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Intracellular RNAs of the Feline Infectious Peritonitis Coronavirus Strain 79-1146

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SUMMARY

In *Felis catus* whole foetus D cells infected with feline infectious peritonitis virus (FIPV), strain 79-1146, six virus-specific, poly(A)-containing RNA species of about 20, 9·6, 5·2, 3·8, 2·8 and 1·6 kb were found. By translation *in vitro* the 3·8 and 2·8 kb RNAs were shown to encode the 25K envelope protein and the 45K nucleocapsid protein, respectively. The partial map of the FIPV genome was compared with genomic maps of porcine, murine and avian coronaviruses. Differences in these maps suggest that transcription units have been lost or gained during coronavirus divergence.

INTRODUCTION

Feline infectious peritonitis (FIP) is a generally fatal disease in domestic and wild Felidae (for review, see Pedersen, 1983). The causative agent, FIP virus (FIPV), has been identified as a coronavirus on the basis of morphology (Ward, 1970; Zook *et al.*, 1968) and antigenic relationship to other coronaviruses, e.g. transmissible gastroenteritis virus (TGEV) of swine, canine coronavirus and human coronavirus E229 (Horzinek *et al.*, 1982; Pedersen *et al.*, 1978).

Although the pathogenesis of FIP is poorly understood, there are indications that the immune system is involved (Horzinek & Osterhaus, 1979). Transferred or actively acquired anti-coronavirus antibodies have been found to make cats more prone to a fatal course of FIP (Pedersen & Boyle, 1980; Weiss & Scott, 1981*a, b*). The clinical features of FIP suggest an immune complex disease (Horzinek & Osterhaus, 1979; Jacobse-Geels *et al.*, 1980, 1982; Pedersen & Boyle, 1980; Weiss & Scott, 1981*a, b*).

Our present knowledge of the molecular biology of coronaviruses primarily stems from studies of murine hepatitis virus (MHV) and avian infectious bronchitis virus (IBV). Coronavirions contain three main structural proteins: a glycosylated peplomer protein (E2), a transmembrane envelope glycoprotein (E1) and a phosphorylated nucleocapsid protein (N). The coronavirus genome is a positive-stranded RNA of at least 20 kb (for reviews, see Siddell *et al.*, 1982; Sturman & Holmes, 1983). During replication a negative strand serves as a template for the synthesis of one genome-sized and multiple subgenomic mRNAs (Lai *et al.*, 1982; Spaan *et al.*, 1981; Stern & Kennedy, 1980). The viral mRNAs have a nested set structure with 3' coterminal ends and sequences extending different lengths in the 5' direction. Of each RNA, only the unique 5' terminal sequences, not present in the next smaller RNA of the set, are translated (Siddell *et al.*, 1982; Sturman & Holmes, 1983). A characteristic feature of coronavirus mRNAs is the presence of a short 5' common 'leader' sequence. 'Body' and 'leader' sequences are not contiguous on the genome, but are joined by discontinuous transcription (Brown *et al.*, 1984; Lai *et al.*, 1984; Spaan *et al.*, 1983, 1984).

For MHV, IBV and recently TGEV, several genes coding for structural and putative non-structural proteins have been mapped (Hu *et al.*, 1984; Jacobs *et al.*, 1986; Rottier *et al.*, 1981;

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Siddell, 1983; Stern & Sefton, 1984) and sequenced (Armstrong *et al.*, 1984; Binns *et al.*, 1985; Bourns & Brown, 1984; Bourns *et al.*, 1984, 1985*a, b*; Skinner & Siddell, 1985; Skinner *et al.*, 1985).

Apart from the polypeptide composition of the virion (Boyle *et al.*, 1984; Horzinek *et al.*, 1982), no data on the molecular biology of FIPV have been reported. However, such studies may be essential for the elucidation of the pathogenesis of FIP and may furthermore expand our knowledge of the molecular biology of coronaviruses. In this paper we describe the isolation and characterization of the intracellular RNAs of the FIPV strain 79-1146. The results enabled us to construct a partial map of the FIPV genome, which is compared with the maps of IBV, MHV and TGEV.

METHODS

Radiochemicals. All radiochemicals were purchased from Amersham.

Cells and virus. *Felis catus* whole foetus (fcwf) D cells (Boyle *et al.*, 1984) and the FIPV strain 79-1146 (McKeirnan *et al.*, 1981) were obtained from Dr N. C. Pedersen (School of Veterinary Medicine, University of California, Davis, Ca., U.S.A.). Fcwf-D cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum (DMEM-10% FCS) and containing penicillin (100 IU/ml) and streptomycin (100 µg/ml).

FIPV 79-1146 was purified twice by endpoint dilution before virus stocks were prepared. In all infection experiments the cells were washed with phosphate-buffered saline (PBS), containing 50 µg/ml DEAE-dextran (PBS-DEAE), and inoculated with FIPV diluted to the desired m.o.i. with the same buffer. Virus adsorption was allowed to proceed for 1 h at 37 °C. Subsequently, the inoculum was replaced by DMEM-10% FCS. Tissue culture supernatants containing cell-free virus were harvested 16 to 20 h after infection (p.i.). Virus titres of 5×10^7 to 7×10^7 TCID₅₀ units/ml were usually obtained.

Virus suspensions were titrated by infecting fcwf-D cell monolayers in microtitre plates (96 wells; Costar) with 25 µl of serial tenfold dilutions in PBS-DEAE. After 2 to 3 days at 37 °C the cytopathic titration endpoints were read. The titres (in TCID₅₀ units/ml) were calculated using the Spearman-Kärber formula.

Kinetics of virus-specific RNA production. The kinetics of viral RNA production were determined essentially according to Spaan *et al.* (1981). Fcwf-D cells, grown to confluence on 10 × 10 mm coverslips, were infected at an m.o.i. of 15 TCID₅₀/cell or mock-infected. After adsorption, the cells were maintained in DMEM-10% FCS containing 1 µg/ml actinomycin D. At 2 h p.i., 10 µCi/ml [5-³H]uridine (27.8 Ci/mmol) was added and incubation was continued. At different times p.i. the incorporation of [³H]uridine was determined by counting the TCA-precipitable radioactivity on the coverslips.

Isolation and characterization of virus-specific intracellular RNAs. Monolayers of fcwf-D cells in 35 mm Petri dishes were infected with FIPV at an m.o.i. of 15 TCID₅₀/cell. At 5 h p.i. the DMEM-10% FCS was replaced by phosphate-free DMEM buffered with 20 mM-HEPES-KOH pH 7.2, containing 2% dialysed FCS and 1 µg/ml actinomycin D. At 6 h p.i. 150 µCi carrier-free [³²P]orthophosphate per ml was added. The cells were lysed at 9 h p.i. and RNA was extracted as described by Spaan *et al.* (1981). The RNAs were poly(A)-selected by oligo(dT)-cellulose chromatography, denatured with glyoxal and analysed by electrophoresis in 1% agarose-6 M-urea gels. A glyoxal-denatured EcoRI-HindIII digest of phage λ DNA was used as a molecular weight marker (McMaster & Carmichael, 1977).

Preparative isolation of virus-specific mRNAs. Approximately 100 µg poly(A)-containing RNA was isolated from 2×10^8 FIPV-infected fcwf-D cells, grown in plastic roller bottles (850 cm²; Corning). This RNA was fractionated by isokinetic sucrose gradient centrifugation as described by Jacobs *et al.* (1986). ³²P-labelled FIPV RNA was added as a marker. The RNAs were recovered by precipitation with ethanol.

Antisera. Ascites fluid from a field case of FIP (ascites 36) was used for the detection of FIPV-specific structural proteins. Two monoclonal antibodies, COR12 and COR15, directed against N and E1, respectively, were a gift from Dr S. Fiscus (Fort Collins, Co., U.S.A.).

Radioactive labelling of viral structural proteins. Monolayers of fcwf-D cells were infected with FIPV as described above. At 6 h p.i. the tissue culture supernatant (DMEM-10% FCS) was replaced by methionine-free MEM, supplemented with 2% foetal calf serum and 150 µCi/ml [³⁵S]methionine. At 12 h p.i. the tissue culture supernatant was harvested; cell debris was removed by centrifugation at 1000 g for 10 min. The virus suspension was layered on top of an 18 ml linear 20 to 50% (w/w) sucrose gradient and centrifuged for 16 h at 30000 g in an SW27.1 rotor. Virus-containing fractions were identified by measuring the radioactivity.

Radioactive labelling of intracellular virus-specific proteins. Fcwf-D cells grown in a 25 cm² tissue culture flask (Costar; 3×10^6 cells) were infected with FIPV and incubated under standard conditions. At 6 h p.i. the DMEM-10% FCS was replaced by methionine-deficient MEM, containing 150 µCi/ml [³⁵S]methionine. At 6.5 h p.i. the

medium was removed and the cells were washed three times with PBS. They were then lysed with 1.5 ml TES buffer (20 mM-Tris-HCl pH 7.5, 1 mM-EDTA, 100 mM-NaCl), containing 1% Triton X-100, 0.5% 1,5-naphthalene disulphonate disodium salt and 2 mM-PMSF. The lysate was cleared by centrifugation for 2 min at 10000 *g* and stored at -20 °C.

In vitro translation of mRNAs in a rabbit reticulocyte cell-free system. Volumes (1 µl) containing about 1 µg of RNA were incubated with 0.5 µl of [³⁵S]methionine (10 µCi) and 4 µl of rabbit reticulocyte lysate (Amersham) for 60 min at 30 °C. The lysates were analysed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation.

Immunoprecipitation. For immunoprecipitation, TES buffer containing 4 µl portions of translation mixtures, 25 µl lysate of FIPV-infected cells, 5 µl ascites 36 or 5 µl monoclonal antiserum and 1% Triton X-100, 0.5% 1,5-naphthalene disulphonate disodium salt and 2 mM-PMSF in a total volume of 300 µl was incubated for 16 h at 4 °C. Subsequently, 50 µl of 3 M-KCl and 50 µl of a 10% suspension of *Staphylococcus aureus* in TES buffer, containing 0.1% Triton X-100, were added. After 30 min incubation at 4 °C the immune complexes were collected by centrifugation for 30 s at 10000 *g*, washed three times with 0.2 ml of TES buffer, 0.1% Triton X-100 and finally dissolved in electrophoresis sample buffer (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue). Immune complexes were analysed by electrophoresis in 12.5% polyacrylamide slab gels.

Northern blot hybridization. Total RNA extracted from FIPV-infected cells was denatured for 30 min by incubation at 56 °C in 50% deionized formamide, 2.2 M-formaldehyde and 0.5 mM-EDTA. The samples were cooled on ice, and after addition of 0.6 vol. sample buffer containing 0.5% SDS, 0.025% bromophenol blue, 25% glycerol and 25 mM-EDTA, the samples were electrophoresed (50 V for 7 h) in a horizontal submerged gel containing 1.1 M-formaldehyde and 1% agarose in 10 mM-phosphate buffer pH 7.0. RNA was blotted from the gel to a membrane filter (Gene Screen; New England Nuclear). Prehybridization was allowed to occur in 50% formamide, 5 × SSC (1 × SSC is 150 mM-sodium chloride, 15 mM-sodium citrate), 10 × Denhardt's solution (1 × Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.05 M-phosphate buffer pH 6.5, 100 µg/ml sonicated salmon sperm DNA and 0.1% SDS for 30 min at 42 °C. Hybridization at 42 °C for 20 h was carried out with the purified 3.5 kb insert of the recombinant plasmid pFIPVB12, which was radiolabelled with ³²P by nick translation. After the incubation period the filter was washed at 42 °C with 2 × SSC three times and once with 0.1 × SSC.

RESULTS

Kinetics of virus growth

To study the replication of FIPV under one-step growth conditions, fcwf-D cells were infected at an m.o.i. of 15 TCID₅₀/cell. In pilot experiments, it was found by indirect immunofluorescence that at this m.o.i. all the cells became infected (data not shown).

The kinetics of virus release into the medium are shown in Fig. 1. Virus secretion started at 6 h p.i. and reached a maximum at 12 h p.i. Approximately 50% of the infectivity remained cell-associated. As early as 6.5 h p.i. the first cytopathological changes (rounding and swelling of cells) could be observed. At 8 h p.i. the cells had formed syncytia.

Isolation and characterization of virus-specific intracellular RNAs

The kinetics of synthesis of intracellular virus-specific RNA was studied by measuring the incorporation of [³H]uridine in the presence of 1 µg/ml actinomycin D in infected and mock-infected cells. Under these conditions, viral RNA synthesis reached a maximum rate during 6 and 9 h p.i. At 9 h p.i., [³H]uridine incorporation was stimulated 15-fold in FIPV-infected cells above the background in mock-infected cells (data not shown). To analyse the FIPV-specific intracellular RNAs, cells were labelled with [³²P]orthophosphate in the presence of actinomycin D. In infected, but not in mock-infected cells, six poly(A)-containing RNA species could be detected, with sizes of approximately 20, 9.6, 5.2, 3.8, 2.8 and 1.6 kb (Fig. 2a). The virus-specificity of these RNA species was confirmed by Northern blotting. RNA extracted from infected and mock-infected cells was hybridized to a recombinant plasmid, pFIPVB12. This plasmid was selected from a cDNA library of FIPV genomic RNA (to be described elsewhere) by screening with the smallest (1.6 kb) RNA species (R. J. de Groot, unpublished results). All the RNA species hybridized with the 3.5 kb insert of pFIPVB12 (Fig. 2b).

In order to determine whether the RNA patterns of antigenically related coronaviruses are similar, we compared the RNAs of the FIPV strain 79-1146 and the TGEV strain Purdue. As reported by Jacobs *et al.* (1986), six RNA species were found for TGEV, and were designated 1

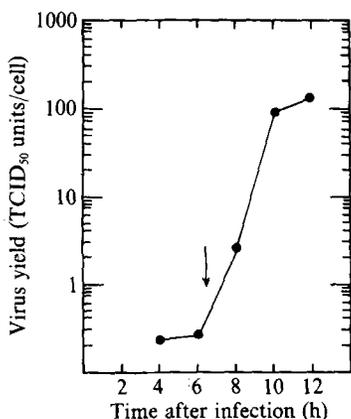


Fig. 1. One-step growth kinetics of FIPV 79-1146 in fcwf-D cells. The cells were infected at an m.o.i. of 15 TCID₅₀/cell. The arrow indicates the onset of cytopathic changes.

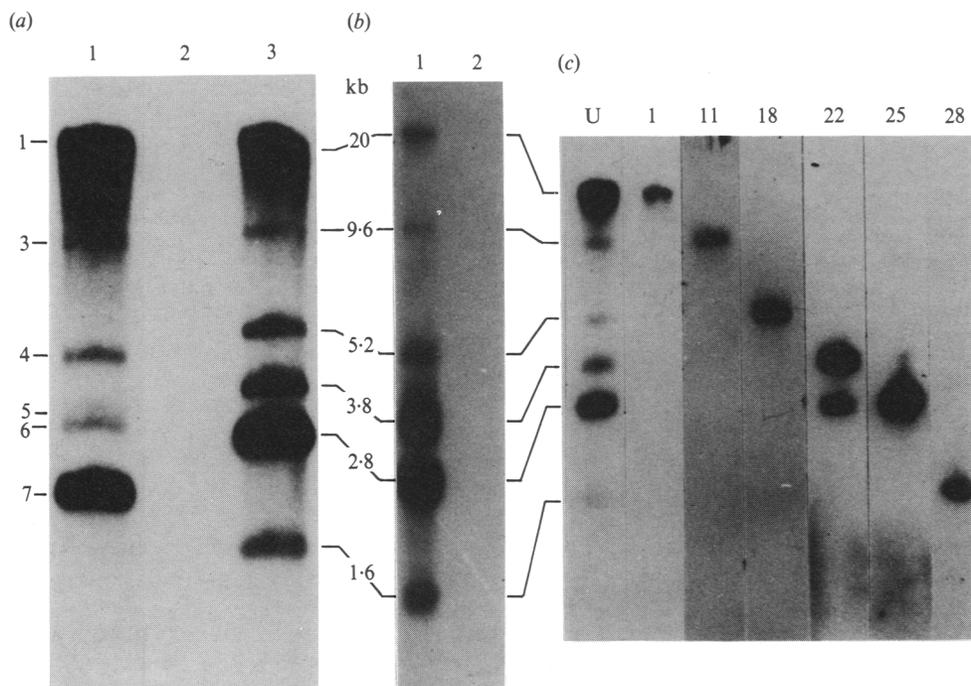


Fig. 2. (a) Electrophoresis of FIPV-specific poly(A)-containing intracellular RNAs. Cells were (lane 2) mock-infected or (lane 3) infected with FIPV 79-1146 and labelled with [³²P]orthophosphate in the presence of 1 µg/ml actinomycin D. At 9 h p.i. RNAs were extracted, purified by oligo(dT)-cellulose chromatography, denatured with glyoxal and analysed by electrophoresis in 1% agarose-urea gels. For comparison, ³²P-labelled TGEV RNAs are shown in lane 1. The TGEV RNAs were isolated and numbered as described by Jacobs *et al.* (1986). (b) Northern blot analysis of FIPV mRNAs. Unlabelled intracellular RNAs from (lane 1) FIPV-infected and (lane 2) mock-infected cells were separated by formaldehyde gel electrophoresis. The RNA was transferred to a membrane filter and hybridized with the radiolabelled insert of pFIPVB12. The autoradiograph was exposed for 6 h at -70 °C with an intensifying screen. (c) Analyses of preparations of separated intracellular viral RNAs. Unlabelled poly(A)-selected RNAs of FIPV-infected cells were purified by isokinetic sucrose gradient centrifugation, ³²P-labelled FIPV RNAs serving as markers. Gradient fractions were analysed by agarose-urea gel electrophoresis. The fractions used for translation *in vitro* are shown together with (U) unfractionated ³²P-labelled FIPV RNAs.

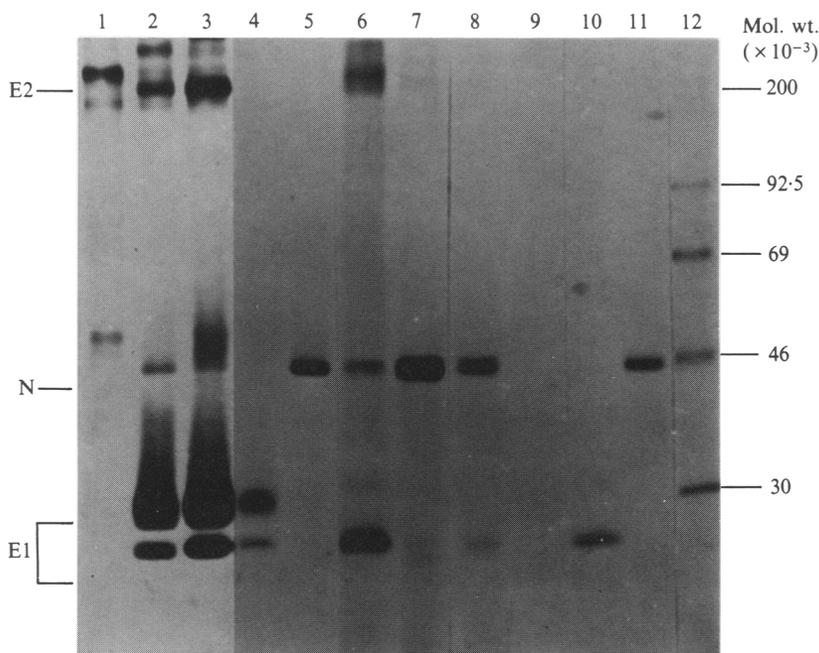


Fig. 3. Translation *in vitro* of FIPV RNAs. Translation products were analysed by electrophoresis in 12.5% SDS-polyacrylamide gels, after immunoprecipitation. Ascites 36 and (lane 1) lysate of mock-infected cells, (lane 2) sucrose gradient-purified FIPV and lysate of FIPV-infected cells (lane 3); (lane 4) infected cell lysate with COR15 and (lane 5) COR12; *in vitro* translation products of (lane 6) fraction 22, (lane 7) fraction 25 and (lane 8) total poly(A)-containing RNA of FIPV-infected cells with ascites 36; (lane 9) *in vitro* translation products of total poly(A)-containing FIPV RNAs with negative cat serum; (lane 10) *in vitro* translation products of fraction 22 with COR15; (lane 11) *in vitro* translation products of fraction 25 with COR12. Lane 12 contains mol. wt. markers.

and 3 to 7. As shown in Fig. 2(a), the RNA patterns of TGEV Purdue and FIPV 79-1146 are clearly different.

Identification of mRNAs coding for the nucleocapsid and envelope protein

As has been found for other coronaviruses, the FIPV virion consists of three main structural proteins: a 210K peplomer protein (E2); a 45K nucleocapsid protein (N) and an envelope protein (E1), which appears in three forms of 32K, 30K and 25K (Boyle *et al.*, 1984).

FIP ascites 36 recognized all three structural proteins in a radioimmunoprecipitation assay using sucrose gradient purified virus or lysates from infected cells (Fig. 3, lanes 2 and 3). The monoclonal antibodies COR12 and COR15 specifically detected the nucleocapsid and envelope protein, respectively (Fig. 3, lanes 4 and 5).

In order to identify the mRNAs encoding the structural proteins, the intracellular RNAs of FIPV 79-1146 were separated by isokinetic sucrose gradient centrifugation, ^{32}P -labelled FIPV RNAs serving as markers. Fractions from the gradient were analysed by urea-agarose gel electrophoresis. All FIPV RNAs could be isolated in a relatively pure state, except for the 3.8 kb species which was always contaminated with either the 5.2 or the 2.8 kb RNA (Fig. 2c). After translation of the RNA fractions in a rabbit reticulocyte lysate, virus-specific products were precipitated with ascites 36 or monoclonal antibodies and analysed by SDS-polyacrylamide gel electrophoresis.

Translation *in vitro* of total poly(A)-selected RNA of FIPV-infected cells resulted in the synthesis of polypeptides of 25K and 45K (Fig. 3, lane 8). These products were also found after translation of fraction 22 (Fig. 3, lane 6), containing both the 3.8 and 2.8 kb RNAs (see Fig. 2c).

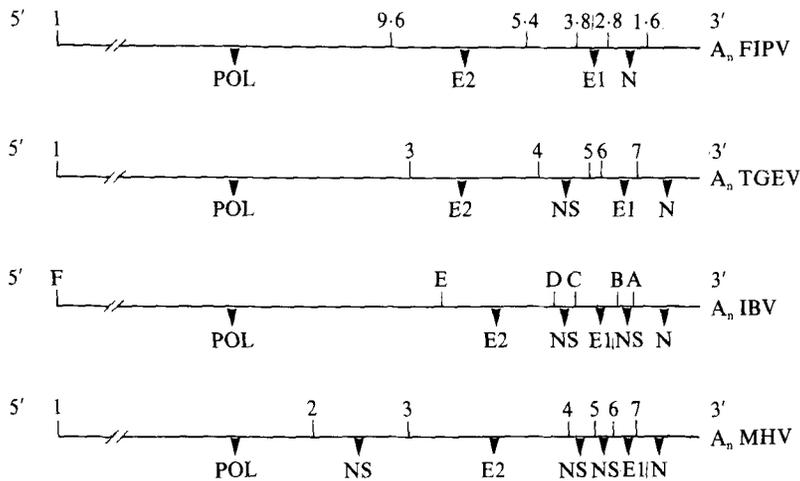


Fig. 4. Genetic organization of FIPV, TGEV, MHV and IBV. The genomic maps are drawn to scale. The positions corresponding to the 5' ends of the intracellular RNAs are marked. Genes encoding the structural proteins are designated E2, E1 and N. Putative non-structural genes and the presumptive polymerase genes are designated NS and POL, respectively. The tentative map of FIPV was determined as discussed in the text. The maps of IBV, TGEV and MHV are based upon the work of others (Armstrong *et al.*, 1984; Binns *et al.*, 1985; Boursnell & Brown, 1984; Boursnell *et al.*, 1984, 1985*a, b*; Hu *et al.*, 1984; Jacobs *et al.*, 1986; Rottier *et al.*, 1981; Siddell, 1983; Stern & Sefton, 1984). The RNAs of TGEV and MHV are indicated by numbers; letters indicate RNAs for IBV.

Fraction 25, containing the 2.8 kb RNA but lacking the 3.8 kb species, directed the synthesis of only the 45K product. The 25K product comigrated with the smallest form of E1 and could be immunoprecipitated using the E1-specific monoclonal antibody COR15 (Fig. 3, lane 10). The 45K polypeptide comigrated with the N protein and reacted with the N-specific monoclonal antibody COR12 (Fig. 3, lane 11). These data indicate that the 3.8 and 2.8 kb RNA species have a messenger function and encode the E1 and N proteins, respectively. In translation mixtures of fraction 22 a high molecular weight product of unknown nature was also observed (Fig. 3, lane 6); this was possibly aggregates of E1 or cellular translation products (see Fig. 3, lane 1).

Translations of fractions containing the other RNA species did not lead to detectable production of polypeptides related to structural proteins of FIPV. Using E2-specific monoclonal antibodies, we could not detect immunoreactive products after translation *in vitro* of total poly(A)-selected RNA or RNA fractions from the experiment depicted in Fig. 2(c).

DISCUSSION

In fcwf-D cells infected with FIPV 79-1146, six different virus-specific RNA species were found, of approximately 20, 9.6, 5.2, 3.8, 2.8 and 1.6 kb. In cells infected with the FIPV strain NOR15 (Evermann *et al.*, 1981) an identical set of RNAs was detected (R. J. de Groot, unpublished results). All the FIPV RNAs bound to oligo(dT)-cellulose, indicating polyadenylation and hence suggesting a messenger function. The existence of sequence homologies between these RNA species was indicated by the fact that they all hybridized to the insert of the FIPV-specific cDNA clone pFIPVB12. We assume that, as in other coronaviruses, the intracellular RNAs of FIPV form a 3' coterminal nested set, and that translation is restricted to the 'unique' 5' terminal segment. Direct proof for a messenger function was obtained for the 3.8 and 2.8 kb RNA species, which directed the synthesis *in vitro* of the E1 and N proteins, respectively.

Although we were not able to identify the mRNA for the E2 protein in the translation assay *in vitro*, the most likely candidate was the 9.6 kb RNA. Attempts to translate this RNA were discontinued when sequence analysis of genomic cDNA clones, corresponding to the 5' terminal segment of this RNA, revealed the presence of an open reading frame with a coding capacity for

a 160K apoprotein. The amino acid sequence of this protein shows a clear homology with the peplomer protein of IBV (R. J. de Groot *et al.*, unpublished observation).

FIPV and TGEV are members of an antigenic cluster of closely related viruses within the coronavirus group. In fact, the structural proteins of FIPV and TGEV are so antigenically similar that they may be regarded as host range variants rather than as individual virus species (Horzinek *et al.*, 1982). Interestingly, their divergence appears to have been accompanied by changes in the RNA pattern. An RNA comparable to the RNA5 of TGEV seems to be absent in FIPV. Furthermore, FIPV-infected cells synthesize an abundant RNA of 1.6 kb, which has no counterpart in TGEV nor in MHV and IBV (Rottier *et al.*, 1981; Stern & Sefton, 1984). Whether the FIPV 1.6 kb RNA is a functional mRNA is not yet known.

The differences in RNA pattern between TGEV and FIPV suggest changes in genomic organization. As shown in Fig. 4, this would not be without precedent in coronavirus evolution. Whereas in IBV, FIPV and TGEV the E2 mRNA is the largest subgenomic RNA yet detected, MHV specifies an additional larger mRNA, RNA2. Another example is the separation of the IBV E1 and N genes by a transcription unit possibly encoding a non-structural protein (Boursnell & Brown, 1984; Stern & Sefton, 1984). This transcription unit is expressed in mRNA B, which is intermediate in length between the mRNAs for E1 and N. No RNA comparable to RNA B has been found for the other coronaviruses. In MHV (Armstrong *et al.*, 1984) and probably also in TGEV and FIPV, the E1 and N genes are adjacent. Finally, as in TGEV two transcription units are located between the E2 and E1 genes of MHV, but in IBV and FIPV only one is found. It appears that transcription units have been lost, gained or perhaps translocated as the coronaviruses diverged.

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