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Expression and cellular localisation of porcine transmissible gastroenteritis virus N and M proteins by recombinant vaccinia viruses

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Summary

Porcine transmissible gastroenteritis virus (TGEV) nucleoprotein and integral membrane protein genes were cloned into the vaccinia virus insertion vector, pGS20, in the correct orientation for expression under the control of the vaccinia $P_{7.5K}$ promoter. Recombinant vaccinia viruses were generated by *in vivo* homologous recombination of the insertion vector with the WR strain of vaccinia virus. Nucleoprotein (N) expressed by both recombinant vaccinia virus and TGEV had a relative molecular mass (M_r) of 47,000 and was susceptible to degradation at the C-terminus yielding discrete breakdown products. The integral membrane protein (M) expressed by a recombinant vaccinia virus and TGEV was sensitive to endoglycosidase H reducing the mature polypeptide of M_r 29,000 to a species of M_r 27,000. Expression of M by recombinant vaccinia virus was inhibited during early infection due to a cryptic vaccinia virus transcriptional termination signal within the TGEV coding sequence. Indirect immunofluorescence showed that both N and M were only localised in the cell cytoplasm of either TGEV or recombinant vaccinia virus infected cells. Antisera from mice infected with recombinant viruses immunoprecipitated specific TGEV antigens from lysates of TGEV infected cells but had little significant TGEV neutralising activity *in vitro*.

Transmissible gastroenteritis virus; Nucleoprotein; Integral membrane protein; vaccinia virus; Porcine

Introduction

TGEV causes gastroenteritis in pigs resulting in a high mortality and morbidity in neonates and belongs to the family Coronaviridae, a group of enveloped viruses with a positive-stranded RNA genome. Coronavirus proteins are expressed from a 'nested' set of subgenomic mRNAs, which have common 3' termini but different 5' extensions. The 5'-terminal region of each mRNA is responsible for the expression of a viral protein. The virion contains three major structural polypeptides; a surface glycoprotein (spike or peplomer protein) with a monomeric M_r 200,000, a glycosylated integral membrane protein (M) observed as a series of polypeptides of M_r 28,000–31,000 and a nucleoprotein (N), a basic phosphorylated protein of M_r 47,000 associated with the viral genomic RNA (Garwes and Pocock, 1975). The genes encoding TGEV N and M have been cloned, sequenced and expressed in bacteria and yeast from a virulent British field isolate, FS772/70 (Britton et al., 1988a, b). We have now extended this work to a vaccinia virus (VV) expression system in order to analyse the structure and function of the N and M proteins in more detail.

The vaccinia virus (VV) expression system was chosen because it has the potential for transporting, processing and folding foreign eukaryotic gene products correctly (for reviews see Mackett and Smith, 1986; Moss and Flexner, 1987). In this paper we describe the construction of recombinant vaccinia viruses (RVV) containing and expressing the TGEV N and M genes under the control of the vaccinia early/late $P_{7.5K}$ promoter. The antigenicity and cellular location of the VV expressed TGEV proteins were compared with the native proteins produced in TGEV infected cells.

Materials and Methods

Viruses and cells

Wildtype VV (WR strain) and RVV were routinely grown on CV-1 cells or human 143 thymidine kinase negative (HTK⁻) cells. Recombinant vaccinia viruses were selected on HTK⁻ cells in the presence of 25 $\mu\text{g}/\text{ml}$ of 5-bromodeoxyuridine (BUdR) (Sigma) as described by Mackett et al. (1985). CV-1 cells and HTK⁻ cells were both grown in medium containing EMEM (Flow Labs.) and 10% heat inactivated foetal calf serum. TGEV strain FS772/70 was grown on adult pig thyroid (APT/2) cell monolayers (Pocock and Garwes, 1975) for neutralisation assays. TGEV antigens were isolated from infected LLC-PK1 cells as described by Garwes et al. (1984).

Preparation of vaccinia virus stocks

Partially purified recombinant and wildtype VV were prepared by pelleting stock virus through an equal volume of 36% w/v sucrose in 10 mM Tris-HCl, 1 mM

EDTA, pH 8.8, in a swing out 55.5 rotor (Kontron Instruments) at $25,000 \times g$ for 80 min at 4°C. Pelleted virus was resuspended in phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.2) for inoculation into mice.

Digestion and analysis of plasmid DNA

Recombinant DNA techniques used were as described by Maniatis et al. (1982). DNA fragments were isolated from agarose gels by freeze phenol elution (Silhavy et al., 1984). Vector DNA was routinely treated with alkaline phosphatase (Boehringer Mannheim) prior to ligation.

Construction of insertion vectors

Recombinant insertion plasmids pGSN1 and pGSIM1 were constructed by removing the TGEV N gene from pBNP5 (Britton et al., 1988a) and the M gene from pBIM3 (Britton et al., 1988b) by digestion with *Bam*HI and ligating these gene cassettes into the *Bam*HI site of pGS20 (Mackett et al., 1984). The orientations of the TGEV genes were checked by restriction mapping and sequencing of the gene/promoter junction. Caesium chloride purified plasmid DNA was directly sequenced after alkali denaturation of double stranded plasmid DNA (Murphy and Kavanagh, 1988). A specific synthetic oligonucleotide primer (5'-CACCCGCTTTT-TATAGTAAGTTTTT-3') that binds 100bp upstream from the *Bam*HI cloning site of pGS20, was used for sequencing by the chain termination method with Sequenase™ (United States Biochemical Corporation) according to manufacturers instructions.

Production of recombinant viruses

Recombinant vaccinia viruses, were generated by transfection of VV-infected CV-1 cells with calcium phosphate precipitates of plasmid vectors pGSN1 and pGSIM1, as described by Mackett et al. (1984).

Viral DNA analysis

DNA was extracted from wildtype and recombinant VV infected CV-1 cells as described by Merchlinsky and Moss (1989). Vaccinia virus DNA and insertion vector plasmid DNA was incubated with *Bam*HI or *Hind*III restriction enzymes in the presence of $0.3 \text{ mg} \cdot \text{ml}^{-1}$ RNase A (Sigma) and DNA fragments separated by electrophoresis on a 0.8% agarose gel. DNA was blotted onto a Biodyne™ A membrane (Pall, P/N BNNG3R 1.2 μm) and treated as described by Britton et al. (1988a). TGEV N or M *Bam*HI gene cassettes were labelled with [α - ^{32}P]dATP using a Multiprime™ kit (Amersham International Plc) and incubated with the blots in prehybridisation buffer (Maniatis et al., 1982) for 16 h at 42°C. Blots were washed four times in $2 \times \text{SSC}$, 0.1% SDS at 25°C, twice in $1 \times \text{SSC}$, 0.1% SDS and twice in $0.1 \times \text{SSC}$, 0.1% SDS both at 68°C, air dried and exposed to X-ray film.

Immunofluorescence

Glass coverslips with confluent monolayer cultures of HTK⁻ or LLC-PK1 cells were infected with wildtype or recombinant vaccinia viruses at an MOI = 1. The infected cells were fixed in cold 80% acetone and probed with mouse monoclonal antibodies (mAbs) DA3 (Garwes et al., 1988) and 3B.B3 (Jimenez et al., 1986) specific to TGEV nucleoprotein and integral membrane protein respectively. Bound antibody was detected with fluorescein-conjugated rabbit anti-mouse IgG antiserum (Nordic Immunology). Fluorescent cells were observed with a Leitz UV microscope and photographed with Ilford XP1 film. Cell surface fluorescence was examined by incubating unfixed infected cells with antiserum at 4°C.

Immunoblot analysis

Infected cell cultures were lysed 24 h p.i. with 0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 0.5% Nonidet P40, 0.1% Aprotinin (Sigma), and proteins were transferred onto nitrocellulose membrane (BA85, Schleicher and Schuell) using a Bio-Rad dot-blot manifold. Infected cell proteins were separated by SDS polyacrylamide gel electrophoresis [SDS/PAGE] (Laemmli, 1970) and transferred to nitrocellulose by electroblotting. The blots were probed using mAbs DA3 and 3B.B3 or TGEV hyperimmune serum.

Immunoprecipitations

L-[³⁵S]methionine labelled viral proteins were produced from TGEV (FS772/70) infected LLC-PK1 cells according to the method of Garwes et al. (1987). The infected cells were lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 1 mM methionine in PBS, pH 7.4). Precipitated proteins were separated on an 8% PAGE gel, treated with 0.8 M sodium salicylate and autoradiographed.

Endoglycosidase H analysis

Infected cell lysates were adjusted to a final concentration of 1% SDS and incubated for 6 h at 37°C in the presence or absence of 5mU of endoglycosidase H (Boehringer Mannheim). Proteins were separated by electrophoresis on a 12% SDS polyacrylamide gel and electroblotted onto nitrocellulose. TGEV proteins were detected with TGEV hyperimmune antiserum and ¹²⁵I-labelled staphylococcal protein A.

Animal inoculations

Wild-type or two separate clones of each recombinant vaccinia virus, purified by sucrose density centrifugation, were inoculated intraperitoneally into groups of five 7-week-old female Balb/C mice ($1-2 \times 10^7$ pfu/animal). At 4 weeks post-vaccina-

tion one mouse from each group was bled and the others were boosted with a second intraperitoneal inoculation with homologous viruses at the same titres and after 8 weeks all remaining mice were bled.

Results

Construction of recombinant *vTN1* and *vTM1*

The TGEV nucleoprotein *BamHI* gene cassette (1585bp) containing the N gene (1149 bp) and the integral membrane protein *BamHI* gene cassette (830 bp) were inserted downstream and under the control of the vaccinia early/late $P_{7.5K}$ promoter in the plasmid insertion vector pGS20 to produce plasmids pGSN1 and pGSIM1 (Fig. 1). The initiation codons of both the TGEV N and M genes were 28 nucleotides from the *BamHI* cloning site in pGS20 (Fig. 1). Restriction endonuclease analysis and nucleotide sequencing confirmed the orientation and promoter/gene junction for the insertion plasmids.

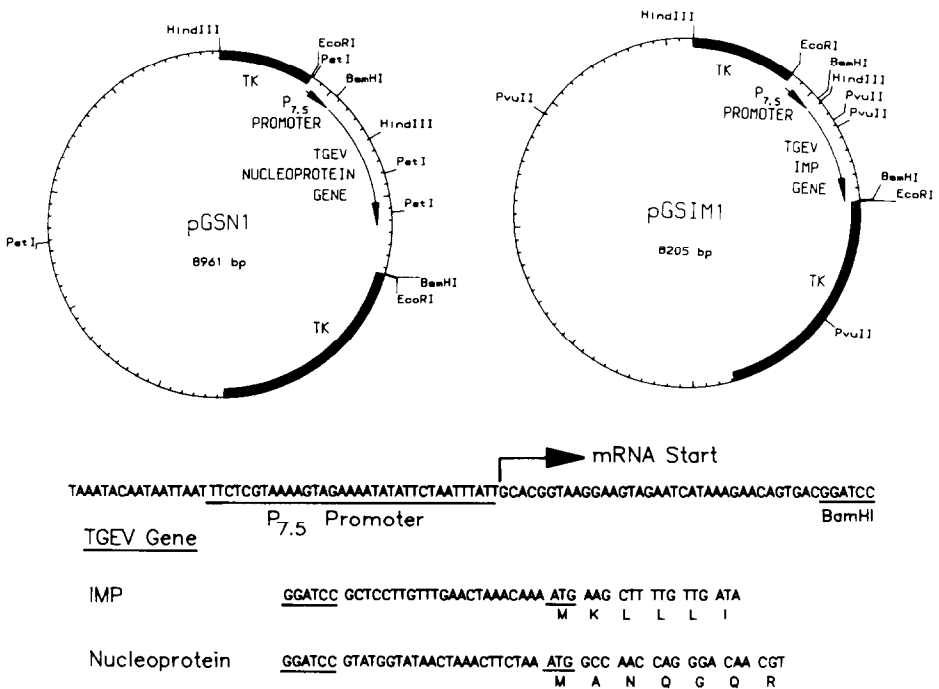


Fig. 1. Plasmid maps of the insertion vectors pGSN1 and pGSIM1 showing the orientation of the nucleoprotein and integral membrane protein (IMP) genes and the nucleotide sequences of the VV promoter/TGEV gene junctions. Initiation codons and cloning restriction enzyme sites are underlined and the transcriptional start site is indicated by an arrow. The *BamHI* site on the promoter sequence and the gene sequences are the same so that the initiation codons are 65 bp from the mRNA start site.

Following transfection of plasmids pGSN1 and pGSIM1 into vaccinia virus infected CV-1 cells, 20% and 1% of the resulting TK⁻ viruses were found to express N or M protein gene products respectively, as determined by immunodot-blot. Recombinant viruses expressing the TGEV antigens were plaque purified three times and the recombinant viruses vTN1, expressing the TGEV nucleoprotein gene, and vTM1, expressing the TGEV integral membrane protein gene, were used for subsequent experiments.

Analysis of recombinant vaccinia virus DNA

Viral DNA was extracted from either wild-type or recombinant virus infected CV-1 cells, digested with *Bam*HI and *Hind*III and analysed by Southern blotting

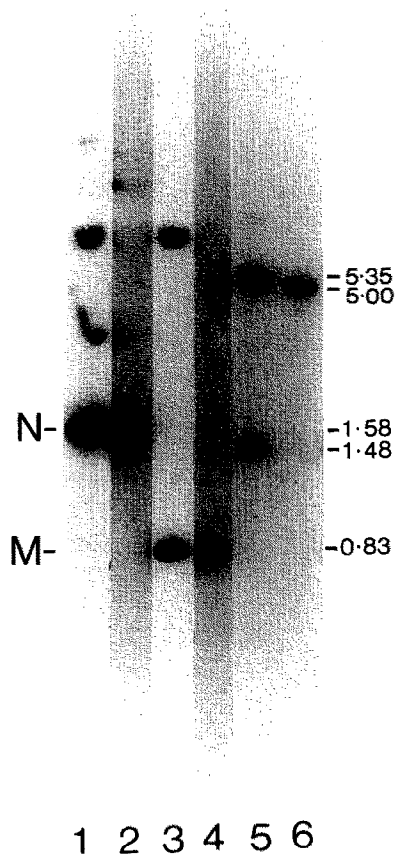


Fig. 2. Analysis of viral and insertion plasmid DNA by Southern blot. The tracks contained DNA from: (1) pGSN1, (2) vTN1, (3) pGSIM1, (4) vTM1 digested with *Bam*HI or (5) vTN1 and (6) vTM1 digested with *Hind*III. The DNA fragments were hybridised with ³²P-labelled cDNA from the N and M *Bam*HI gene cassettes.

(Fig. 2). Digestion of vTN1 with *Bam*HI produced a 1.58 Kbp fragment (Fig. 2, lane 2) that comigrated with the *Bam*HI fragment of pGSN1 (Fig. 2, lane 1). Digestion of vTN1 with *Hind*III cleaved the N gene into two fragments, a 1.48 Kbp fragment containing the first 439 bp of the N gene and a 5.35 Kbp fragment containing the rest of the N gene (Fig. 2, lane 5). Similarly, the M gene fragment was detected as a complete 830 bp *Bam*HI fragment in pGSIM1 (Fig. 2, lane 3) and vTM1 (Fig. 2, lane 4). Digestion of vTM1 DNA with *Hind*III should produce a 5.00 Kbp fragment as well as a smaller 1.07 Kbp fragment containing TGEV cDNA. The 1.07 Kbp fragment was not observed because cleavage with *Hind*III provides this fragment with only 30 bp of M gene sequence, insufficient for hybridisation with the probe. Only the 5.00 Kbp fragment was detected (Fig. 2, lane 6) because it contained 800 bp of M gene sequence. Results from the *Hind*III digestion data and the TK⁻ phenotype of the recombinant viruses confirms that the TGEV gene sequences are integrated into the vaccinia TK gene within the *Hind*III J fragment.

Expression of the nucleoprotein and integral membrane protein genes

The cellular localisation of the TGEV genes expressed by recombinant vaccinia viruses were determined by indirect immunofluorescence. Acetone fixed vTN1 infected cells probed with the anti-N mAb DA3 (Garwes et al., 1988) produced characteristic nucleoprotein staining in the cell cytoplasm (Fig. 3c), like that seen for TGEV (Fig. 3a). Nucleoprotein could not be detected in association with either the cell nucleus or on the cell surface of unfixed vTN1 infected cells (Fig. 3b). Acetone fixed vTM1 infected HTK⁻ cells probed with 3B.B3 showed M protein as having a polar cell distribution (Fig. 3g). LLC-PK1 cells infected with TGEV (Fig. 3d) or vTM1 (Fig. 3e) produced a more general cytoplasmic staining with foci of fluorescence throughout the cell cytoplasm. Integral membrane protein was not found on the cell surface of vTM1 infected or TGEV infected unfixed cells by indirect immunofluorescence, using either the mAb 3B.B3 or TGEV hyperimmune serum, implying that M protein for both viruses is retained on internal membranes. TGEV infected LLC-PK1 cells treated with mouse anti-vTN1 or mouse anti-vTM1 serum produced similar fluorescent staining (not shown) as that obtained with mAbs.

RVV early and late gene expression for both N and M was investigated by incubating infected cells in the presence or absence of 40 μgml^{-1} arabinosylcytosine (araC; Sigma), an inhibitor of VV DNA synthesis and therefore late gene function (Cochran et al., 1985). Production of the TGEV antigens was assessed by titrating total cell lysates with TGEV hyperimmune serum. N gene expression was observed to be about 4-fold lower in the presence of araC (Fig. 4). This showed that N protein was still expressed by the early gene function of the $P_{7.5K}$ promoter. However, expression of M in the presence of araC was almost abolished when compared to early/late gene expression (Fig. 4). This implied that M protein expression by vTM1 was by late gene function only or that early gene expression was very low.

The gene products were further characterised by immunoblot analysis. Sequence studies predict that the nucleoprotein gene product has a M_r of 43,483 (Britton et

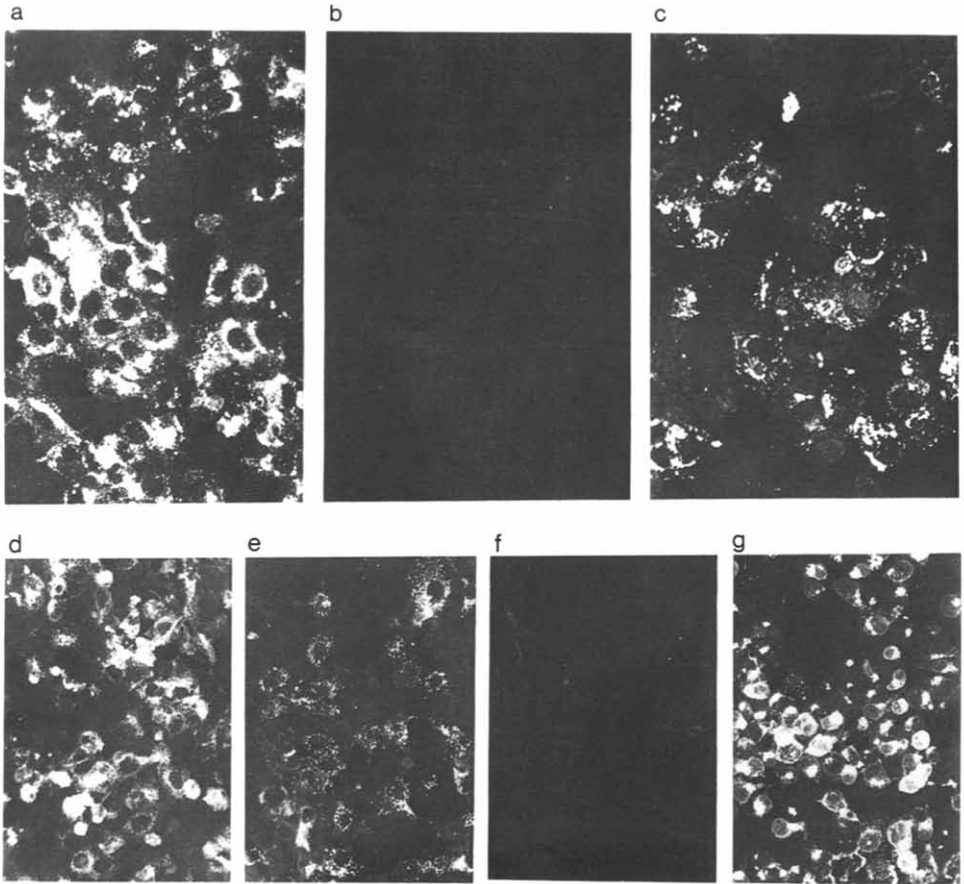


Fig. 3. Location of TGEV N and M proteins in RVV infected HTK⁻ or TGEV infected LLC-PK1 cells. LLC-PK1 cells (a, d, e) were infected with TGEV (a, d) for 10 h, or vTM1 (e) for 24 h. HTK⁻ cells (b, c, f, g) were infected with vTN1 (b, c) or vTM1 (f, g) for 16 h. Cells (a, c, d, e, g) were either fixed or washed unfixed (b, f) and probed with mAbs DA3 (a, b, c) or 3B.B3 (d, e, f, g) and detected by indirect immunofluorescence using a rabbit anti-mouse FITC conjugate.

al., 1988a), but migrates as a protein of M_r 47,000 by SDS/PAGE. The difference between predicted and observed molecular size may be due to phosphorylation on the serine residues (Garwes et al., 1984), which represent 11% of the amino acids in the N protein. Nucleoprotein breakdown products have been observed in TGEV LLC-PK1 cell lysates, notably a product of M_r 42,000 that is also phosphorylated (Garwes et al., 1984). Smaller nucleoprotein breakdown products are only detected with polyclonal serum and not with the anti-nucleoprotein mAb DA3 (Fig. 5) the binding of which has been mapped to a linear sequence within the last 25 amino acids of the protein's C-terminus (F. Parra, personal communication). Infection of LLC-PK1 cells with vTN1 also produced a polypeptide of M_r 42,000 which is not recognised with DA3 antiserum. Immunoblot analysis of TGEV infected cell

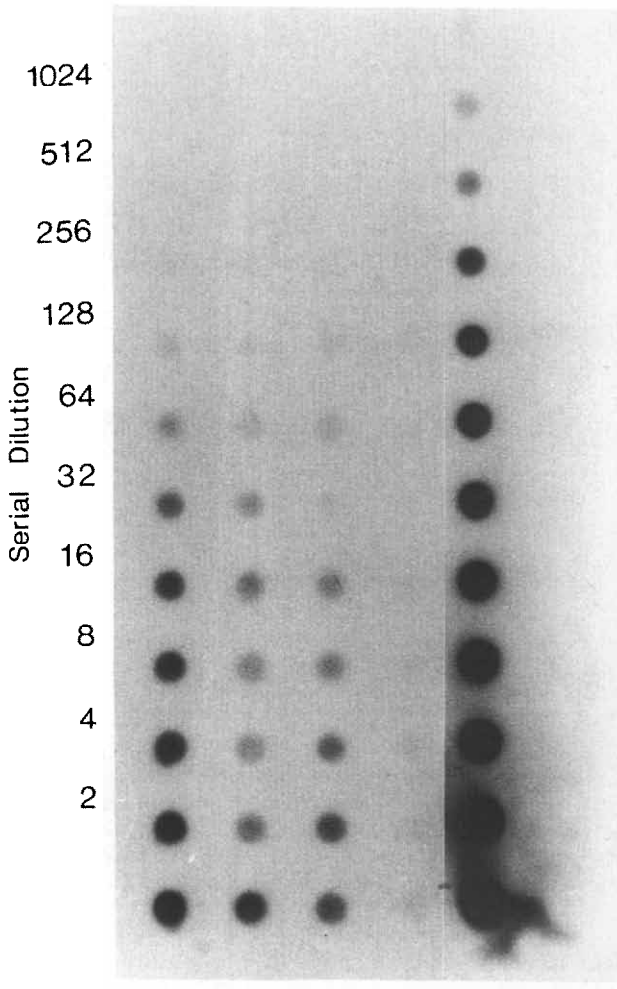


Fig. 4. Early and early/late gene expression of TGEV N and M proteins by RVV. Monolayers of CV-1 cells were infected with 10 pfu/cell of vTN1, vTM1 or wild-type virus in the presence or absence of $40 \mu\text{g}\cdot\text{ml}^{-1}$ arabinosylcytosine (araC) and lysed 24 h p.i. Serial twofold dilutions of cytoplasmic extracts were spotted onto nitrocellulose and incubated to saturation with TGEV hyperimmune antiserum, washed and incubated with ^{125}I -labelled staphylococcal protein A. The autoradiograph shows expression from: (1) vTN1; (2) vTN1 + araC; (3) vTM1; (4) vTM1 + araC; (5) TGEV infected LLC-PK1 cells 10 h p.i.; (6) wild-type VV.

material contains a range of smaller nucleoprotein species (M_r 38,000–42,000) (Fig. 5, lane 1). A major N breakdown product of M_r 40,000 seen in TGEV cells also appears to occur in CV-1 cells infected with vTN1 (Fig. 5, lane 4). This indicates that when nucleoprotein is expressed in large amounts the pattern of degradation products appear to be characteristic of the cell type infected.

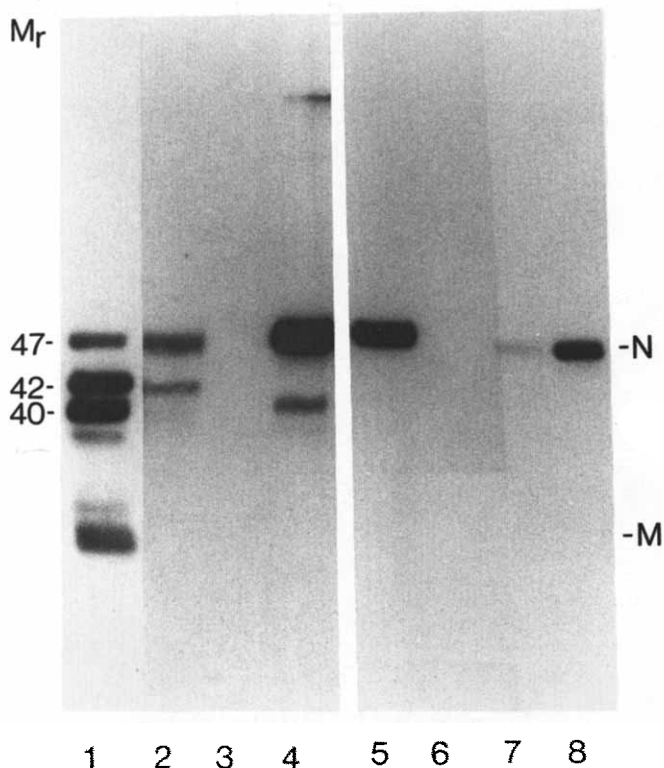


Fig. 5. Characterisation of N protein expressed by TGEV and vTN1 using immunoblotting. Proteins from infected cell lysates were separated on a 10% SDS polyacrylamide gel and transferred onto nitrocellulose. Lanes 1 and 8, TGEV infected LLC-PK1 cells; lanes 2 and 7, vTN1 infected LLC-PK1 cells; lanes 3 and 6, WR infected CV-1 cells; lanes 4 and 5, vTN1 infected CV-1 cells. Blots were incubated with TGEV hyperimmune polyclonal serum (lanes 1-4) or mAb DA3 (lanes 5-8).

The M protein is observed as a series of polypeptides, M_r 28,000-31,000, in TGEV infected cells (Garwes and Pocock, 1975). Such a series of M protein polypeptides was not observed for vTM1 infected monkey (CV-1) cells (Fig. 6) or in human (HTK⁻) and porcine (LLC-PK1) cells (not shown). Cells infected with vTM1 contain a major polypeptide species of M_r 29,000 (Fig. 6), identical in size to the most abundant M polypeptide species found in TGEV infected cells. Digestion of M polypeptides synthesised from vTM1 or TGEV infected cells, with endoglycosidase H (Fig. 6), reduced the mature M protein to a molecule of M_r 27,000, for both viruses.

Animal inoculations

Antisera from mice infected with two clones of each RVV, vTN1 or vTN2 and vTM1 or vTM2, were used to immunoprecipitate antigens from L-[³⁵S]methionine labelled TGEV infected LLC-PK1 cell lysates (Fig. 7). The mouse sera contained

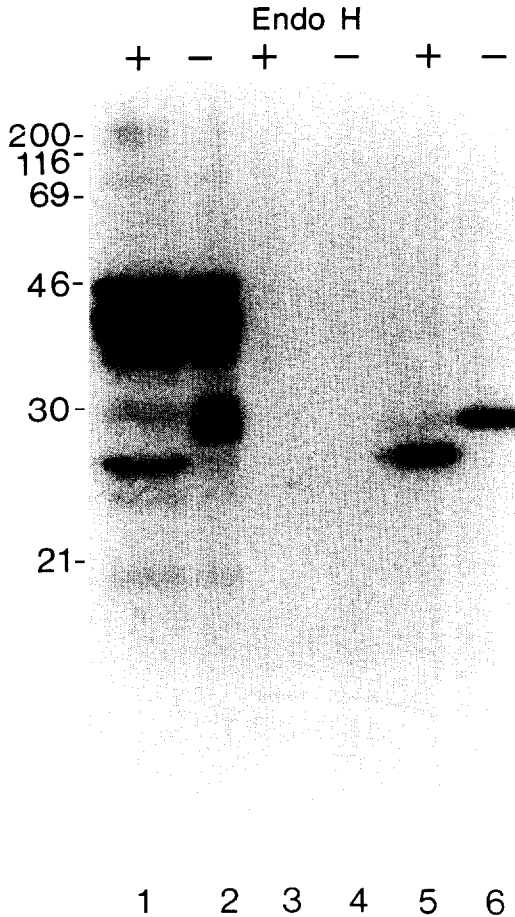


Fig. 6. Endoglycosidase H analysis of TGEV M protein. The lanes show lysates from: (1 and 2) TGEV infected LLC-PK1 cells; (3 and 4) VV (WR) or (5 and 6) vTM1 infected CV-1 cells. Lysates in lanes: (1, 3 and 5) were treated with endoglycosidase H and (2, 4 and 6) were untreated. Position of protein molecular weight markers (Sigma, SDS6H) are indicated on the left margin.

antibodies specific to the individual TGEV structural proteins but had very low TGEV neutralising activity as measured with an in vitro plaque reduction assay. Analysis of the mouse sera for VV neutralising antibody levels demonstrated that all mice infected with wild-type or recombinant vaccinia viruses had seroconverted to similar titres.

Discussion

The TGEV N and M protein genes from strain FS772/70 have been singularly inserted into the VV TK locus and expressed from the vaccinia $P_{7.5K}$ promoter.

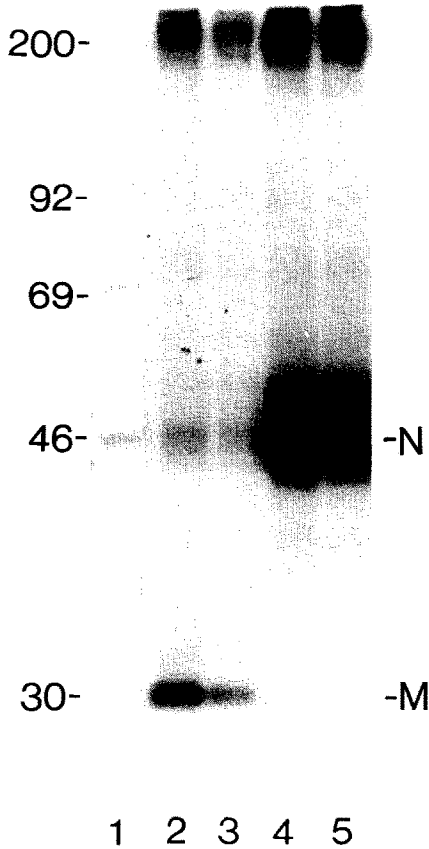


Fig. 7. Fluorograph showing the immunoprecipitation of ^{35}S -labelled TGEV polypeptides with mouse antiserum raised against: (2) vTM1; (3) vTM2; (4) vTN1; (5) vTN2. Lane 1 contains ^{14}C high molecular weight markers (Amersham).

Using mAbs previously raised against the TGEV proteins the RVV expressed proteins were found to be of a similar size and antigenicity to their TGEV counterparts.

The low levels of early M gene expression during vTM1 infection is probably due to the presence of a VV early transcriptional termination signal sequence, TTTT-TAT (Rohrmann et al., 1986), 190 bp downstream of the M gene initiation codon (Britton et al., 1988b). Browne et al. (1986) demonstrated that the human papilloma virus type 16 L1 coding region was poorly expressed by the $P_{7.5\text{K}}$ promoter because it contained two VV early transcriptional termination signals. The presence of the TTTTTAT sequence in the TGEV M gene probably explains the different levels of product synthesised between early and early/late gene expression for this protein.

The structural characteristics of the nucleoprotein from TGEV and RVVs were compared by analysis with immunoblot and immunoprecipitation. Garwes et al. (1984) showed that TGEV, strain FS772/70, infected LLC-PK1 cells contained a

phosphorylated polypeptide of M_r 42,000 that appeared late in infection, was not incorporated into virions and was derived from the nucleoprotein. The polypeptide of M_r 42,000 was shown to be co-precipitated with full size N using the mAb, DA3, but could not be detected by immunoblot with the same mAb. Welch and Saif (1988) have also co-precipitated smaller products with an anti-N mAb including a product of M_r 44,000 from TGEV strain P115 and a polypeptide of M_r 46,000, from the M6 strain, along with the full size M_r 48,000 molecule. Zhirnov and Bukrinskaya (1981) have shown that two forms of the influenza virus major nucleocapsid protein, NP₅₆ (M_r 56,000) and NP₅₃ (M_r 53,000), appear at late times of infection and in varying amounts depending on the cell type. Peptide mapping demonstrated that the NP₅₆ was cleaved to form NP₅₃ and virions preferentially contained the full length form. Cells infected with RVVs expressing the TGEV N protein also produce breakdown products. It seems unlikely that these smaller N products are the translational consequence of deleted transcripts arising from TGEV defective interfering particles, as vTM1 infected LLC-PK1 cells also synthesise these smaller molecules (Fig. 5, lane 2). The presence of smaller nucleoprotein products could be due to the release of proteolytic enzymes in dying cells in the late stages of infection. Variations in the quantity of nucleoprotein breakdown products, in different cell types, could be explained by a variable resistance of host cells to viral-induced damage as has been implicated for influenza A nucleoprotein (Zhirnov and Burkinskaya, 1981).

Characterisation of the recombinant M protein by immunoblot and immunoprecipitation studies have demonstrated that it is antigenically similar to TGEV M protein. Both the TGEV and vTM1 expressed forms of the M protein are endoglycosidase H sensitive, giving a polypeptide of M_r 27,000, implying that it is glycosylated with N-linked glycans. The predicted molecular weight for the M protein is M_r 29,459 (Britton et al., 1988b) as deduced from its gene sequence. Because the M protein in TGEV and vTM1 infected cells is glycosylated it is quite likely that the potential 16 amino acid N-terminal sequence of the M protein is cleaved. Laude et al. (1987) sequenced the N-terminus of the M protein from purified virions confirming that, for the Purdue strain, the M protein signal sequence is cleaved.

Indirect immunofluorescence has demonstrated that M protein was restricted to the cytoplasm of both vTM1 and TGEV infected cells. Coronavirus membrane proteins are transported and retained at the Golgi apparatus, where they associate with nucleoprotein in coronavirus assembly (Tooze et al., 1985). The similar distribution of M protein in TGEV and vTM1 infected LLC-PK1 cells, observed by indirect immunofluorescence, supports the theory for M protein transport to the Golgi apparatus of the cell. The distribution of M protein in HTK⁻ cells, a human osteomyeloma cell line, was distinctly polar.

Monospecific mouse serum to N and M proteins reacted with single TGEV polypeptides by immunoprecipitation demonstrating that the foreign gene products were antigenically similar. TGEV M protein mAbs can neutralise TGEV in the presence of high concentrations of guinea pig complement (Woods et al., 1987), but not with swine complement. Complement-mediated antibody neutralisation has not

been implicated as a natural protective mechanism in the pig gut and so the monospecific mouse polyclonal serum was heat treated to destroy any complement and titrated using a plaque reduction assay. Very little residual TGEV neutralising antibody was detected for these two proteins, but high neutralising antibody titres were obtained for antiserum raised to a RVV expressing the FS772/70 spike gene (Pulford et al., 1990).

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