homologous recombination between type I FCoV and CCoV (27, 55). As a result of this recombination, the spike (S) gene and adjacent regions of type I FCoV were replaced by the corresponding part of the CCoV genome. The presence of different S genes is reflected by different growth characteristics in vitro. For type II FCoV, the feline aminopeptidase N (fAPN) has been identified as a cellular receptor (53). While type II FCoV can be easily propagated in cell culture, type I FCoV isolates usually grow only poorly in cell culture. Consequently, most investigations in the last years concentrated on type II FCoV (12,

14, 21, 44), although their prevalence is much lower than that of type I.

Interestingly, both serotypes exist as two biotypes; the rather benign feline enteric CoV (FECV) causes subclinical, often persistent infections, whereas feline infectious peritonitis virus (FIPV) is highly virulent and causes FIP, a fatal, systemic granulomatous disease. In the development of the disease FIPV-infected monocytes are thought to play an important role in the dissemination of the virus throughout the body (20, 36, 39). It has been suggested that virulent FIPV arises by mutation from parental FECV in the individual, persistently infected host (54). However, despite the high prevalence of FCoV infection in the cat population, FIP develops in only about 5% of these animals. It is not yet clear which alterations in the FCoV genome are responsible for the generation of FIPV from FECV, but mutations in accessory genes and the S gene have been proposed to be associated with the generation of FIP (33, 54).

Although a reverse genetic system based on targeted recombination has already been established for type II FIPV 79-1146 (21), the differences between FECV and FIPV have not yet been delineated on the genetic level. It could be shown that the accessory genes are not required for in vitro replication, but they are important for the virulence in vivo (21, 22). Nevertheless, a major obstacle of these studies is the fact that mutagenesis by targeted recombination is, for technical reasons, restricted to the 3' third of the genome. Therefore, the 5' untranslated region (UTR) and the entire replicase gene are not amenable to mutagenesis. There is actually accumulating evidence that some CoV replicase gene products are involved in virus-host interactions and represent important pathogenicity factors (48, 60). Thus, it is desirable to extend FCoV reverse genetics to the genetic manipulation of the entire FCoV genome and to the much more widely distributed type I FCoV serotype.

In this study we report the complete sequence and genome organization of the type I FCoV strain Black (4). We have developed a versatile reverse genetic system for this virus using

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a vaccinia virus cloning vector. This system allows the genetic manipulation of the entire genome including the 5' UTR and the replicase gene. The rescued recombinant FCoV Black (recFCoV) exhibits growth characteristics in vitro similar to the parental FCoV strain Black. Replacement of accessory 3abc protein genes by green fluorescent protein (GFP) and *Renilla* luciferase (RL) genes allowed us to demonstrate that replication of type I FCoV in monocytes, macrophages, and dendritic cells (DCs) is limited.

MATERIALS AND METHODS

Viruses and cells. *Felis catus* whole fetus 4 cells (FCWF-4) and baby hamster kidney cells (BHK-21) were originally purchased from the American Type Culture Collection and obtained from the diagnostic laboratory of our institute. Monkey kidney (CV-1) cells were purchased from the European Collection of Cell Cultures. D980R cells were a kind gift from G. L. Smith, Imperial College, London, United Kingdom. BHK-Tet/ON cells were a kind gift from N. Tautz, Justus Liebig Universität, Giessen, Germany. All cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml). The fowlpox virus was a kind gift from P. Britton, Institute for Animal Health, Compton, United Kingdom. The FCoV Black strain was a kind gift from R.J. de Groot (Utrecht University, The Netherlands). FCoV Black and recombinant FCoV were propagated in FCWF cells. The vNotI/tk vaccinia virus, recombinant vaccinia viruses, and fowlpox virus were propagated, titrated, and purified as described previously (52).

Plasmid construction and generation of recombinant vaccinia viruses. The complete sequence of type I FCoV strain Black was determined by sequencing overlapping cDNA fragments generated from viral RNA. On the basis of the consensus sequence, a set of seven plasmid clones (A, BC-1, 2, 3, D, E, and F) spanning the entire FCoV Black genome were generated. Plasmid pA is based on pGem T-easy (Promega) and contains sequences corresponding to nucleotides (nt) 1 to 5083 of the FCoV Black genome, preceded by a G nucleotide, a T7 promoter, and a Bsp120I cleavage site and followed by a BsaI cleavage site with reverse orientation. Plasmids pBC-1, pBC-2, pBC-3, and pD are based on pGem T-easy and contain sequences corresponding to nt 4389 to 8769, 8161 to 12680, 12031 to 16404, and 15775 to 20672 of the FCoV Black genome, respectively. Plasmid pE is based on pGem T-easy and contains sequences corresponding to nt 20568 to 24873 of the FCoV Black genome followed by a BsmBI cleavage site with reverse orientation. Plasmid pF contains a BsmBI cleavage site upstream of the cloned FCoV Black sequence corresponding to nt 24844 to 29228 followed by a synthetic poly(A) tail, a ClaI cleavage site, a hepatitis delta ribozyme sequence, and a Bsp120I restriction site.

Introduction of the full-length FCoV cDNA in the vaccinia virus genome was carried out in two steps. First, fragments A, D, E, and F were ligated. DNA fragment A is derived from plasmid pA after Bsp120I and BsaI digestion, treatment with alkaline phosphatase, and gel purification. DNA fragment D resulted from digestion of the plasmid pD with BsmBI and gel purification. DNA fragment E resulted from cleavage of the plasmid pE with BsmBI and gel purification. DNA fragment F was generated from the plasmid pF after BsmBI and Bsp120I digestion, treatment with alkaline phosphatase, and gel purification. About 100 to 500 ng of each DNA fragment (A, D, E, and F) was in vitro ligated using T4 DNA ligase, and the resulting product was ligated without further purification to the NotI-cleaved vNotI/tk vaccinia virus DNA in the presence of NotI enzyme. The resulting products from this ligation were used directly for transfection of fowlpox virus-infected CV-1 cells. The recombinant vaccinia viruses (vrecFCoV-ADEF) were isolated as described previously (52). Second, the missing part of the FCoV Black genome (nt 5083 to 15775) was introduced by two rounds of vaccinia virus-mediated homologous recombination using guanosine-phosphoribosyl-transferase (GPT) as a positive and negative selection marker, as described previously (8, 28). For this purpose DNA fragments BC-1 and BC-3, corresponding to FCoV Black nt 4389 to 8769 and 12031 to 16404, respectively, were cloned upstream and downstream of the *gpt* gene in the plasmid pGPT-1 (28). The resulting plasmid (pGPT-BC-1/3) contains fragment BC-1 upstream and fragment BC-3 downstream of the *gpt* gene and was used for vaccinia virus-mediated homologous recombination. The resulting *gpt* gene-containing recombinant virus vrecFCoV-ABC was selected using GPT as a positive selection marker. To obtain vrecFCoV containing the complete FCoV Black cDNA, vrecFCoV-ABC-2 was used for vaccinia virus-mediated homologous recombination with plasmid pBC-2 containing FCoV Black nt 8161 to 12680. The resulting recombinant virus vrecFCoV was selected using GPT as a negative selection marker.

The recombinant vaccinia virus vrecFCoV was used to replace the accessory 3abc genes by the GFP or RL gene by two rounds of vaccinia virus-mediated homologous recombination. First, plasmid pGPTΔ3abc was constructed to encode FCoV Black nt 24224 to 24812 and 25768 to 26378 upstream and downstream of the *gpt* gene. Recombination of vrecFCoV with plasmid pGPTΔ3abc resulted in the isolation of the recombinant vaccinia virus vrecFCoV-GPTΔ3abc. To construct the recombinant GFP-encoding vaccinia virus vrecFCoV-GFP, vaccinia virus vrecFCoV-GPTΔ3abc was used for recombination with the plasmid pFCoV-GFP, encoding FCoV Black nt 24224 to 24812 upstream and FCoV Black nt 25768 to 26378 downstream of the GFP sequence. The resulting recombinant vaccinia virus vrecFCoV-GFP was obtained after *gpt* negative selection. To construct the recombinant RL-encoding vaccinia virus vrecFCoV-RL, vaccinia virus vrecFCoV-GPTΔ3abc was used for recombination with the plasmid pFCoV-RL, encoding FCoV Black nt 24224 to 24812 upstream and FCoV Black nt 25768 to 26378 downstream of the RL sequence. The resulting recombinant vaccinia virus, vrecFCoV-RL, was obtained after *gpt* negative selection.

Sequence analysis and Northern blotting. The sequences of all generated plasmids and insert DNAs of recombinant vaccinia viruses were verified by sequence analysis.

Northern blot analysis was done using poly(A)-containing RNA isolated from FCoV Black-infected (multiplicity of infection [MOI] of 0.1) FCWF cells and electrophoresis on formaldehyde 1% agarose gel. After a blotting step, a ³²P-labeled probe directed against the 7ab genes of the FCoV Black genome was used to detect genomic RNA and subgenomic RNAs (52).

To determine the leader-body junctions of FCoV-Black subgenomic mRNAs, poly(A)-containing RNA from FCoV Black-infected FCWF cells was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) with primers specific for the unique region of a subgenomic mRNA, followed by a PCR using a leader- and a body-specific primer pair. The PCR products were subjected to standard sequence analysis using an ABI 310 Prism Genetic Analyzer.

Generation of a BHK-21-derived cell line that stably expresses the FCoV strain Black N protein. The cell line BHK-FCoV-N expressing the FCoV Black nucleocapsid (N) protein is based on BHK-Tet/ON cells that were a kind gift from N. Tautz, Justus Liebig Universität, Giessen, Germany. A plasmid encoding the FCoV Black N protein was transfected into BHK-Tet/ON cells, and single colonies were collected after hygromycin selection (300 μg/ml). Expression of FCoV strain Black N protein was assessed by Western blot analysis 16 h after the addition of doxycycline (2 mg/ml) to the culture medium.

Recovery of the recombinant FCoV. DNA from vaccinia virus vrecFCoV-, vrecFCoV-GFP-, or vrecFCoV-RL-infected BHK-21 cells was prepared as described previously (52). After ClaI digestion, phenol extraction, and ethanol precipitation, the DNA was used as a template for in vitro transcription, as described previously (52). A total of 10 μg of the resulting RNA was electroporated into BHK cells that express the FCoV strain Black N protein (BHK-FCoV-N). The electroporated cells were cocultivated with FCWF cells, and after a 48-h incubation the supernatant was harvested and added to fresh FCWF cells. At 24 to 48 h postinfection cytopathic effect was observed, and the cell supernatant containing recombinant FCoV was collected and used for further characterization. Virus supernatant obtained after recFCoV rescue was used without plaque purification.

Measurement of RL expression. recFCoV-RL-infected FCWF cells (MOI of 0.1) were harvested at different time points using lysis buffer from an RL assay system kit (Promega). Intracellular RL expression was measured according to the manufacturer's instructions.

Isolation of blood monocytes and differentiation to DCs. Peripheral blood mononuclear cells were purified by Ficoll-Paque separation from 15 ml of heparinized blood obtained from specific-pathogen-free cats. Anti-human CD14 magnetic beads (Miltenyi Biotec) were used to obtain CD14⁺ feline monocytes. To obtain differentiated feline macrophages and DCs, CD14⁺ feline monocytes were cultured for 7 days using either human recombinant granulocyte macrophage colony-stimulating factor (hrGM-CSF) or hrGM-CSF together with human recombinant interleukin-4 (IL-4), respectively, as described previously (49).

Nucleotide sequence accession number. The complete genomic sequence of FCoV strain Black was deposited in GenBank database under accession number EU186072.

RESULTS

Genome organization of type I FCoV strain Black. The virus used in our experiments was originally isolated in the 1970s from an FIPV-infected cat and was then named Tennessee strain (TN-406). It was maintained by animal passage and

FIG. 1. FCoV type I genome organization. (a) The type I FCoV genome, ORFs, and TRS elements are shown together with the characteristic nested set of viral sgRNAs. Boxes of viral mRNAs indicate the predicted translationally active “unique” region of each particular mRNA. L, FCoV leader sequence; (A)_n, poly(A) tail. (b) Northern blot analysis from poly(A)-containing RNA isolated from type I FCoV strain Black-infected FCWF cells. A ³²P-labeled probe directed against the 7ab genes of the type I FCoV genome was used to detect genomic RNA and sgRNAs. (c) Alignment of type I FCoV strain Black TRS elements that direct the synthesis of sgRNAs. Nucleotides matching the leader TRS are underlined. The conserved TRS core is shaded in gray.

stored as a liver homogenate. John Black was able to successfully propagate the virus in feline cell culture, and this virus was later named the Black strain (4). The complete genomic sequence of type I FCoV strain Black was determined by sequencing overlapping cDNA fragments generated by reverse transcription-PCR (RT-PCR) from viral RNA. The FCoV strain Black genome has a size of 29,228 nt, excluding the 3' poly(A) tail, and shows typical genome organization for CoVs (Fig. 1a). The 5' UTR consists of 311 nt including the leader

sequence (nt 1 to 92) and the transcription-regulatory sequence (TRS) with the core TRS 5'-CUAAAC-3' (nt 93 to 98) which controls mRNA synthesis. Like type II FCoV 79-1146, the 5' UTR of strain Black harbors a short open reading frame (ORF; nt 116 to 127) potentially encoding the tripeptide Met-Lys-Pro. The 3' UTR comprises 275 nt followed by the poly(A) tail. Approximately two-thirds of the genome is covered by the replicase gene comprising ORFs 1a (nt 312 to 12407) and 1b (nt 12362 to 20407). Translation of the CoV replicase gene

TABLE 1. Predicted protease cleavage sites of FCoV strain Black replicase polyproteins

Cleavage product	Polyprotein	Position in polyprotein (amino acid residues)	Size (aa) ^a
nsp-1	pp1a/pp1ab	1Met-Gly110	110
nsp-2	pp1a/pp1ab	111Ala-Gly879	769
nsp-3	pp1a/pp1ab	880Gly-Gly2402	1523
nsp-4	pp1a/pp1ab	2403Ser-Gln2892	490
nsp-5	pp1a/pp1ab	2893Ser-Gln3194	302
nsp-6	pp1a/pp1ab	3195Ser-Gln3488	294
nsp-7	pp1a/pp1ab	3489Ser-Gln3571	83
nsp-8	pp1a/pp1ab	3572Ser-Gln3766	195
nsp-9	pp1a/pp1ab	3767Asn-Gln3877	111
nsp-10	pp1a/pp1ab	3878Ala-Gln4012	135
nsp-11	pp1a	4013Gly-Asp4032	19
nsp-12	pp1ab	4013Gly-Gln4942	929
nsp-13	pp1ab	4943Ala-Gln5541	599
nsp-14	pp1ab	5542Ala-Gln6060	519
nsp-15	pp1ab	6061Ser-Gln6399	339
nsp-16	pp1ab	6400Ser-Pro6699	300

^a aa, amino acids.

gives rise to the primary translation products polyprotein 1a (pp1a) and pp1ab, which are processed by virus-encoded proteinases. Based on sequence comparison with other group I CoV replicase genes, we have identified three putative papain-like proteinase cleavage sites and 11 putative main proteinase cleavage sites. Thus, we predict that proteolytic processing of the FCoV strain Black pp1a/1ab gives rise to 16 processed end products, nonstructural protein 1 (nsp-1) to nsp-16 (Table 1). Downstream of the replicase gene we identified the canonical four structural genes encoding the

proteins S (nt 20404 to 24798), envelope (E) (nt 25826 to 26074), membrane (M) (nt 26085 to 26876), and N (nt 26889 to 28022). FCoV strain Black contains sequences corresponding to the known five additional ORFs coding for the accessory proteins 3abc (nt 24810 to 25839) and 7ab (nt 28027 to 28954). There is a large deletion of 62 nt at the border of the accessory 3b and 3c genes, leading to translational readthrough of ORFs 3b and 3c, as described previously for FIPV Black (54). The 7b gene contains a nonsense mutation at nt 28442 (C to T) resulting in early termination of the 7b protein compared to other FCoV isolates (data not shown). CoV structural and accessory proteins downstream of the replicase gene are translated from a set of subgenomic RNAs (sgRNAs) that contain the leader sequence at the 5' end fused to the TRS and the so-called body sequence. To visualize the FCoV strain Black mRNAs, Northern blot analysis was performed, and the predicted six subgenomic mRNAs (sgmRNAs) were identified (Fig. 1b). The exact leader-body junctions of FCoV Black sgmRNAs were determined by leader-body-specific RT-PCRs and sequencing analysis (Fig. 1c).

Cloning of a full-length cDNA of FCoV strain Black. For the generation of an infectious clone of FCoV strain Black, we used vaccinia virus as a cloning vector. It has previously been shown that vaccinia viruses are suitable vectors for the cloning of full-length cDNA of HCoV 229E, avian infectious bronchitis virus, and mouse hepatitis virus (5, 8, 52).

The introduction of the full-length FCoV cDNA into vaccinia virus genome was carried out in two steps as illustrated in Fig. 2. First, we constructed four plasmids, pA, pD, pE, and pF, comprising segments which covered the major part of the ge-

FIG. 2. Schematic diagram for the introduction of the full-length FCoV strain Black cDNA into the vaccinia virus genome. In the first phase, fragments A, D, E, and F (gray boxes) derived from plasmids pA, pD, pE, and pF were ligated into the vaccinia virus (VV) genome (white boxes). In the second phase, to complete the full-length FCoV strain Black cDNA, fragments BC-1, -2, and -3 were introduced using two rounds of vaccinia virus-mediated homologous recombination with *gpt*-positive and *gpt*-negative selection using the plasmids pGPT-BC1/3 and pBC-2.

TABLE 2. Silent nucleotide changes between vrecFCoV and FCoV strain Black

Position in the vrecFCoV genome (nt)	Change of nucleotide from FCoV Black
485	T → C
2784	C → T
4285	T → C
7091	C → T
7326	T → C
8036	A → G
10973	T → C
13717	A → G
16498	T → A
17914	T → C
18673	A → G
21597	T → C
25776	T → C
26648	T → C

nome. DNA fragments A, D, E, and F prepared from plasmids were used for in vitro ligation, and the resulting product was ligated without further purification into NotI-cleaved vaccinia virus DNA in the presence of NotI enzyme. Fowlpox virus-infected CV-1 cells were transfected with this DNA to recover recombinant vaccinia virus (vrecFCoV-ADEF) as described previously (52). The identity of vrecFCoV-ADEF was verified by sequencing the FCoV insert, and it was used as parental virus for the introduction of the missing FCoV genome segment (nt 5083 to 15775) in a second step by two rounds of vaccinia virus-mediated homologous recombination using the *Escherichia coli gpt* gene as a positive or negative selection marker, as described previously (8, 28). The vaccinia virus vrecFCoV-ΔBC-2 was generated from vrecFCoV-ADEF by introducing the BC-1, *gpt*, and BC-3 fragments encoded by the plasmid pGPT-BC-1/3. After three rounds of plaque purification under *gpt*-positive selection, the recombinant vaccinia virus clone vrecFCoV-ΔBC-2 was obtained, and the identity was confirmed by sequence analysis. To obtain the full-length FCoV cDNA-containing vaccinia virus vrecFCoV, the *gpt* gene

was replaced by fragment BC-2. This involved the recombination of vrecFCoV-ΔBC-2 with plasmid pBC-2 and *gpt*-negative selection. Finally, the sequence of the full-length FCoV insert was verified by sequence analysis. The nucleotide differences between vrecFCoV sequence and the initially determined FCoV strain Black sequence are depicted in Table 2. In total, we introduced 14 silent nucleotide changes, which served as diagnostic markers to identify recombinant FCoV Black (see below).

Generation of recombinant FCoV strain Black. To rescue the recombinant FCoV strain Black, genomic DNA from purified vaccinia virus vrecFCoV was prepared, digested with ClaI enzyme, and in vitro transcribed using bacteriophage T7 RNA polymerase. It has been previously shown for other CoVs that rescue of recombinant CoVs from cloned cDNA is facilitated by N protein expression (5, 8, 59). Therefore, we generated a stable BHK cell line expressing the FCoV strain Black N protein (BHK-FCoV-N). The in vitro transcribed recombinant FCoV strain Black genomic RNA was electroporated into BHK-FCoV-N cells, and the cells were subsequently cocultivated with FCWF cells. At 2 to 3 days posttransfection, the supernatant was harvested and transferred to fresh FCWF cells. After 2 to 3 days of incubation, cytopathic effect was observed, and the supernatant containing recombinant FCoV strain Black viruses was collected for further analysis. First, the identity of recFCoV strain Black was confirmed by RT-PCR sequencing analysis using poly(A)-containing RNA from recFCoV strain Black-infected FCWF cells. DNA fragments amplified by RT-PCR contained the diagnostic mutations which allowed discrimination between parental and recombinant FCoVs (Fig. 3a). This result demonstrated that we had indeed rescued a recFCoV strain Black. Second, the phenotype of recFCoV was analyzed in tissue culture. The recombinant virus has growth kinetics similar to the wild-type virus, replicates to the same titer (Fig. 3b), and leads to the same plaque morphology (data not shown).

Generation of recFCoV Black encoding reporter genes. Efficient expression of foreign genes by CoVs has been previously described (9, 11, 16, 45, 47). In particular, the introduction of

FIG. 3. Analysis of recFCoV. (a) Sequence analysis of the FCoV strain Black and recFCoV in a region containing a silent marker mutation (at nt 485; indicated by an asterisk). (b) Growth kinetics of FCoV strain Black or recFCoV after infection of FCWF cells (MOI of 0.1). wt, wild type; p.i., postinfection.

FIG. 4. Generation and analysis of recFCoV expressing reporter genes. (a) The structural relationship of the FCoV strain Black and the recombinant viruses recFCoV-GFP and recFCoV-RL is shown. ORFs are indicated as boxes. The insertion site of the heterologous reporter gene sequence is depicted together with TRS elements and start and stop codons. (b) Growth kinetics of recFCoV-GFP and recFCoV-RL compared to the parental FCoV strain wt Black (wt) after infection of FCWF cells (MOI of 0.1). (c) Detection of GFP expression in recFCoV-GFP-infected (MOI of 0.1) FCWF cells 24 h postinfection (magnification, $\times 100$; Leica DM IL fluorescence microscope). (d) Kinetics of RL expression in recFCoV-RL-infected (MOI of 0.1) FCWF cells. p.i., postinfection; RLU, relative light units.

reporter genes into CoV genomes or replicon RNAs has been demonstrated to greatly facilitate the studies of CoV replication and transcription in target cells (28, 51). We replaced the accessory 3abc genes by genes encoding the GFP and RL, resulting in the recombinant viruses recFCoV-GFP and recFCoV-RL, respectively (Fig. 4a). Both viruses replicated efficiently in FCWF cells, displayed the same plaque morphology, and reached nearly the same titers as the parental FCoV strain Black (Fig. 4b). Furthermore, the heterologous genes were stably retained for at least six passages in FCWF cells (data not shown). To demonstrate reporter gene expression, FCWF cells were infected with recFCoV-GFP and recFCoV-RL. After recFCoV-GFP infection, green fluorescent cells could easily be detected by fluorescence microscopy, confirming the expression of GFP (Fig. 4c). recFCoV-RL-infected cells were harvested at different time points, and RL-activity was detectable in cell lysates. The level of RL expression was found to correlate with increasing viral titers during the course of infection (Fig. 4d).

Infection of feline monocytes and DCs with recombinant FCoV encoding reporter genes. FCoVs are known to cause in their natural host subclinical, persistent infection as well as lethal disease. It has been speculated that the ability of FCoV to efficiently infect and replicate in monocytic cells determines the severity of disease (13, 44, 50). Feline monocytes have been shown previously to be susceptible to type II FCoV infection, although the number of infected cells was usually very low (13). In order to investigate whether type I FCoV strain Black can infect and replicate in monocytes, we isolated feline CD14⁺ monocytes from peripheral blood using anti-human CD14 magnetic beads (49). First, feline CD14⁺ monocytes were infected (MOI of 0.1) with the parental FCoV strain Black and recFCoV-RL. Interestingly, we were not able to detect efficient replication of FCoV strain Black in monocytes, and even infection with the recFCoV-RL virus did not result in the detection of RL expression (data not shown). This may indicate that feline monocytes are not susceptible to type I FCoV strain Black infection. Alternatively, only a very limited, minor frac-

FIG. 5. recFCoV-GFP infection of feline monocytic cells. Shown are images of recFCoV-GFP-infected (MOI of 0.1) feline CD14⁺ monocytes (a) and feline DCs (b) at 24 h postinfection (Leica DM IL fluorescence microscope; magnification, $\times 100$).

tion of monocytes was infected, and RL expression or titers were too low for detection. To clarify this point, we infected feline CD14⁺ monocytes with recFCoV-GFP and monitored GFP expression by fluorescence microscopy. As shown in Fig. 5a, we were able to identify GFP-expressing monocytes; however, the number of green fluorescent cells was extremely low ($<0.01\%$). This low number of infected cells would explain why we could not demonstrate luciferase activity. To further assess the ability of FCoV strain Black to infect and replicate in monocyte-derived cells, we prepared feline macrophages and DCs from the CD14⁺ monocytes using hrGM-CSF or hrGM-CSF together with human recombinant IL-4, respectively (49). After CD14⁺ monocytes were cultured in the presence of hrGM-CSF for 6 days, about 30% of the cells displayed typical macrophage morphology. Again, infection with FCoV Black or recFCoV-RL did not result in the detection of efficient replication. However, after recFCoV-GFP infection, we were able to detect cells displaying green fluorescence but only a very low number; however, those cells did not show the typical macrophage morphology. Finally, infection of DCs was analyzed, and, similar to the results described above, no efficient replication was observed. We were, however, able to detect green fluorescent cells that displayed the characteristic DC morphology (Fig. 5b). Taken together, the susceptibility of CD14⁺ monocytes, macrophages, and DCs to type I FCoV infection appears to be limited, and only the use of a recombinant GFP-expressing FCoV enabled us to demonstrate type I FCoV replication in a small subset of these cells.

DISCUSSION

Type I FCoV causes approximately 80% of the natural infections of cats, while the other 20% are due to type II FCoV. Until now most studies on FCoVs focused on type II viruses, mainly because, in contrast to type I FCoV, they can be easily propagated in cell culture. Here, we have analyzed the genome organization and replication of a type I FCoV, the Black strain. We have cloned the full-length FCoV strain Black cDNA into a vaccinia virus cloning vector which allowed us to generate recombinant type I FCoV. Finally, we have addressed to what extent monocytic cells are susceptible to type I FCoV infection.

Our sequence analysis revealed the characteristic CoV genome organization. The replicase gene comprises two large ORFs; within the polyprotein pp1a/1ab, 14 protease cleavage sites were predicted with 16 nsps as end products. The analysis

of the structural gene region revealed that type I FCoV strain Black encodes four structural proteins in the order S-E-M-N. Between the S and E genes and downstream of the N gene, we have identified the accessory 3abc and 7ab genes, respectively. A 62-nt deletion in the ORF 3bc region results in the fusion of ORFs 3bc, and a nonsense mutation at nt 28441 leads to premature termination of the ORF7b gene product. FCoV strain Black expresses a characteristic set of six nested sgRNAs. The determination of the leader-body junctions allowed us to identify the core TRS of strain Black. In contrast to published type II FCoV genome sequences (14), we did not find any evidence for a recombination event between FCoV and CCoV (or any other CoV).

We have chosen the FCoV strain Black for several reasons. First, it is a type I FCoV, the most prevalent type of natural FCoV infections. Second, this isolate can be propagated in cell culture with peak titers of up to 10^5 times the 50% tissue culture infective dose in FCWF cells. Third, we have chosen the high-passage (HP) FCoV strain Black stock to generate our reverse genetic system in order to facilitate the recovery of recombinant viruses. We have obtained low-passage (LP) and high-passage (HP) stocks of the original FCoV strain Black. According to the available literature, the LP virus is virulent and causes FIP in experimentally infected cats, while the HP virus is attenuated and no longer able to induce FIP (42). Because it is not documented whether our HP Black strain represents the HP stock that was used in previous *in vivo* experiments, its pathogenicity remains to be determined. Our preliminary sequencing analyses of the LP virus stock revealed a number of nucleotide differences, mainly within the accessory gene regions (unpublished data). Moreover, we observed that the LP virus stock contains a mixture of viruses including the HP virus that we have cloned in vaccinia virus. The reverse genetic system for the type I FCoV strain Black will now allow the reconstitution of LP virus(es) and its phenotypic analysis *in vitro* and *in vivo*. Since our reverse genetic system for type I FCoV is based on cloned full-length cDNA, the entire genome is now amenable to mutagenesis. Thus far, the genome of an FCoV could be modified only within the 3' third of the genome (21). However, there is now accumulating evidence for a role of replicase gene product(s) in the pathogenesis of CoVs (48, 60). Accordingly, the reconstitution of LP virus(es) may include replicase gene product(s) (i.e., regions of the 5' two-thirds of the genome).

The reverse genetic system also allows the identification of type I FCoV target cells. Along this line we have generated recFCoV encoding GFP or RL reporter proteins (recFCoV-GFP and recFCoV-RL). The GFP and RL genes were stably retained in the rescued viruses, and their efficient growth in feline cells confirmed the general observation that accessory genes are dispensable for CoV replication *in vitro* (6, 10, 21, 22, 40, 46, 58). We have chosen to evaluate the infection of feline CD14⁺ monocytes, macrophages, and DCs with FCoV strain Black, since these cell types most likely represent important target cells for FCoV and other CoVs *in vivo* (3, 7, 35, 37, 41, 56). For FCoV infection, monocytes are considered to contribute to the dissemination of the virus throughout the host organism. Our analyses revealed that efficient replication of type I FCoV can be observed in only very few monocytes, macrophages, and DCs. Notably, the detection of type I FCoV-

infected cells was possible only through the expression of GFP by a recombinant virus produced with our reverse genetic system. It has previously been reported that less than 1% of the monocytes could be infected in vitro with the most commonly investigated type II FCoV, strain 79-1146 (13). Since type II 79-1146 grows in FCWF cells to 100-fold higher titers than the type I FCoV strain Black (data not shown), a direct comparison of both types is required to assess if there are differences in the susceptibility of monocytes between type II and type I FCoV infections. Further studies are also required to evaluate if there are differences between type II and type I FCoV replication in feline macrophages and DCs. Notably, the use of commercially available human cytokines (hrGM-CSF and human recombinant IL-4) enabled us to generate feline DCs from CD14⁺ monocytes. To our knowledge, the detection of recFCoV-GFP-mediated GFP expression in feline DCs represents the first demonstration that FCoV is able to infect and replicate in DCs. DCs have been shown to represent important target cells for a number of CoVs. In the murine system it has been shown that murine hepatitis virus can infect DCs and that plasmacytoid DCs are important for the induction of type I interferon responses (7). Furthermore, besides their function in innate immune responses, DCs are known to be key mediators in shaping the emerging adaptive immune response. Thus, the study of FCoV target cells represents an important aspect of FCoV research and may help provide more insight into the pathogenesis of FCoV-induced disease.

The limited susceptibility of monocytes, DCs, and macrophages to type I FCoV infection observed in this study raises the question of which receptor(s) is involved in FCoV infection. It has been previously reported that type II FCOVs use fAPN (feline CD13) as a receptor (53). Nevertheless, it should be noted that type II FCoV strains can also display differences in the ability to replicate in macrophages (13, 44, 50), and it has been proposed that the type II FCoV spike protein is the major determinant of macrophage tropism. Detailed analyses revealed that the type II FCoV spike-binding domain resides within fAPN residues 670 to 840, a region that is also important for entry of porcine transmissible gastroenteritis virus and CCoV into permissive cells (23, 57). Interestingly, the type II FCoV sequence indicates that recombination between type I FCoV and CCoV led to the replacement of the type I FCoV S gene by the CCoV S gene. Thus, it appears questionable whether type I FCOVs also use fAPN as a receptor (15, 32). Further studies are required to resolve which cellular receptor is used for type I FCoV entry and whether a pronounced macrophage tropism correlates with more virulent type I FCoV phenotypes in vivo. Clearly, the reverse genetic system for type I FCoV will greatly facilitate addressing these questions.

In summary, we have analyzed the genome organization and replication of a type I FCoV and established a reverse genetic system for this FCoV serotype. The system is based on cloned, full-length cDNA and enables the manipulation of the entire genome. The reverse genetic analysis of type I FCoV will lead to new insights into important aspects of FCoV target cell tropism, receptor usage, and pathogenicity. Finally, this system will enable the construction of recombinant FCoV vaccines designed to protect against homologous and heterologous virus-induced diseases of cats.

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