The integral membrane protein from a virulent isolate of transmissible gastroenteritis virus: molecular characterization, sequence and expression in *Escherichia coli*

P. Britton, R. S. Cármenes, K. W. Page and D. J. Garwes
Division of Microbiology, AFRC Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire RG16 0NN, UK.

Summary
Subgenomic mRNA from a virulent isolate of porcine transmissible gastroenteritis virus (TGEV) was used to produce cDNA clones. Part of a new clone and a previously reported clone were sequenced and used to construct the viral gene for integral membrane protein. A single open reading frame (ORF) encoding a polypeptide of 262 amino acids, relative molecular mass (Mr) 29459, was identified. The positive identification of the polypeptide as the integral membrane protein was demonstrated by the production in *E. coli* of a chimaeric protein comprising most of the ORF encoding the M^29459 polypeptide and β-galactosidase. The chimaeric protein reacted with a specific monoclonal antibody to viral integral membrane protein and antibodies raised against the chimaeric protein immune precipitated the viral protein. Comparison with the sequence of an avirulent isolate indicates amino acid residues that may be important in pathogenicity.

Introduction
TGEV is a coronavirus that causes gastroenteritis in pigs, resulting in a high mortality in neonates. TGEV belongs to the family Coronaviridae, a large group of pleomorphic enveloped viruses with a positive-stranded RNA genome. The coronavirus proteins are expressed from a 'nested' set of subgenomic mRNAs which have common 3' termini but different 5' extensions. The region of each mRNA responsible for the expression of a protein appears to correspond to the 5'-terminal region that is absent on the preceding smaller species. Mouse hepatitis virus (MHV) and infectious bronchitis virus (IBV) mRNA species contain identical short non-coding sequences at their 5' ends which appear to be joined to the sequences encoding the viral genes by discontinuous transcription. A consensus sequence identified upstream of each gene/ORF may act as a binding site for the RNA polymerase-leader complex (Spaan et al., 1983; Brown et al., 1984; Lai et al., 1984; Budziowicz et al., 1985; Shieh et al., 1987). It has been postulated that a heptameric sequence, ACTAAC (Britton et al., 1988), or a hexameric sequence, CTAAC (Kapke and Brian, 1986; Rasschaert et al., 1987a; 1987b), may be involved in the binding of the TGEV RNA polymerase-leader complex for the transcription of the mRNA species from the negative form of the RNA genome.

The TGEV virion contains three major structural polypeptides: a surface glycoprotein (spike or peplomer protein) with a monomeric M, of 200,000, a glycosylated integral membrane protein observed as a series of polypeptides of M, 28000-31000 and a basic phosphorylated protein (the nucleoprotein) of M, 47000 associated with the viral genomic RNA (Garwes and Pocock, 1975). TGEV-infected cells have, in addition to the genomic RNA, six species of subgenomic mRNA (Britton et al., 1986). Expression and sequencing studies have shown that the two smaller species (1.7 kb and 0.7 kb) contain the nucleoprotein gene and a gene encoding a polypeptide of M, 9000 (Britton et al., 1986; Kapke and Brian, 1986; Rasschaert et al., 1987b; Britton et al., 1988). The mRNA species of 2.6 kb and the largest mRNA species (8–11.2 kb) appear to encode the integral membrane protein and the peplomer protein (Britton et al., 1986; Jacobs et al., 1986; Kapke et al., 1987; Laude et al., 1987; Rasschaert and Laude, 1987; Rasschaert et al., 1987a; 1987b). The other mRNA species of 3 kb and 3.9 kb (Britton et al., 1986; Jacobs et al., 1986) have had no product assigned to them from either infected cells or virions. Jacobs et al. (1986) identified a product of M, 24000 from *in vitro* translation of the 3.9 kb mRNA species (Purdue strain) in rabbit reticulocyte lysate.

The integral membrane proteins from other coronaviruses, including IBV (Boursnell et al., 1984), MHV (Armstrong et al., 1984; Pfleiderer et al., 1986), bovine coronavirus (BCV) (Lapps et al., 1987) and the avirulent Purdue strain of TGEV (Kapke et al., 1987; Laude et al., 1987) have been sequenced. The N-terminal region of the
proteins from MHV and BCV contain O-linked glycans in which the sugar moieties are joined to serine and threonine residues (Niemann and Klenk, 1981; Niemann et al., 1984; Lapps et al., 1987). The integral membrane proteins from TGEV (Garwes et al., 1984; Jacobs et al., 1986) and IBV (Stern and Sefton, 1982a) have been shown to have N-linked glycans. The integral membrane proteins from MHV, IBV and BCV do not appear to have an N-terminal cleaved signal sequence (Rottier et al., 1986; Stern and Sefton, 1982b; Lapps et al., 1987). However, Laude et al. (1987) demonstrated that the integral membrane protein from the Purdue strain of TGEV contained a cleaved 17-amino-acid signal sequence. A model for the membrane topology of the integral membrane protein for coronaviruses has been postulated from a combination of biochemical and primary sequence data from WHV (Armstrong et al., 1984; Rottier et al., 1986).

Here we describe the cloning and sequence of the integral membrane protein gene of a virulent field isolate of TGEV, provide experimental confirmation that the gene encodes the integral membrane protein and compare its primary structure with that of three other coronaviruses.

Results

Cloning from TGEV mRNA species

TGEV poly(A)-containing mRNA species were isolated from virus-infected LLC-PK1 cells and used for the synthesis of cDNA. The production of plasmid pF4F-36 was described by Britton et al. (1988). Plasmid pTG22 was produced using a synthetic oligonucleotide as primer for cDNA synthesis. Plasmid pF4F-36 was found to hybridize, by Northern blot analysis, to all the TGEV mRNA species except the 0.7 kb species, postulated to express a polypeptide of Mr 9000 found at the 3' end of the viral genome (Britton et al., 1988). Plasmid pTG22 did not hybridize to either the 0.7 kb or 1.7 kb mRNA species, indicating that it originated within the 2.6 kb mRNA species previously shown, by in vitro translation in rabbit reticulocyte lysates, to express the TGEV integral membrane protein (Britton et al., 1986; Jacobs et al., 1986). From the size and position of the cDNA insert on the TGEV genome, pF4F-36 was deduced to contain about 80% of the integral membrane protein. Plasmid pTG22 is 2.5 kbp long and from its restriction map was shown to overlap pF4F-36 (Fig. 1) and extend 4.6 kb into the TGEV genome, thus completing the cloning of the integral membrane protein gene.

Construction of the TGEV integral membrane protein gene

A 0.44 kbp NsiI fragment from pTG22, containing restriction sites at one end that overlapped with pF4F-36, was purified and ligated to a 0.5 kbp NsiI-BalI fragment from pF4F-36 to produce a 0.94 kbp contiguous piece of TGEV cDNA (Fig. 2). The 0.94 kbp fragment was digested using HgiAl to produce a 0.83 kbp fragment containing the complete integral membrane protein gene. The HgiAl site, 22 bp upstream of the integral membrane protein gene initiation site, deduced from sequence data, was end-repaired using the Klenow fragment of E. coli DNA polymerase I, and BamHI linkers were added. The resulting BamHI cassette was cloned into pBR322 as described by Britton et al. (1988) for the nucleoprotein gene. A
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Restriction fragments obtained from plasmids pF4F-36 and pBIM3 were subcloned into M13mp vectors and sequenced in both directions. The cDNA was translated in all six reading frames and translation in the virus sense strand revealed an ORF of 786 bp. The corresponding DNA sequence, 21 bp from the 5' end of the ORF to the start of the TGEV nucleoprotein gene, present in pF4F-36 (Britton et al., 1988), is illustrated in Fig. 3. Two other ORFs were found in the viral sense strand: one was composed of 20 amino acids positioned between nucleotides 57–116, and the other comprised 43 amino acids between nucleotides 141–269, within the integral membrane protein gene. Three ORFs were identified, in the complementary strand; one of 45 amino acids (nucleotides 78–212), one of 62 amino acids (nucleotides 246–431), and another of 33 amino acids (nucleotides 519–617), within the integral membrane protein gene. None of the internal ORFs were preceded by the potential RNA polymerase-leader complex binding site.

The 786 bp ORF, initiating from the ATG at position 22, overlapped both cDNA inserts and encoded a polypeptide of 262 amino acids with a M, 29,459. The 5' end of this ORF mapped at 2.5 kb from the 3' end of the viral genome and, from its length and position, corresponded to the unique region present in the 2.7 kb mRNA species. The difference between molecular weights of the deduced polypeptide and those found in infected cells and virions is due to post-translational processing. The integral membrane protein is often found as a series of polypeptides when analysed by SDS-polyacrylamide gel electrophoresis.

The nucleotide sequence (Fig. 3) revealed the presence, near the 5' end of the 786 bp ORF, of the heptameric sequence, ACTAAAC, also found 5' of the ATG sequences at the start of the TGEV nucleoprotein gene and the ORF encoding the polypeptide of M, 9000 (Britton et al., 1988). The sequence context, CAAAATGA, about the first ATG of the 786 bp ORF, downstream of the ACTAAAC sequence, is favourable for initiation by eukaryotic ribosomes ((CC)ACCATGG; Kozak, 1983; 1986). A second ATG, found 60 bp from the first ATG, lies in a less favourable context.

The M, 29,459 polypeptide, encoded by the 786 bp ORF, contains about 46% hydrophobic amino acid residues spread over five domains. The sequence is about 96% identical to that described by Kapke et al. (1987) and Laude et al. (1987), who sequenced the integral membrane protein gene from the avirulent Purdue strain of TGEV and postulated that the protein was the TGEV integral membrane protein because of its homology to the...
integral membrane protein genes sequenced from IBV (Boursnell et al., 1984) and MHV (Armstrong et al., 1984).

Laude et al. (1987) identified a 17-amino-acid sequence at the N-terminal end of the protein as a putative signal sequence that is lost upon incorporation of the TGEV integral membrane protein into cell membranes. The same 17-amino-acid sequence, except for a conservative substitution of an isoleucine for a leucine residue at amino acid residue 3 resulting from a change in the first base of the codon, is encoded from our cDNA sequence. Cleavage of the leader sequence results in a polypeptide of 245 amino acids with a predicted Mr of 27712, of which 44% of the residues are hydrophobic; this polypeptide has an overall charge of +5 at neutral pH (identical to the Purdue strain but distributed over different amino acids). The size of the cleaved product is in agreement with a polypeptide of Mr 26000 found by Garwes et al. (1984) for the expression of TGEV mRNA species in rabbit reticulocyte lysates or in TGEV-infected cells in the presence of tunicamycin; and with a polypeptide of Mr 25000 identified by Jabobs et al. (1986) using the same analyses.

Expression of the 786bp ORF as a gene fusion in E. coli

In order to prove that the polypeptide of Mr 29459 encoded by the 786bp ORF was the viral integral membrane protein, HindIII linkers were added to a 0.88kb FspI fragment from plasmid pBIM3 containing 95% of the complete gene. This was then ligated into the HindIII site of the lacZ gene in plasmid pUR290. The 0.88kb FspI fragment consisted of the TGEV integral membrane protein gene 39bp downstream from the start of the gene to the FspI site 113bp from the BarnHI site in pBR322. This resulted in removal of DNA encoding most of the amino acids from the signal sequence at the N-terminal end of the protein. The recombinant plasmid pURIM-2 expressed a chimaeric protein of Mr 140000, upon induction with IPTG, which consisted of part of the TGEV integral membrane protein fused to the C-terminal end of β-galactosidase. A specific monoclonal antibody to the integral membrane protein from the Purdue strain of TGEV, 3BB3, reacted with the chimaeric protein by immunoblot analysis (Fig. 4A). The chimaeric protein was purified from SDS-polyacrylamide gels and used to raise antibodies in mice. The antibodies immune-precipitated the TGEV integral membrane protein, further confirming that the cDNA encoded the viral integral membrane protein gene (Fig. 4B).

Discussion

The complete integral membrane protein gene (from a British field isolate of TGEV) was cloned over two cDNA fragments and then sequenced. A major ORF of 786bp was identified in the viral-sense strand. The initiation codon of the ORF was preceded by the heptameric
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Sequence, ACTAAAC, which was previously described as preceding both the TGEV nucleoprotein gene and an ORF encoding a polypeptide of M, 9068 at the 3' end of the viral genome. The 786bp ORF encoded a primary translation product of 262 amino acids with a M, 29459. The first 17 amino acids fulfil the criteria of being a eukaryotic signal sequence, having a net charge following the N-terminus and a hydrophobic uncharged region of 14 amino acids (McGeoch, 1985; Von Heijne, 1986). The cleavage site, identified by Laude et al. (1987) for the Purdue strain of TGEV, is located between a glutamic acid and an arginine residue. The signal sequence is predicted to fall between the glycine (16) and glutamic acid (17) residues (score = 10.2) by the weight-matrix rule of Von Heijne (1986), which predicts 75 to 80% of eukaryotic signal sequences.

The ORF encoding the polypeptide of M, 29459 was confirmed as the TGEV integral membrane protein gene by constructing a fusion between most of the 786bp ORF and the E. coli lacZ gene in a similar way to that described for the TGEV nucleoprotein gene (Britton et al., 1987). The gene fusion resulted in the synthesis, in E. coli cells, of a polypeptide of M, 140000 composed of β-galactosidase with most of the TGEV integral membrane protein gene attached to the C-terminal end. A monoclonal antibody, 3BB3, specific for TGEV integral membrane protein interacted with the chimaera polypeptide, and antibodies raised against the chimaera precipitated the integral membrane protein from TGEV-infected cells. This confirmed that the 786bp ORF, predicted to direct the synthesis of a polypeptide of M, 29459, was the viral integral membrane protein gene.

Comparison of the M, 29459 product, using a dot-matrix analysis program (DIAGON; Staden, 1982), with the integral membrane proteins from MHV, IBV and BCV in a pairwise manner revealed remarkable homology between the proteins (Fig. 5). There are four major areas of homology between the integral membrane proteins of TGEV (FS772/70), MHV (A59) and BCV (Mebus), whereas there are only two regions of homology between TGEV and IBV as also seen between IBV and either MHV or BCV (Fig. 5). The homology between MHV and BCV is almost 100%, indicating that the two viruses probably share recent common ancestral evolution. From the homologies between the integral membrane proteins, this paper, and the nucleoproteins, Britton et al. (1988), it is clear that TGEV and MHV share a more recent ancestral evolution than either virus with IBV and it will be interesting to compare homologies with other coronaviruses once their gene sequences have been completed. Direct alignment of the integral membrane proteins from TGEV strains FS772/70 (virulent) and Purdue (avirulent) with MHV (A59), BCV (Mebus) and IBV (Beaudette) using the GAP program (Devereux et al., 1984) identified the position of identical amino acids between different viruses or in some cases between all four viruses (Fig. 6), which are indicated on the deduced amino acid sequence of TGEV integral membrane protein (Fig. 3). The optimal alignment identified a perfectly conserved eight-amino-acid sequence, SWWSFNPE, between all four viruses (Figs 3 and 6). The alignment of the amino acid sequences allowed the identification of the three membrane-spanning regions for TGEV by deduction from the regions identified for MHV (Rottier et al., 1986). These are shown as thick black lines.
Fig. 5. Comparison of the sequences of coronavirus integral membrane proteins (from four different viruses) that fall into three antigenically distinct subgroups, using DIAGON (Staden, 1982) A, MHV A59 and TGEV FS772/70; B, BCV Mebus and TGEV FS772/70; C, IBV Beaudette and TGEV FS772/70; D, MHV A59 and IBV Beaudette; E, BCV Mebus and IBV Beaudette; F, BCV Mebus and MHV A59. MHV A59 (Armstrong et al., 1984), IBV Beaudette (Boursnell et al., 1984) and BCV Mebus (Lapps et al., 1987). The comparisons used a window of 15 residues with a score of 180.

The coronavirus integral membrane protein is glycosylated and has been reported to be of the N-linked type for TGEV (Garwes et al., 1984; Jacobs et al., 1986) and IBV (Stern and Sefton, 1982a), whereas MHV and BCV are of the O-linked type (Niemann et al., 1984; Lapps et al., 1987). There are three potential N-glycosylation sites within the integral membrane protein: at asparagine positions 32, 55 and 251, as deduced from the DNA sequence for TGEV. The first two are at the amino terminal of the polypeptide (Fig. 3) although the second site (Asn-55) falls within the first predicted membrane-spanning region (Figs 3 and 6), as determined from alignment with MHV, and so it may not be glycosylated. The third potential glycosylation site is at the carboxy-terminal end of the polypeptide, predicted to be found inside the virion, and so it may not be glycosylated. The prediction that only the first N-glycosylation site is functional would give rise to a polypeptide of M, 29,000–30,000, a size close to the major species identified by SDS polyacrylamide gel electrophoresis of...
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**Experimenal procedures**

**Preparation of viral RNA**

TGEV mRNA was prepared from LLC-PK1 cells infected with TGEV strain FS772-70 and purified from other RNA species on poly(U)-Sepharose, as described previously (Britton et al., 1987; 1988).

**Digestion and analysis of plasmid DNA**

Standard recombinant DNA methods were used (Maniatis et al., 1982) with enzymes purchased from New England Biolabs (CP Laboratories, Bishop’s Stortford, UK) unless otherwise stated in the text. DNA fragments were isolated from agarose gels by freeze-phenol elution (Silhavy et al., 1984). Ligation reactions were carried out as described by Britton et al. (1984). E. coli cells were routinely transfected using the RbCl method developed by V. Simanis (Hanahan, 1985). E. coli strain DH1 was used for routine plasmid construction and JM101 for expression of the chimaeric protein. E. coli transformants were selected on LB plates containing ampicillin (100 μg ml⁻¹). Vector DNA was routinely treated with alkaline phosphatase prior to ligation.

**cDNA synthesis**

cDNA synthesis was carried out as described by Britton et al. (1988) except that a synthetic oligonucleotide, 5' - GCCATTA-GAAGTTTAGT-3', was used to prime first-strand synthesis and 1200 U of Motoney murine leukaemia virus (M-MLV) reverse transcriptase (BRL) was used for the production of cDNA. The primer corresponded to a complementary sequence 13bp upstream from the 5' end of the nucleoprotein gene, derived from the sequence data reported by Britton et al. (1988), and was synthesized by the phosphorimidate method using an Applied Biosystems model 381A DNA synthesizer. Second-strand synthesis was carried out as described by Britton et al. (1988). Following second-strand synthesis, oligo-dC tails were added to the cDNA molecules using 25U terminal transferase at 15°C for 2 min. The dC-tailed cDNA molecules were annealed to oligo(dG)-tailed pUC9 (Pharmacia) at 65°C for 10 min. After 4 h at 58°C, they were transformed into competent DH1 cells. Transformants containing TGEV cDNA were identified by colony hybridization, as described by Britton et al. (1988) using a [³²P]-labelled TGEV cDNA fragment that hybridized to all of the TGEV mRNA species larger than the 2.7 kb mRNA species.

**Subcloning for M13 sequencing**

Various restriction endonuclease fragments from plasmids pF4F-36 and pBIM3 deduced from restriction maps were cloned into M13mp vectors. DNA sequencing and sequence analysis were carried out as described by Britton et al. (1988).

**Production of a β-galactosidase-TGEV integral membrane protein chimera**

A TGEV cDNA fragment containing 95% of the integral membrane protein gene, was ligated into the expression vector pUR290
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