GENOMIC ORGANIZATION AND EXPRESSION OF THE 3' END OF THE CANINE AND FELINE ENTERIC CORONAVIRUSES

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ABSTRACT

The genomic organization at the 3' end of canine coronavirus (CCV) and feline enteric coronavirus (FECV) was determined by sequence analysis and compared to that of feline infectious peritonitis virus (FIPV) and transmissible gastroenteritis virus (TGEV) of swine. Comparison of the latter two has previously revealed an extra open reading frame (ORF) at the 3' end of the FIPV genome, lacking in TGEV, now designated ORF 6b. Both CCV and FECV possess 6b-related ORFs. The CCV ORF 6b is colinear with that of FIPV, but the predicted amino acid sequences are only 58% identical. The FECV ORF 6b contains a large deletion compared to that of FIPV, reducing the colinear part to 60%. The sequence homologies were highest between CCV and TGEV on the one hand and between FECV and FIPV on the other. The expression product of the CCV and the FECV ORF 6b can be detected in infected cells by immunoprecipitation.

INTRODUCTION

Canine coronavirus (CCV), feline enteric coronavirus (FECV), feline infectious peritonitis virus (FIPV), and transmissible gastroenteritis virus (TGEV) of swine belong to one antigenic cluster (1). Sequence analysis revealed a close genetic relatedness between FIPV and TGEV (2, 3, 4). FIPV contains an extra open reading frame (ORF) in the 3'-terminal region of its genome (3). It is the second ORF of mRNA 6, currently designated ORF 6b. The 6b gene product was detected in FIPV-infected cells (5). It is a secreted nonstructural glycoprotein. In cats it induces antibodies during FIPV infections. The 6b protein provides an antigenic distinction between FIPV and TGEV. The aim of the present study was to examine whether this distinction could be extended to CCV and FECV.

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MATERIALS AND METHODS

Cells and viruses

FIPV strain 79-1146, FECV strain 79-1683 (6), and CCV strain K378 (Dutch field isolate) were grown in *Felis catus* whole fetus cells (fcwf-D). Recombinant vaccinia virus vTF7-3 (7) infections were carried out in HeLa cells. Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories) containing 5% fetal bovine serum.

Cloning and sequence analysis of the 3' end of CCV

cDNA libraries were prepared of intracellular poly(A)$^+$ RNA from CCV infected fcwf-D cells as described elsewhere (J. Wesseling manuscript in preparation). Clones containing sequences derived from the 3' end of the genome were selected by colony hybridization with restriction fragments of FIPV cDNA clone B12 (3) as probes. Nucleotide sequencing was performed on double stranded DNA using a bacteriophage T7 DNA polymerase based kit (Pharmacia LKB). Sequence data were analyzed using the computer programs of Devereux et al. (8).

cDNA synthesis and PCR amplification of the 3' end of FECV

Synthesis of cDNA on total RNA isolated from FECV infected fcwf-D cells was performed as described (9) by priming specifically with synthetic oligonucleotide 5'-CCAGTTTTAGACATCGGG-3' which binds to a sequence in the 3' non-coding region of FIPV, downstream of ORF 6b. Oligonucleotide 5'-GATCCAGACGTTAGCTC-3', was used to prime cDNA synthesis from a position closer to the 3' end. Amplification of cDNA was performed by the polymerase chain reaction (PCR) as described (9), after the addition of synthetic oligonucleotide 5'-GATGACACACAGGTTGAG-3', which is located at the 3' end of the nucleocapsid (N) protein gene of FIPV. PCR amplified cDNA fragments of FECV were cloned after homopolymer tailing in dG-tailed pUC9 (Pharmacia LKB) and sequenced as described above.

Radio immunoprecipitation assays (RIPA)

Lysates from coronavirus-infected fcwf-D cells or recombinant vaccinia virus-infected HeLa cells were prepared after metabolic labeling with L-[^{35}S]cysteine (ICN Biomedicals, Inc.). Lysis, RIPA with ascites fluid from a field case of FIP and endo-ß-*N*-acetylglucosaminidase H (endo H; Boehringer Mannheim Biochemicals) treatment were carried out as described (10). Analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (11).

RESULTS

Sequence analysis of the 3' end of the CCV genome

CCV cDNA clones were prepared, selected and sequenced as described under materials and methods. We obtained a contiguous sequence of 2.5 kb from the 3' end. The sequence data are available under the accession number X66717. Translation of the nucleotide sequence revealed three ORFs corresponding to the N protein and ORFs 6a and 6b of FIPV. The ORF 6b predicts a short hydrophobic amino-terminus,

which may function as a signal sequence. In contrast to the FIPV ORF 6b, the CCV ORF 6b contains no potential N-glycosylation site.

PCR amplification of cDNA derived from the 3' end of the FECV genome

We performed cDNA synthesis followed by PCR amplification on total RNA from FECV- and CCV-infected cells using primers which flanked the coding region of mRNA 6. The CCV PCR product had the expected size of 1 kbp. The FECV product was considerably smaller, being approximately 750 bp. The controls with RNA from mock-infected cells and without RNA were both negative. Sequence analysis revealed that the FECV ORF 6b extended into the sequences used to design the PCR primers. Therefore, cDNA-PCR was repeated with a primer hybridizing 36 nucleotides upstream of the poly(A)-tail. This resulted in a fragment of approximately 950 bp (data not shown).

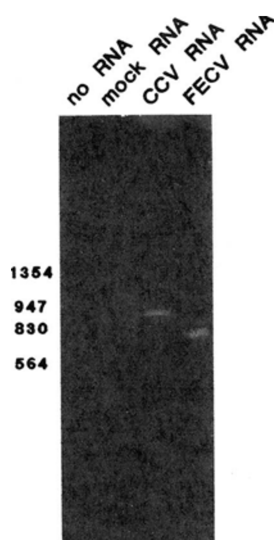

Figure 1. Agarose gel analysis of PCR amplified CCV and FECV cDNA. The RNA sources used for cDNA preparation are indicated above the lanes. Marker indicated on the left is bacteriophage lambda DNA digested with EcoRI and HindIII.

Analysis of genomic sequences of FECV near the 3' end

The PCR products of FECV were cloned and sequenced, leading to a contiguous sequence of 957 nucleotides. The sequence data are available under the accession number X66718. Comparison with the corresponding sequence of FIPV showed a single deletion of 238 nucleotides and an overall sequence identity of 93.6%. Translation of the nucleotide sequence revealed the presence of two ORFs similar to the FIPV ORFs 6a and 6b. The deletion is located in ORF 6b. The FECV and FIPV 6b sequences are colinear for the amino-terminal 123 amino acid residues. The deletion results in a shift to the -1 reading frame which extends 53 codons. The ORF specifies a polypeptide with a total length of 176 amino acid residues and a predicted mol wt of 20,300. A short hydrophobic amino-terminus, probably acting as a signal sequence, and one N-glycosylation site are predicted.

Sequence comparison and genomic organization

The genomic organizations of the CCV and FECV 3' ends are similar to FIPV, containing an ORF 6b and the extra 69 nucleotides in ORF 6a as compared to TGEV ORF 7 (Fig. 2). Paired alignments of the colinear parts of the amino acid sequences revealed that CCV is closely related to TGEV and FECV to FIPV (Table 1). The same was found when the nucleotide sequences were compared. The 6b amino acid sequences of CCV and FIPV were only 58% identical and several small insertions in the CCV sequence were found. Nevertheless, the hydrophobicity plots of the putative 6b proteins were remarkably similar (not shown). The same plot for FECV 6b showed divergence in the C-terminal 40%.

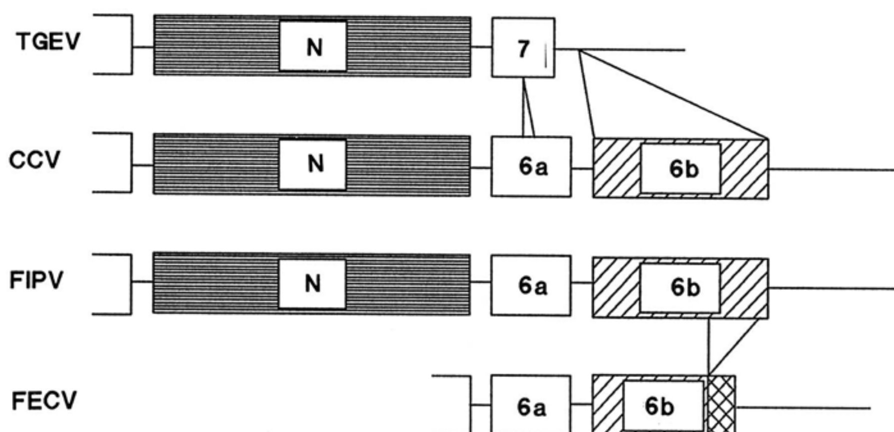

Figure 2. Schematic comparison of the genomic organization of the 3' terminal regions of TGEV, CCV, FIPV and FECV. Boxes represent the relevant open reading frames. The cross hatched box at the C-terminal end of FECV ORF 6b indicates the divergent part.

Table 1. Paired comparisons of the colinear parts of the amino acid sequences, in percentages identical residues.

		FIPV		CCV			TGEV	
		6a	6b	N	6a	6b	N	7
FECV	6a	99	-	-	80	-	-	77
	6b		89	-	-	51	-	-
FIPV	N			78	-	-	76	-
	6a				79	-	-	77
	6b					58	-	-
CCV	N						93	-
	6a							96

Identification of the CCV and FECV 6b proteins

The FIPV 6b protein was readily detected in lysates of FIPV-infected cells (5). To identify the CCV and FECV 6b proteins their 6b ORFs were recloned in a T7 expression vector. The resulting constructs pTC6b and pTE6b, respectively, and pTF6b containing the FIPV 6b gene (5) were used to transfect HeLa cells infected with recombinant vaccinia virus vTF7-3, which produces T7 RNA polymerase (7). The expression products were analyzed by metabolic labeling with [^{35}S]cysteine, RIPA, and endo H treatment followed by SDS-PAGE (Fig. 3). The CCV and FECV 6b proteins appeared to be slightly smaller than the FIPV 6b protein, the FECV 6b protein being the smallest. Digestion with endo H which cleaves high mannose N-linked oligosaccharides, resulted in an approximately 2,000 mol wt reduction of the FECV and FIPV 6b protein. The CCV 6b protein, was not affected and was also insensitive to digestion by endoglycosidase F, which cleaves complex N-linked sugars (data not shown). This indicates that the 6b proteins of FECV and FIPV are glycoproteins while CCV 6b is not. The shift in molecular weight of the FECV and FIPV 6b proteins is consistent with the removal of 1 sugar side chain (12). This is in agreement with the predicted numbers of glycosylation sites in the amino acid sequences. The observed molecular weights of the CCV 6b protein and of the FECV 6b protein after deglycosylation are also in agreement with those predicted from the amino acid sequences. Similar proteins were detected in CCV-, FECV-, and FIPV-infected cells, analyzed in the same way (Fig. 3). The lanes of FECV were overexposed to reveal the 6b protein band. This indicates that the expression level of the FECV 6b protein was lower than that of CCV and FIPV.

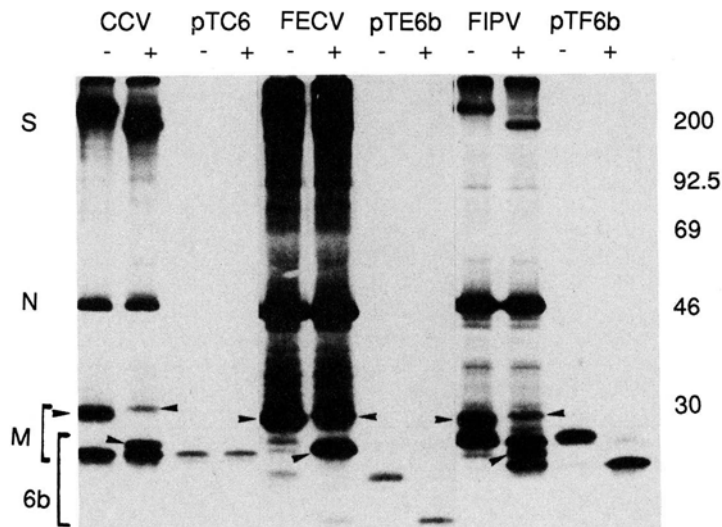

Figure 3. Radio immunoprecipitation and SDS-PAGE analysis of lysates from CCV-, FECV-, FIPV-, and vTF7-3-infected cells. Recombinant vaccinia virus vTF7-3-infected cells were transfected with the plasmid DNAs pTC6b, pTE6b and pTF6b, as indicated above the lanes. One half of each sample was treated with endo H, the other half was mock treated (indicated with + and -, respectively). Structural proteins (S, N and M) are indicated. In addition, the M protein bands are indicated with arrowheads. The region of the gel in which the 6b protein bands appear overlaps with that of M protein bands.

DISCUSSION

The genomic organization of the 3' end of FIPV differs from that of TGEV in that it contains an additional ORF (3). Recently, we identified the expression product of this extra gene, designated 6b (5). These observations prompted us to study CCV and FECV of the same antigenic cluster. Sequence analysis showed that their genomic organization in the 3' terminal region is similar to that of FIPV. Therefore, the presence rather than the absence of ORF 6b appears to be the common theme, suggesting that TGEV has lost the corresponding ORF by deletion. The same inference probably holds true for the 69 nucleotides that are present in all 6a ORFs but not in the corresponding ORF 7 of TGEV. Alignment of nucleotide and amino acid sequences of TGEV, CCV, FIPV and FECV allowed division into two pairs on the basis of their homologies; TGEV and CCV on the one hand and FIPV and FECV on the other.

Both in CCV- and in FECV-infected cells 6b proteins are produced. This observation appears to compromise their antigenic distinction from FIPV. However, the differences between the 6b proteins may allow discrimination using e.g. monoclonal antibodies. The deletion of 238 nucleotides in FECV 79-1683 was revealed by cDNA-PCR, allowing discrimination from CCV (Fig. 1) and FIPV 79-1146 (data not shown). It remains to be determined whether this is a universal distinguishing property of FIPV and FECV.

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