cDNA Cloning and Sequence Analysis of the Gene Encoding the Peplomer Protein of Feline Infectious Peritonitis Virus

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(Accepted 22 June 1987)

SUMMARY

The peplomer gene of feline infectious peritonitis virus (FIPV) strain 79-1146 was isolated from a genomic cDNA library by differential hybridization with RNA 2 and 3 as probes. From the nucleotide sequence a primary translation product of 1452 residues (Mr 160472) was predicted, containing an N-terminal signal sequence, a C-terminal transmembrane segment and 35 potential N-linked glycosylation sites. By S1 nuclease analysis the 5' end of the presumptive RNA 2 body was located at about 30 nucleotides upstream from the initiating AUG codon. At approximately the same position a nine nucleotide sequence ACUAAACUU was found, which was also present 37 nucleotides downstream from the open reading frame. Comparison of the sequences of the FIPV, murine hepatitis virus and infectious bronchitis virus peplomer proteins showed about 27% overall homology, with most conservation in the C-terminal half.

INTRODUCTION

Feline infectious peritonitis (FIP) is a virus-induced, mostly fatal condition in domestic and wild Felidae (for a recent review, see August, 1984). The causative virus (FIPV) is a member of the family Coronaviridae, a group of enveloped RNA viruses with a single-stranded, positive-sense genome (for reviews, see Siddell et al., 1983; Sturman & Holmes, 1983). The pathogenesis of FIP is thought to be the result of complex immunologically mediated phenomena (Horzinek & Osterhaus, 1979). Apparently, the humoral response is not protective. In fact, the presence of anti-coronavirus antibody may accelerate and aggravate the course of the disease. A possible explanation for this phenomenon is antibody-dependent enhancement (ADE) of virus uptake by the presumptive main target cell, the macrophage (Pedersen & Boyle, 1980; Weiss et al., 1980; Weiss & Scott, 1981a, b).

To elucidate the pathogenetic mechanism with the aim of developing an effective vaccine, we have begun investigating the molecular biology of FIPV. In a previous report (de Groot et al., 1987a) we have shown that in FIPV-infected cells six virus-specific RNAs are produced with sizes ranging from 1.6 to at least 20 kb. Data on the RNAs and their coding assignment are summarized in Fig. 1(a). The mRNAs of murine hepatitis virus (MHV) and infectious bronchitis virus (IBV) arise by discontinuous transcription and consist of a 'leader' sequence, containing the coding information, and a short 5' 'leader' sequence (Spaan et al., 1983; Lai et al., 1984; Brown et al., 1986). We assume that the FIPV RNAs have a similar structure.

The FIPV virion consists of three main structural proteins: a 45K nucleocapsid protein, a 25K to 32K transmembrane glycoprotein (E1) and a 210K peplomer glycoprotein (E2) (Horzinek et al., 1982; Boyle et al., 1984; de Groot et al., 1987a). The peplomer protein mediates the binding of virions to the host cell receptor and is involved in membrane fusion (Sturman & Holmes, 1983). Moreover, in the case of MHV (Hasony & Macnaughton, 1981; Collins et al., 1982), IBV (Cavanagh et al., 1986b) and porcine transmissible gastroenteritis virus (TGEV) (Garwes et al., 1978), the E2 appears to be the major inducer of protective immunity. The role of the E2 protein...
in the pathogenesis of FIP is unclear. Although it seems the most suitable candidate for the development of a vaccine, it may also carry the epitopes responsible for ADE.

In this report, we describe the cDNA cloning and sequence analysis of the E2 gene of FIPV strain 79-1146. We present evidence that the gene is located at the 5’ end of a 9.6 kb RNA (RNA 2), indicating that this RNA is the E2 messenger.

METHODS

**Virus growth and cells.** The FIPV strain 79-1146 (McKeirnan et al., 1981) was grown in Felis catus whole foetus (fcwf)-D cells as described previously (de Groot et al., 1987a).

**Isolation of viral RNA.** Fcwf-D cells grown in plastic roller bottles (850 cm²; Corning) were infected at a m.o.i. of 0.01 TCID₅₀. At 20 h after infection the tissue culture supernatant was harvested. FIPV genomic RNA was isolated as described by Spaan et al. (1981). About 5 µg of genomic RNA was obtained from 2 × 10⁸ infected cells.

**Cloning of FIPV genomic RNA.** cDNA synthesis was primed with DNase I-derived calf thymus DNA pentamers (Pharmacia) on methylmercuric hydroxide-denatured FIPV genomic RNA (1 µg pentanucleotides/0.5 µg RNA in 10 µl). First and second strand synthesis was carried out as described previously (Niesters et al., 1986). The cDNA was tailed with dC residues, annealed to G-tailed, PstI-digested pUC9 (Pharmacia), and used for transformation of Escherichia coli strain JM109 (Yanisch-Perron et al., 1985) as described by Hanahan (1983). The recombinant plasmids contained inserts with lengths up to 6 kb (mean length 2 kb).

**Labelling of nucleic acids.** RNA and DNA probes were prepared according to Maniatis et al. (1982). Hybridization conditions were described previously (de Groot et al., 1987a).

**DNA sequencing.** Restriction fragments were purified by agarose gel electrophoresis, isolated by binding to NA-45 paper (Schleicher and Schüll) and subsequently recloned in bacteriophage M13 vectors. Sequencing was carried out using the dideoxynucleotide chain-termination procedure of Sanger et al. (1977). Both a universal M13 sequencing primer as well as internal oligonucleotide primers complementary to FIPV sequences were used. The latter were synthesized as described by Niesters et al. (1986). The data were analysed on a DEC 20/60 computer, using the programs of Staden (1982) and Lipman & Pearson (1985).
Sequence of the peplomer protein of FIPV

Fig. 2. (a) Restriction map of the genomic region containing the FIPV E2 gene: \(\nabla\), EcoRI; \(\blacktriangle\), PstI; \(\blacktriangleup\), HindIII; \(\blacktriangleleft\), XbaI. The position of the inserts of plasmids B1, H6 and III-3 is also shown. The E2 coding region is represented as a black bar. (b) Sequencing strategy using \(\bullet\), B1; \(\bigcirc\), H6; \(\blacksquare\), III-3. (b, c) S1 nuclease mapping of the 5' end of the RNA 2 'body'. The 1400 bp PstI fragment of plasmid B1 was used for the preparation of a probe 899 nucleotides in length as described in the text. This probe was hybridized to 60 \(\mu\)g \(E.\ coli\) tRNA (lane 1), 60 \(\mu\)g \(E.\ coli\) tRNA + 0.1 \(\mu\)g genomic RNA (lane 2) or 60 \(\mu\)g \(E.\ coli\) tRNA + 0.1 \(\mu\)g RNA of a sucrose gradient fraction enriched for RNA 2 (lane 3). The samples were digested with S1 nuclease and analysed on a 2% agarose gel. An EcoRI–HindIII digest of phage \(\lambda\) DNA was used as molecular weight marker. Values on the right are in kb.

S1 nuclease analysis. The PstI 1400 bp fragment of plasmid pUC-FIPV-B1, recloned in M13mp8, was used as a template for the synthesis of uniformly labelled complementary DNA. A synthetic 17-mer oligonucleotide was used as an internal primer. The reaction mixture contained 100 ng DNA, 4 ng primer, 10 mM-Tris-HCl pH 8, 50 mM-NaCl, 5 mM-MgCl2, 10 mM-dithioerythritol, 50 \(\mu\)M-dGTP, -dCTP and -dTTP, 1 \(\mu\)M-dATP, 2 units Klenow DNA polymerase (Boehringer) and 10 \(\mu\)Ci \([\alpha-32P]dATP\) (3000 Ci/mmol, Amersham) in a total volume of 25 \(\mu\)l. Synthesis was for 15 min at room temperature, followed by a 5 min chase after the addition of 5 \(\mu\)l chase mix (dATP, dCTP, dGTP and dTTP, 1.25 mM each). The reaction was stopped by phenol extraction and unincorporated dNTPs were removed by isopropanol precipitation. The pellet was washed twice with 70% ethanol and subsequently resuspended in 100 \(\mu\)l Tris-EDTA.

For S1 analysis, 1 \(\mu\)l DNA (about 104 c.p.m.) was hybridized in the presence of 60 \(\mu\)g \(E.\ coli\) tRNA with either 100 ng FIPV genomic RNA or 100 ng RNA from a sucrose gradient fraction (fraction 11; de Groot et al., 1987a), enriched for RNA 2. Hybridization conditions and S1 nuclease digestion were as described by Berk & Sharp (1977). Samples were analysed in 2% agarose gels.

RESULTS

Cloning of the peplomer gene

To clone the peplomer gene we prepared a cDNA library of FIPV genomic RNA by random priming of cDNA synthesis with calf thymus DNA pentamers. Our selection procedure for recombinants containing E2-specific sequences was based on the typical 3' coterminal 'nested'
Sequence of the peplomer protein of FIPV

**Fig. 3.** DNA sequence of the genomic region encoding the peplomer protein of FIPV 79-1146. A nine nucleotide repeat, flanking the gene, is indicated by asterisks. The amino acid sequence is shown in the one-letter code. Nucleotide and amino acid residue numbers are given on the right. The presumptive N-terminal signal sequence (according to von Heijne, 1986) and transmembrane segment (identified according to Eisenberg et al., 1984) are indicated by black bars. Potential glycosylation sites are indicated by boxed asparagine residues.

set structure of coronaviral mRNAs. The coding information of each mRNA is contained within the 5' terminal segment that is not present in the next smaller RNA of the set. Hence, selection may be achieved by differential hybridization.

Because of its large 'non-overlapping' coding capacity, we considered the FIPV RNA 2 most likely to encode the peplomer protein (Fig. 1a). Therefore, Southern blots of 20 randomly selected recombinant cDNA clones were hybridized to RNA fractions labelled in vitro, enriched for RNA 2 or RNA 3. Fractions were obtained by isokinetic sucrose gradient centrifugation of total poly(A)-containing RNA of FIPV-infected cells (de Groot et al., 1987a). A recombinant, III-3, which hybridized with RNA 2 but not with RNA 3 was identified (Fig. 1b) and subsequently used to screen the library for additional recombinant clones. The plasmids B 1 and H6, containing inserts of 5.2 and 5.0 kb respectively, were selected for further study. Southern blot analysis using the RNA 3 probe indicated that most of the insert corresponded to the 'unique' 5' terminal segment of RNA 2 (not shown).

**Characterization of the cloned sequences**

Restriction maps of the inserts of plasmids B1, H6 and III-3 and the sequence strategy are shown in Fig. 2(a). A translational open reading frame (ORF) of 4356 bases was identified,
predicting a primary translation product of 1452 amino acid residues \( (M_r 160,472) \). The deduced amino acid sequence showed a clear homology to the peplomer proteins of IBV and MHV (see Discussion), providing sufficient evidence that the ORF encoded the FIPV peplomer protein.

To determine the position of the peplomer gene on RNA 2 we employed S1 nuclease analysis. The 1400 bp \( PstI \) fragment of plasmid B1, recloned in M13mp8, was used as a template for the synthesis of \(^{32}\)P-labelled DNA complementary to FIPV plus-strand RNA. A 17-mer oligonucleotide, TCATTCCACTCAAGACC, hybridizing 552 nucleotides downstream of the AUG start codon was used as an internal primer. Thus, a FIPV-specific probe of 899 nucleotides in length was generated (Fig. 2b). Hybridization of this probe to FIPV genomic RNA, followed by S1 nuclease digestion, resulted in the protection of a fragment of about 900 bp. After hybridization with RNA 2, a fragment of approximately 580 bp was protected (Fig. 2c), indicating that the 5' end of the body of RNA 2 was located about 30 nucleotides upstream from the start codon. At this position (+33 to +25), a sequence, ACUAAACUU, was found, which was also present 37 nucleotides downstream from the stop codon (indicated by asterisks in Fig. 3).

**DISCUSSION**

The gene encoding the peplomer protein of FIPV strain 79-1146 was isolated from a genomic cDNA library by differential hybridization with RNA 2 and 3 as probes. S1 nuclease analysis indicated that the 5' end of the presumptive RNA 2 body was located about 30 residues upstream of the ORF. A nine nucleotide sequence, ACUAAACUU, which flanked the peplomer gene, was present at approximately the same position. Interestingly, this sequence has also been found at the 5' end of the nucleocapsid gene of TGEV (Kapke & Brian, 1986). Most probably the nine nucleotide sequence is similar to the 'regions of homology', preceding the transcription units of MHV and IBV (Budzilowicz et al., 1985; Brown et al., 1986).

From the nucleotide sequence of the peplomer gene a primary translation product of 1452 residues was deduced, 290 and 217 residues larger than the peplomer apoproteins of IBV strain M41/M42 (Binns et al., 1985; Niesters et al., 1986) and MHV strain JHM (Schmidt et al., 1987), respectively. The FIPV E2 protein displays several features previously described for these peplomer proteins (Fig. 3). The amino acid sequence predicts an N-terminal signal sequence (Wickner & Lodish, 1985), which is probably cleaved between serine 19 and threonine 20 (von Heijne, 1986). Furthermore, a conserved hydrophobic segment near the C-terminus probably represents the transmembrane anchor. As in the peplomer proteins of IBV and MHV, the presumptive transmembrane domain is followed by a cysteine-rich region. Finally, the sequence of the FIPV E2 protein contains 35 potential N-glycosylation sites (Fig. 3). These sites have a distribution similar to that in the peplomer protein of IBV (Binns et al., 1985; Niesters et al., 1986) and are predominantly found in the N-terminal part and in a region immediately upstream of the presumed transmembrane anchor. Under the assumption that all potential glycosylation sites carry a carbohydrate group of 2K (Neuberger et al., 1972) and that a signal sequence is cleaved off, we predict a fully processed FIPV E2 protein of 216K. This agrees with the \( M_r \) of 210K found in SDS-PAGE for the mature protein (Boyle et al., 1984; de Groot et al., 1987a).

Computer-aided comparison (Lipman & Pearson, 1985) of the amino acid sequences of the FIPV, IBV and MHV peplomer proteins indicated an overall homology of only about 27% in all three cases (not shown). This degree of conservation is comparable to values reported for the nucleocapsid proteins of IBV, MHV and the FIPV-related TGEV (Kapke & Brian, 1986). An alignment of the peplomer sequences of FIPV 79-1146, IBV M41 (Niesters et al., 1986) and MHV A59 (W. Luytjes et al., unpublished results), is presented elsewhere (de Groot et al., 1987b). Most conservation is in the C-terminal part of the peplomer proteins. Insertions or deletions in the non-homologous N-terminal regions largely account for the considerable differences in length. Furthermore, the C-terminal segments contain two regions with heptad repeat periodicity (residues 1067 to 1149 and 1335 to 1380 for the FIPV E2), indicative of the presence of long \( \alpha \)-helices involved in a coiled-coil structure (Cohen & Parry, 1986). As for the influenza virus haemagglutinin spike (Wilson et al., 1981) a coiled-coil structure could stabilize the E2 oligomer and provide an explanation for its elongated shape (de Groot et al., 1987c).
The peplomer proteins of IBV (Cavanagh, 1983) and MHV (Sturman et al., 1985) undergo a host-dependent proteolytic cleavage, which in the case of MHV appears to be essential for fusion activity. It is of interest that the FIPV peplomer protein is not cleaved (at least not in the fcw-D cell system) and yet is capable of inducing cell fusion (Boyle et al., 1984; de Groot et al., 1987a). For IBV M42 and MHV A59, a basic stretch of five amino acids (RRFRR, position 534, and RRAHR, position 717, respectively) was identified at the cleavage site (Cavanagh et al., 1986a; W. Luytjes et al., unpublished results). In the region formed by residues 730 to 950 of the FIPV E2 protein no sequence related to the cleavage site of IBV and MHV was found, except perhaps for the tripeptide RTR at position 788.

Cloning of the FIPV peplomer gene is an important step towards pinpointing the role of the peplomer protein in FIP pathogenesis. We are in the process of constructing vaccinia virus recombinants containing a functional peplomer protein gene. These recombinants will be used to test whether the E2 is capable of inducing protective immunity in cats or whether it triggers ADE.

We thank Willem Luytjes for help with the computer analysis and Diane Ruyzendaell (Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, The Netherlands) for preparation of oligonucleotide primers. This work was supported by a research grant from Duphar BV, Weesp, The Netherlands.

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(Received 23 March 1987)