The Polypeptide of $M_r$ 14000 of Porcine Transmissible Gastroenteritis Virus: Gene Assignment and Intracellular Location

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SUMMARY

Synthetic oligopeptides, corresponding to an amino acid sequence encoded by a potential $M_r$ 9000 product's open reading frame (ORF-4) at the 3' terminus of the transmissible gastroenteritis virus genome, were used to generate rabbit antiserum. These antibodies produced immune complexes with an $M_r$ 14000 (14K) polypeptide in infected cells. The 14K product was shown by immune fluorescence to become associated with the cell nucleus, correlating with the onset of nuclear vacuolation, and suggesting a role in pathogenesis for the ORF-4 gene.

Analysis of the viral subgenomic mRNA species synthesized in cells infected with the porcine coronavirus transmissible gastroenteritis virus (TGEV) (Britton et al., 1986; Jacobs et al., 1986), together with data from base sequence analysis (Kapke & Brian, 1986; Rasschaert et al., 1987; Britton et al., 1988), suggests that several non-structural polypeptides are encoded by the viral genome in addition to the three major structural proteins. By analogy with the avian coronavirus causing infectious bronchitis, there is sufficient coding capacity for one or two high $M_r$ molecules of RNA polymerase at the 5' terminus of the genome. An open reading frame, designated ORF-4, capable of encoding a polypeptide of 9K has been identified at the 3' terminus of the genome (Britton et al., 1988). The ORF is preceded by the putative RNA polymerase recognition sequence, ACTAAAC, thought to be required for mRNA synthesis (Britton et al., 1988) and an mRNA species of 0.7 kb, capable of encoding a polypeptide of 9K, is found in low abundance in TGEV-infected cells (Britton et al., 1986).

A polypeptide of $M_r$ 17000 (17K) was reported to be synthesized in TGEV-infected cells (Wesley & Woods, 1986) and it was suggested that it could be found in the virion, although serological data suggested that it was not exposed at the surface of the viral envelope. The same authors subsequently revised their estimate of the $M_r$ of the polypeptide to 14K and showed, by immune precipitation, that it was cell-associated (Wesley et al., 1987).

Confluent monolayer cultures of LLC-PK1 cells in 25 cm$^2$ plastic flasks were infected with the British FS772/70 strain of TGEV at an input m.o.i. of 5 to 10 p.f.u./cell for 2 h at 37 °C or left uninfected to serve as controls (Garwes et al., 1984). The inoculum was removed and 5 ml methionine-free Eagle's MEM supplemented with 50 mM-HEPES, 0.014% sodium bicarbonate and penicillin, streptomycin and mycostatin at 100 units, 100 µg and 25 units per ml respectively was added for a further 2 h at 37 °C. This was then replaced with 1.5 ml of the same medium but containing 250 µCi of $[^{35}S]$methionine (> 1000 Ci/mmol, Amersham). After a further 6 h incubation, the cells were drained and solubilized in 2% SDS, 5% 2-mercaptoethanol, 25% glycerol in 62.5 mM-Tris–HCl pH 6.8 (Laemmli, 1970) and equivalent amounts of lysate from uninfected and infected cells were electrophoresed in 12 to 20% exponential gradient polyacrylamide gels. Following autoradiography, a number of polypeptide bands were seen to be unique to the infected cells (Fig. 1a, lanes 1 and 2). Three of these corresponded to the virion structural proteins (Garwes & Pocock, 1975) with apparent $M_r$ values of 200K (peplomer), 47K (nucleoprotein) and 30K (integral membrane complex). In addition, there were two other unique bands not seen in infected cells, having apparent $M_r$ of 17K and 14K (Fig. 1a, lane 2).
Fig. 1. Autoradiograph of polypeptides of TGEV labelled with [35S]methionine. (a) Intracellular polypeptides from uninfected (lane 1) and infected (lane 2) cell lysates. (b) Immune precipitation of TGEV polypeptides with preimmune rabbit serum (lane 1), rabbit anti-12A/86 (lane 2) and anti-12B/86 (lane 3) serum and with mouse anti-TGEV serum (lane 4).

Data from the proposed amino acid sequence for ORF-4 (Britton et al., 1988) were used to synthesize three oligopeptides (produced by Dr T. Doel, AFRC Institute for Animal Health, Pirbright Laboratory, U.K.) as shown in Fig. 2. These corresponded to Thr 37 to Lys 55, peptide 12A/86, Gly 19 to Lys 55, peptide 12B/86, and Gly 19 to Lys 36, peptide 1/87. As can be seen, peptides 1/87 and 12A/86 formed the N and C termini of peptide 12B/86 respectively. The peptides were coupled to glutaraldehyde-activated keyhole limpet haemocyanin (KLH) (Calbiochem) for 5 h at 20°C in 0·15 M-NaCl, 0·06 M-phosphate buffer pH 7·8 at the rate of 1 mg peptide/1 mg KLH, emulsified in Freund's complete adjuvant and inoculated into pairs of rabbits, 1 ml intramuscularly and four subcutaneous injections of 0·25 ml. Four weeks later, the rabbits were reinoculated with peptide–KLH conjugates emulsified in Freund's incomplete adjuvant, 0·25 ml intramuscular plus 0·25 ml subcutaneous and serum samples were obtained 4 weeks later.

TGEV-infected cells were labelled with [35S]methionine as above, and lysed at 10 h post-infection with RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0-1% SDS, 1 mM-PMSF and 1 mM-methionine in phosphate-buffered saline pH 7·2). Cell lysates were centrifuged at 15,000 g for 30 min in a microcentrifuge to remove virus particles and aggregates. The
supernatants were adsorbed with formalinized *Staphylococcus aureus* cells (Immunoprecipitin; BRL-Gibco) for 20 min at 20 °C, centrifuged to remove the bacteria and aliquots were mixed with 1/100 dilutions of sera from rabbits before and after immunization with peptides or with 1/1000 dilutions of mouse anti-TGEV serum. After 2 h at 20 °C, *S. aureus* were added for a further 1 h and the adsorbed immune precipitates were sedimented at 10000 g for 1 min and washed by four rounds of resuspension and sedimentation in RIPA buffer. The final pellets were resuspended in 50 μl of PAGE sample buffer, held at 100 °C for 2 min then sedimented at 10000 g for 2 min. The supernatants were analysed by PAGE with 12 to 20% exponential gradient gels.

Fig. 1 (b) shows the immune precipitates generated by preimmune rabbit serum (lane 1), rabbit anti-peptide 12A/86 (lane 2) and 12B/86 (lane 3) or mouse anti-TGEV serum (lane 4). Specific antibodies could not be detected in serum from rabbits inoculated with peptide 1/87. A small amount of the host cell antigen was precipitated by preimmune rabbit serum whereas mouse anti-TGEV serum detected the virion proteins as well as small amounts of other proteins. By comparison with prestained *M*ₚ standards in an adjacent lane (not shown), the native 14K protein was the only product specifically precipitated by the anti-12B/86 serum. The same protein was also precipitated by anti-12A/86, but to a much lesser extent and requiring longer exposure for it to be visualized (not shown).

To determine the intracellular location of the 14K protein, infected and uninfected cells were fixed with acetone at 2 h intervals during a 10 h incubation period and examined by immune fluorescence with 1/100 dilutions of serum samples from rabbits before and after immunization with peptide 12B/86. The first evidence of specific immune fluorescence in the TGEV-infected cells was seen 5 to 7 h after infection as a faint fluorescent stippling in the nucleus (Fig. 3 b). By 8 to 10 h after infection, this was intensified to a marked granular antigen fluorescence in the nuclei of most infected cells (Fig. 3 c) and was not seen in the uninfected cells nor with preimmune serum. Following 10 h, the infected cells underwent a marked cytopathology and the fluorescence was less obvious due to degradation of the nucleus. Duplicate cells, stained with Giemsa stain to show their morphology, showed the extent of the nuclear vacuolation in the infected cells in the last 1 to 2 h of the infection (Fig. 3 e).

Translation of the experimentally determined sequence of ORF-4 would result in a polypeptide with several interesting features, as previously described (Britton et al., 1988). The polypeptide has 78 amino acids with an *M*ₛ of 9068. The difference in size between that predicted and the 14K observed by PAGE could be accounted for by the fact that the protein is very hydrophobic, containing 36% leucine. Reduction and carboxymethylation of the protein by iodoacetamide resulted in a band with an electrophoretic mobility corresponding to approximately 11K (not shown). Whether this represents the monomeric form of a 14K dimer is difficult to assess but it seems more likely to reflect a change in secondary structure induced by the treatment. Attempts to demonstrate the presence of covalently linked carbohydrate in the 14K protein have failed but the possibility cannot be ruled out that the 9K primary translation product is subsequently glycosylated. There is a potential *N*-linked glycosylation site at Asn 30 and potential *O*-linkage sites at the two serine and four threonine residues throughout the molecule. The eventual localization of the molecule in the nucleus may suggest *N*-acetylglucosamine linkage to the serine or threonine, as recently reviewed for nuclear proteins by Hart et al. (1988), but it seems unlikely that this mechanism, even if it were occurring, would account for an increase in apparent *M*ₛ of the magnitude seen.

The nuclear location of the 14K protein at the end of the viral growth cycle is consistent with the cell association previously reported by Wesley and coworkers (1987) and the hydrophobic nature of the protein. Information about the nature of nuclear signals on proteins is insufficient to be sure whether such a signal is present on the molecule although there is some evidence of a 'leucine zipper' motif (Landschultz et al., 1988). This theory postulates that the presence of a heptad repeat of leucine residues may result in an α-helical conformation which can interdigitate with a similar molecule to form a dimer. The leucine zipper motif is a characteristic property of some DNA-binding proteins and the leucine residues at positions 21, 28 and 35 in ORF-4 form a potential zipper motif.
Following the detection of the protein in the nucleus, there was a marked disruption of the nuclear structure but whether this was caused by the 14K product, another viral protein or was a consequence of general cytopathology cannot be ascertained at present. There has been some debate on the requirement for the cell nucleus in coronavirus replication. Evans & Simpson (1980) demonstrated that avian infectious bronchitis virus replication was blocked in cells from which the nucleus had been removed or inactivated and suggested a requirement for transcriptional factors similar to those essential for influenza virus. Two other groups working with murine hepatitis virus, however, showed that the virus grew efficiently in enucleated cells (Brayton et al., 1981; Wilhelmsen et al., 1981). TGEV will replicate in the presence of actinomycin D, as originally demonstrated by Clarke (1968), indicating that nuclear transcription is not required. The role of the TGEV 14K protein in the nucleus, therefore, may be to overcome host resistance in the animal rather than to be essential for the replication and maturation of progeny virus in cell culture and the gene product therefore warrants further investigation.

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REFERENCES


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