Expression of novel genes encoded by the paramyxovirus J virus

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Characterization of the J virus or, in keeping with recent nomenclature recommendations, J paramyxovirus (JPV) genome revealed a unique genome structure, consisting of eight genes in the order 3'-N-P/V/C-M-F-SH-TM-G-L-5'. The small hydrophobic (SH) protein and the transmembrane (TM) protein genes are predicted to encode proteins 69 and 258 aa in size, respectively. The 4401 nt attachment (G) protein gene, much larger than most other paramyxovirus attachment protein genes sequenced to date, encodes a putative 709 aa attachment protein and contains distally a second open reading frame (ORF-X) 2115 nt long. Experiments undertaken in this study were intended to confirm the sequence-based gene allocation of JPV and to determine if proteins encoded by the SH gene, the novel TM gene and ORF-X are expressed. Northern blot analyses carried out on mRNA purified from JPV-infected cells indicated that the putative transcription initiation and termination sequences flanking the SH and TM genes are functional, consistent with their allocation as discrete genes, although a high level of read-through was observed across almost all transcriptional boundaries. Probes specific to the G protein coding region and ORF-X both identified an mRNA species corresponding to the predicted length of the G gene, confirming sequence-based predictions. While the SH and TM proteins were both detected in infected cells, no evidence was found for the expression of ORF-X. Preliminary studies indicate that the novel TM protein is a type II glycosylated integral membrane protein, orientated with its C terminus exposed at the cell surface.

INTRODUCTION

J virus or, in keeping with recently introduced recommendation for paramyxovirus nomenclature, J paramyxovirus (JPV) was isolated by using kidney autoculture from moribund mice (Mus musculus) trapped in 1972 in northern Queensland, Australia, during a study investigating the pathology of feral rodents in the region (Jun et al., 1977; Mesina et al., 1974). It was reported that the four mice from which the virus was isolated had extensive haemorrhagic lung lesions (Jun et al., 1977). Syncytium formation was observed in kidney autoculture monolayers, and electron microscopy revealed virion morphology and nucleocapsid structure typical of the paramyxoviruses. Characterization of the full-length genome of JPV revealed a genome structure unique within the family Paramyxoviridae (Jack et al., 2005). At 18954 nt in length, the JPV genome conforms to the 'rule-of-six' (Kolakofsky et al., 1998), and contains eight genes in the order 3'-N-P/V/C-M-F-SH-TM-G-L-5'. The two genes located between the fusion (F) and attachment (G) protein genes, named the small hydrophobic (SH) protein and transmembrane (TM) protein gene, encode putative proteins of 69 and 258 aa, respectively. The substantial increase in genome size over that of most other paramyxovirus genomes (15.1–15.9 kb) is largely due to the presence of these two additional genes, as well as an extensive second open reading frame (termed ORF-X) within the G gene (Fig. 1). Recently, the genome of a newly discovered paramyxovirus, Beilong virus or Beilong paramyxovirus (BeiPV), has been shown to be of similar size and organization to that of JPV (Li et al., 2006). Sequence alignments and phylogenetic analyses also indicated that these two viruses are more closely related to one another than to other members of the subfamily Paramyxovirinae and it has been proposed that they should constitute a new genus within the subfamily.
The TM gene of JPV and BeiPV does not have an analogue in any of the other paramyxoviruses sequenced to date. The TM gene shares some basic features with the attachment protein gene of members of the subfamily Pneumovirinae, including genome position and size and predicted membrane association of the encoded protein, although the significance of these similarities is unclear. The HMMTOP server, an automatic server for predicting topology of TM proteins (http://www.enzim.hu/hmmtop/) (Tusnády & Simon, 1998, 2001), predicted that the 258 aa JPV TM protein is a type II TM protein with a TM domain from aa 58 to 81 and a 177 aa C-terminal extracellular domain. Although the BeiPV TM protein only shares 35% sequence identity with the JPV TM protein, the hydrophobicity profiles of the two proteins are very similar and the location of the putative TM domain is almost identical. The TM gene of JPV and BeiPV does not have an analogue in any of the other paramyxoviruses sequenced to date. The TM gene shares some basic features with the attachment protein gene of members of the subfamily Pneumovirinae, including genome position and size and predicted membrane association of the encoded protein, although the significance of these similarities is unclear. The HMMTOP server, an automatic server for predicting topology of TM proteins (http://www.enzim.hu/hmmtop/) (Tusnády & Simon, 1998, 2001), predicted that the 258 aa JPV TM protein is a type II TM protein with a TM domain from aa 58 to 81 and a 177 aa C-terminal extracellular domain. Although the BeiPV TM protein only shares 35% sequence identity with the JPV TM protein, the hydrophobicity profiles of the two proteins are very similar and the location of the putative TM domain is almost identical. The JPV TM protein contains two potential N-linked glycosylation sites (Jack et al., 2005).

The JPV genome contains a 2115 nt ORF (ORF-X) that commences immediately after, and in frame with, the stop codon for the putative G protein. Within the 704 aa protein encoded by ORF-X, the first methionine residue is the 30th aa residue. Translation of ORF-X, either independently or in tandem with ORF-G as a 1414 aa ‘G–X’ protein, would rely on the use of an unconventional mechanism. An alternative possibility could be that the stop codon separating ORF-X from ORF-G is the result of a relatively recent mutation, prior to which an attachment protein of 1414 aa was translated. No significant level of amino acid sequence identity was identified between the putative X protein and any sequenced protein in a BLASTP search. BeiPV also has a very large G gene (4527 nt) and contains two overlapping ORFs distal to the G protein ORF, although neither show sequence similarity to ORF-X (Li et al., 2006).

The studies described here were intended to investigate the expression of the SH and TM genes and ORF-X in infected and transfected cells, at both the transcriptional and translational levels.

**METHODS**

**Cells and virus.** JPV supplied in RK13 cells by the Queensland Department of Primary Industries (passage history unknown) was passaged once in Vero cells prior to three rounds of plaque purification in Vero cells. Vero cells, cultured in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES and penicillin/streptomycin (100 U ml$^{-1}$/100 μg ml$^{-1}$), were used for all subsequent JPV propagation.

**Northern blot analysis.** Cells were infected with JPV at an m.o.i. of 0.005 TCID$_{50}$ and at 2 days post-infection (p.i.) cells were washed in PBSA (Dulbeco’s PBS without calcium or magnesium), trypsinized and pelleted by centrifugation at 3000 g for 5 min. Total RNA was extracted using the RNAasy Midi kit (Qiagen), quantified using the GeneQuant II RNA/DNA Calculator (Pharmacia), and mRNA was purified from equal quantities of total RNA using the Oligotex mRNA Mini kit (Qiagen), in accordance with the manufacturer’s instructions. PCR products specific to the JPV N, P/V/C, M, F, SH, TM and L genes, and to ORF-G and ORF-X within the G gene, were generated from random-hexamer-primed JPV cDNA prepared as described previously (Jack et al., 2005). The Rediprime II random priming labelling system (Amersham) was used to generate random-primed $^{32}$P-labelled DNA probes from the purified gene/ORF-specific PCR products, following the manufacturer’s guidelines. The NorthernMax-Gly kit (Ambion) was used for electrophoresis of purified mRNA samples, mRNA transfer to a positively charged nylon membrane (BrightStar-Plus; Ambion), membrane–probe hybridization and membrane washing, in accordance with the manufacturer’s instructions. Hybridization signals were visualized by autoradiography.

**Preparation of monospecific antisera.** Gene fragments encoding aa 93–258 of the TM protein, aa 30–274 of the X protein and the full-length SH protein were amplified by PCR and cloned into the pRSET B vector (Invitrogen) for expression of (His)$_6$-tagged proteins and into the pDW363 vector (Tsao et al., 1996) for the production of biotin-tagged proteins. The SH gene was also cloned into the pCDI

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**Fig. 1.** Schematic diagram of the JPV genome. The 3′-leader and 5′-trailer regions are shaded black, predicted protein-coding regions (ORFs) are shaded grey with the designations of the encoded proteins provided inside the box, non-coding regions are indicated by diagonal stripes and the trinucleotide intergenic regions are represented by vertical black lines. For genes with a different name from their ORF, the name of the gene is provided below. The black bars beneath the genes represent probes used for Northern hybridization analysis, one for each predicted gene with the exception of the G gene, which has two probes, for ORF-G and ORF-X, respectively. The numbers at the top are sizes of predicted transcripts for each gene, and the scale below indicates the genome size.
vector (Wang et al., 1996) for expression of the GST fusion proteins. All clones were verified by DNA sequencing. Expression of recombinant proteins in *Escherichia coli* was conducted as in Yu et al. (2008). Cells were lysed by treatment with BugBuster protein extraction reagent (Novagen), in accordance with the manufacturer’s instructions, then sonicated on ice five times for 30 s. Pellets were resuspended in native binding buffer (NBB; 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.6). Recombinant (His)_6-tagged X protein was purified on ProBond resin (Invitrogen) under native conditions, in accordance with the manufacturer’s recommendations. Other recombinant proteins used in this study, present predominantly within the insoluble protein fractions, were purified by preparative SDS-PAGE as described previously (Tachedjian et al., 2006).

A peptide corresponding to aa 29–48 of the putative JPV SH protein was commercially synthesized and conjugated to keyhole limpet haemocyanin (KLH) (Auspep). Lyophilized KLH–SH peptide conjugate was reconstituted in PBSA at 1 mg ml⁻¹.

Three-month-old female specific-pathogen-free New Zealand White rabbits were used to generate antisera. Purified proteins in Montanide ISA 50V (Seppic) adjuvant were prepared according to the manufacturer’s instructions. Rabbits were inoculated on days 0, 31 and 52 with 50 μg (His)_6-tagged X or TM protein and antibody responses specific for the X and TM protein fragments were monitored against biotin-tagged X or TM proteins, respectively, by Western blot. To generate antiserum to the SH protein, one rabbit was inoculated on days 0 and 31 with 200 μg KLH–SH, on days 56 and 77 with 400 μg KLH–SH, and on days 98 and 119 with 100 μg purified GST-tagged SH protein, in a Montanide ISA 50 emulsion. Antibody responses were monitored by Western blot using antibodies against recombinant (His)_6-tagged and biotin-tagged SH proteins and by ELISA against free SH peptide.

**Western blot analysis of viral proteins.** For identification of the putative TM, X, G and G–X proteins, lysates from infected or transfected cells were separated on 10–15 % Tris/HCl polyacrylamide gels under reducing conditions (Laemmli, 1970). For identification of the putative SH protein, lysates from infected cells were separated on 4–20 % Tris–HEPES–SDS–PAGE (Gradientipore) following the manufacturer’s instructions. Proteins were transferred to 0.45 μm PVDF membrane (Micron Separations), blocked with 5 % skimmed milk in TBS-T (0.01 M Tris/HCl pH 8.0, 0.15 mM NaCl, 0.05 % Tween 20) and probed with rabbit antisera. Bound antibodies were detected using a goat anti-rabbit alkaline phosphatase conjugate (Chemicon), followed by colour development using the BCIP/NBT substrate solution (Promega).

**Indirect immunofluorescence assay.** Cells were grown at 37 °C in eight-well chamber slides (Nalge Nunc). Upon reaching approximately 80 % confluency, medium was removed and replaced with either 400 μl medium containing 300 TCID₉₀ JPV (JPPV-infected wells) or 400 μl medium only (mock-infected wells). For staining of permeable cells, at 2 days p.i. slides were fixed in ice-cold methanol for 10 min and air-dried. Slides were incubated at room temperature for 10 min in PBSA containing 1 % BSA. One hundred microlitres of primary antibody solution [rabbit antisera raised against recombinant TM protein, pre-immune rabbit serum or mouse monoclonal antibody to alpha tubulin (Amersham)] was added to each well and incubated in a humidified atmosphere at 37 °C for 60 min. Slides were washed with PBSA then incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG or FITC-labelled goat anti-mouse IgG (ICN Immunologicals) at 37 °C for 30 min. Slides were mounted in 90 % glycerol:10 % PBSA and examined at ×400 magnification using a Polyvar microscope (Reichert-Jung). For surface staining of cells, incubation with primary antibody was carried out prior to methanol fixation.

**Construction of mammalian expression plasmids.** The expression vector pCI-neo (Promega) was used in this study. All primers used contained either an MluI or a NcoI restriction site. For expression of the TM, G and X ORFs, the PCR fragments obtained were cloned into the MluI and NcoI sites of pCI-neo. For expression of the G–X fusion protein, PCR-based site-directed mutagenesis was used to replace the stop codon (TAA) of the JPV G ORF with a serine codon (TCA) in the pc neo-G plasmid. This mutated ORF-G was joined with ORF-X to create the pc neo-G–X plasmid. All clones were verified by DNA sequencing.

**Expression of TM in transfected cells.** Vero cells were seeded into six-well plates at 3.5 × 10⁶ cells per well. Cells were transfected with either pc neo-TM or pc neo alone using Fugene transfection reagent (Roche) according to the manufacturer’s instructions and 2 μg plasmid per well. DNA/Fugue mixtures were added to cells and incubated overnight at 37 °C in a humidified 5 % CO₂ atmosphere. Medium was then removed, cells washed once with PBSA and infected with a recombinant T7 polymerase-encoding vaccinia virus, vTF7-3 (Fuerst et al., 1986), at an m.o.i. of 10. Plates were incubated overnight under the same conditions. Cells were washed three times with PBSA and lysed on ice in ice-cold lysis buffer [1 % Triton X-100, 0.1 M NaCl, 0.1 M Tris/HCl, pH 8.0, 1 Complete Mini Protease Inhibitor Cocktail tablet (Roche) per 10 ml lysis buffer]. Nuclei were removed by centrifugation.

**Deglycosylation of TM proteins.** Cell lysates from mock- and TM-transfected cells prepared as described above were treated with Peptide-N-glycosidase F (PNGaseF) (New England Biolabs) following the manufacturer’s recommendations. Briefly, cell lysates were denatured by the addition of 10 × glycoprotein denaturing buffer and heating at 100 °C for 10 min. Lysates were then cooled on ice, followed by the addition of 10 × G7 reaction buffer, NP-40 to a final concentration of 1 % and 1000 U PNGaseF prior to incubation at 37 °C overnight. Cell lysates were also mock treated, by incubation in buffers without the addition of PNGaseF. Following incubation, lysates were separated by 15 % SDS–PAGE and analysed by Western blot using anti-TM rabbit serum.

**Surface biotinylation and immunoprecipitation of biotinylated TM proteins.** TM-transfected cells were prepared as described above. Prior to lysis cells were washed three times with ice-cold PBS and incubated in 0.25 mg EZ-Link NHS-Biotin (Pierce) ml⁻¹ in PBS for 1 h at 4 °C. Cells were then washed three times with ice-cold PBSA and lysed as described above. Cell lysates were incubated with protein G–Sepharose (20 % slurry) (Sigma-Aldrich) for 45 min. Protein G–Sepharose was removed and either 2 μl anti-TM antiserum, 2 μl monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich) or 2 μl goat anti-human angiotensin-converting enzyme 2 (ACE2) ectodomain antibody (R&D Systems) was added. Samples were rotated at 4 °C for 1 h. Sixty microlitres of fresh protein G–Sepharose (20 % slurry) was added and samples were further rotated for 45 min at room temperature. Samples were washed three times with lysis buffer. Precipitated proteins were boiled in reducing sample buffer [0.006 % (w/v) bromophenol blue, 1 % (w/v) SDS, 30 mM Tris/HCl pH 6.5, 7.5 % (w/v) glycerol, 2.5 % (w/v) 2-β mercaptoethanol] and separated using 15 % SDS–PAGE. Proteins were transferred to a 0.45 μm PVDF membrane and probed with a streptavidin-AP conjugate (Chemicon) diluted 1:1000.

**Radio-immunoprecipitation of SH proteins from JPV-infected cells.** Cell monolayers were infected with JPV at an m.o.i. of 1.0 or 0.01 TCID₉₀ and incubated for 16 or 40 h p.i., respectively. Medium was then removed and the monolayers washed three times with PBSA prior to incubation in EMEM without L-glutamine, methionine and cysteine (ICN Biomedicals) for 30 min at 37 °C. Cells were then radiolabelled with 200 μCi ml⁻¹ (7.4 MBq) Tran³S-Label (ICN Biomedicals) for
5 h at 37 °C, washed three times in ice-cold PBSA and lysed on ice in ice-cold lysis buffer as above for TM-transfected cells.

Dynabeads Protein A (Dynal) were washed once in NBB (pH 8.1), then once in 0.5% Triton X-100 buffer (0.5% Triton X-100, 0.15 M NaCl, 0.05 M Tris/HCl pH 8.0). Anti-SH and pre-immune rabbit sera were diluted with 0.5% Triton X-100 buffer and incubated with washed Dynabeads Protein A for 30 min at room temperature with slow tilt rotation. Beads were then washed twice with 0.5% Triton X-100 buffer. JPV-infected radiolabelled lysates were combined and incubated with the protein A bead–IgG complexes at room temperature for 1 h with slow tilt rotation. Beads were washed three times with 0.5% Triton X-100 prior to elution of the IgG-antigen complexes in 0.1 M citric acid (pH 2.8). Tris/HCl (2 M, pH 8.0) was added to each eluate for neutralization prior to separation on a 4–20% Tris–HEPES–SDS-polyacrylamide mini gel (Gradipore) and autoradiography.

Expression of X, G and G–X proteins in transfected cells. Transfections of pCIneo-X, -G and -G–X were carried out using Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer’s instructions. For Western blot analysis using anti-X antiserum, transfected cells were resuspended in NBB (pH 8.1) prior to being sonicated twice on ice for 10 s. Sonicated cell lysates were separated using 12% SDS-PAGE and transferred to 0.45 μm PVDF membranes, as described above, prior to probing with anti-X antiserum diluted 1:100. For radio-immunoprecipitation analysis using anti-JPV antiserum (raised against inactivated whole virus, kindly provided by Gary Crameri of the Australian Animal Health Laboratory), radiolabelled transfected cells were lysed on ice in ice-cold lysis buffer. Nuclei were removed by centrifugation and cell lysates were pre-incubated with protein G–Sepharose (20% slurry) (Amersham) for 45 min. After removing protein G–Sepharose beads, 2 μl anti-JPV antiserum was added. Samples were rotated at 4 °C for 1 h. Sixty microlitres of fresh protein G–Sepharose (20% slurry) was added and samples were further rotated for 45 min at room temperature. Beads were washed three times with lysis buffer. Precipitated proteins were boiled in reducing sample buffer, separated using 12% SDS-PAGE and visualized by autoradiography.

RESULTS

Northern blot analysis

Poly(A) + mRNA purified from mock- and JPV-infected cells was hybridized with probes specific to each of the JPV N, P/V/C, M, F, SH, TM and L genes and to regions within the G gene corresponding to ORF-G and ORF-X. None of the probes resulted in a positive signal for mRNA purified from mock-infected cells. In hybridizations with mRNA purified from JPV-infected cells, each probe detected multiple species (Fig. 2). With the exception of the SH probe, the smallest mRNA species detected by each probe corresponds to the predicted size of monocistronic mRNA for that gene (all denoted by black arrowheads in Fig. 2, see predicted transcript sizes in Fig. 1). An mRNA molecule specific to ORF-X alone was not identified, instead the smallest species detected by both the G- and X-specific probes was approximately 4400 nt, the predicted size of mRNA transcribed from the full-length G gene. Even when the autoradiograph was overexposed, no band in the region of 366 nt, the predicted size of monocistronic SH mRNA, could be identified. The smallest mRNA band identified by the SH probe was approximately 1350 nt in size. As a band of the same size was detected by the TM probe, it is likely that both represent the dicistronic SH–TM mRNA (1362 nt). Bands corresponding in size to dicistronic N–P (3356 nt), P–M (2874 nt) and F–SH (2119 nt) mRNA species were also identified (denoted by grey arrowheads in Fig. 2). The interpretation of the putative N–P and P–M species is not straightforward; however, a faint band was apparent in the M probe hybridization at the approximate level of the putative N–P dicistronic species and a faint band was apparent in the N probe hybridization at the approximate level of the putative P–M species. Therefore, the possibility that these bands represent fortuitously sized, co-purified JPV genome fragments containing the P gene and differing fractions of the N and M genes, cannot be discounted. A band at approximately 3000 nt was common to the F, SH and TM hybridizations (denoted by open arrowheads in Fig. 2), possibly representing a tricistronic F–SH–TM mRNA, which has a predicted size of 3118 nt. Bands consistent with the predicted size of multicistronic N–P–M (4570 nt) and M–F–SH–TM (4332 nt) mRNA species were also identified. Most probes recognized multiple bands above 5000 nt in size, possibly representing a mixture of further multicistronic mRNA species and copurified fragments of the viral genome.

Identification and localization of the TM protein in infected and transfected cells

When total cell lysate of JPV-infected cells was analysed by Western blot using anti-TM antiserum, several bands were detected in the range of ~25–45 kDa (data not shown),
indicating potential glycosylation of the TM protein. To characterize better these bands, a transient expression system with a higher level of expression was used. Similar to the band pattern observed from JPV-infected cells, at least three bands were present in the transfected cells, which reacted specifically with the anti-TM antibodies (indicated by open arrowheads in Fig. 3). After treatment with PNGaseF, the upper bands disappeared and a single sharp band appeared at approximately 30 kDa (indicated by the black arrowhead), consistent with the predicted molecular mass of 29.1 kDa for the non-glycosylated form of the TM protein (Jack et al., 2005). The extra band (indicated by an asterisk) present only in lanes 3 and 6 was also present when only the PNGaseF enzyme solution was loaded in a separate Western blot analysis (data not shown). The size of the band matches the predicted molecular mass of PNGaseF, 36 kDa (Lemp et al., 1990).

Immunofluorescence assays were carried out on fixed and permeabilized JPV-infected cells to determine cell-surface exposure of the TM protein. In JPV-infected cells probed with anti-TM antiseraum, intense perinuclear staining and diffuse cytoplasmic staining were observed (Fig. 4a). No significant fluorescence was detected in either mock-infected cells probed with anti-TM antiserum (Fig. 4b) or JPV-infected cells probed with pre-immune serum (data not shown). In order to determine if the TM protein is present at the surface of infected cells, cells were incubated with anti-TM antiserum prior to fixing and permeabilizing. Post-fixation, cells were incubated with FITC-conjugated anti-rabbit IgG. In JPV-infected cells, a peripheral or membrane staining pattern was observed (Fig. 4c), whereas no significant staining was observed in mock-infected cells (Fig. 4d) or JPV-infected cells similarly incubated with pre-immune serum (data not shown). This pattern of fluorescence is consistent with the presence of TM protein on the cell surface. To ensure that the cells used for surface staining were non-permeable, a monoclonal antibody against alpha tubulin (an intracellular protein) was used to probe both permeable and non-permeable cells and, as expected, only permeable cells were stained (data not shown).

The surface exposure of TM protein was further confirmed by surface biotinylation of intact cells, followed by specific precipitation of biotinylated TM proteins using the anti-TM antiserum (Fig. 5). Under the same conditions, a known surface-exposed protein, ACE2, with a predicted molecular mass of 93 kDa (Tipnis et al., 2000), was also biotinylated, whereas the intracellular protein GAPDH, of 36 kDa (Arcari et al., 1984), was not.

![Fig. 3. Western blot analysis of TM proteins expressed in transfected cells. Mock- and TM-transfected cell lysates were treated with PNGaseF and probed with the rabbit anti-TM antiserum. Lanes: 1 and 4, untreated lysate; 2 and 5, lysate incubated with buffer in the absence of PNGaseF; 3 and 6, lysate treated with PNGaseF as detailed in Methods. TM-specific protein bands in the untreated sample are indicated by open arrowheads. The TM-specific band in the treated sample is indicated by a black arrowhead. The cross-reactive band present only in lanes 3 and 6 is indicated by an asterisk. Sizes of the molecular markers are shown at the left.](image)

![Fig. 4. Indirect immunofluorescent staining of JPV- and mock-infected cells. (a) Permeable JPV-infected cells probed with anti-TM antiserum. (b) Permeable mock-infected cells probed with anti-TM antiserum. (c) Surface staining of JPV-infected cells probed with anti-TM antiserum. (d) Surface staining of mock-infected cells probed with anti-TM antiserum.](image)
Expression of the SH protein in JPV-infected cells

Different approaches were used to investigate the expression of SH in JPV-infected cells. On Western blot analysis, anti-SH antiserum detected a distinct protein band in JPV-infected cell lysate with an apparent molecular mass of between 5 and 14 kDa (Fig. 6a), corresponding with the molecular mass of 7.7 kDa predicted for the SH protein. A similar band was also detected using radio-immunoprecipitation (Fig. 6b). No specific staining was observed when JPV-infected cells were probed with the same anti-SH antiserum in immunofluorescent antibody tests, probably due to the very small size of the predicted surface-exposed domain, which could be masked by adjacent protein, carbohydrate or lipid moieties. The subcellular localization of the SH protein therefore could not be determined during this study.

Detection of X protein in infected and transfected cells

JPV-infected cell lysates were analysed using the anti-X antiserum by Western blot and radio-immunoprecipitation assay using the same conditions as described above for SH and TM proteins. No specific band was detected by either of the approaches (data not shown) in the region of 75 kDa, the predicted molecular mass of an X protein that commences at the first methionine residue encoded by ORF-X, or 155 kDa, the predicted molecular mass of a G–X fusion protein. Attempts to identify the putative X protein in JPV-infected cells using indirect immunofluorescence were also unsuccessful.

As all attempts to identify the X protein in infected cells failed, it was important to determine if the antiserum raised against (His)₆-tagged X protein was in fact capable of detecting X protein expressed in mammalian cells. To achieve this, cells transiently expressing X were analysed by Western blot. The anti-X antiserum identified a protein consistent with the predicted molecular mass of the X protein in transfected cells (Fig. 7a). The anti-X antiserum was also able to react with the X-protein region (Fig. 7b) when it was expressed as a G–X fusion protein from a mutated G gene in which the single TAA stop codon between ORF-G and ORF-X was changed to the TCA codon encoding serine. The identity of the expressed G–X fusion protein was independently confirmed by radio-immunoprecipitation with anti-JPV antiserum, targeting the G-specific protein sequence (Fig. 7c).

DISCUSSION

Characterization of the JPV genome not only allowed the classification of JPV as a member of the subfamily...
**Paramyxovirinae**, but also revealed a unique genome structure. In addition to the presence of two genes between the fusion and attachment protein genes, an extensive second ORF was identified within the attachment protein gene. From the sequence data it was predicted that the SH and TM protein genes, each flanked by conserved transcriptional control and intergenic sequences, were likely to be expressed in JPV-infected cells. In contrast, the potential for expression of ORF-X was unclear.

To investigate the nature of the mRNA species transcribed from the JPV genome, Northern blot hybridizations were carried out on mRNA purified from JPV-infected cells. Probes specific to the N, P, M, F, TM and L genes each identified mRNA species consistent with the size of monocistronic mRNA predicted from the JPV genome sequence and, as expected, both the G- and X-specific probes identified an mRNA species at approximately 4400 nt. Of the eight JPV genes, the only gene for which a monocistronic mRNA species was not identified was the SH gene. As putative dicistronic F–SH and SH–TM species were identified, indicating that both the SH transcriptional initiation and termination signals are functional, it can be deduced that the failure to identify a monocistronic SH species is most likely a result of low levels or instability of the monocistronic mRNA molecule in infected cells. Interestingly, of the eight transcription termination signals within the JPV genome, only the F and SH gene termination signals vary from the consensus sequence at within the JPV genome, only the F and SH gene junctions. Although uncertainty exists with respect to the identification of putative dicistronic mRNA species in the N, P and M hybridizations, if these bands do in fact represent N–P and P–M mRNA molecules, their relative abundance could not be directly attributed to non-consensus N and P gene transcription termination signals. It is possible that the JPV consensus transcription termination sequence itself is suboptimal, resulting in a high frequency of read-through at gene junctions and a consequent abundance of dicistronic and multicistronic mRNA molecules.

When JPV-infected and TM-transfected cell lysates were probed with anti-TM antiserum, at least three specific protein bands were identified. This, together with the diffused pattern of the top band and the presence of two potential N-glycosylation sites in the TM protein sequence, suggested that the TM protein could be glycosylated. This was confirmed by the shift of bands after deglycosylation. Sequence analysis predicted that the TM protein is a type II integral membrane protein with an extracellular C terminus. Immunoreactivity assays and direct biotinylation of surface-expressed TM independently confirmed the surface expression of the TM protein on non-permeable cells. As the anti-TM antiserum was raised to the C-terminal region of the putative TM domain, these results support the sequence-based prediction.

When used to probe JPV-infected cell lysates with Western blot and radio-immunoprecipitation, anti-SH antiserum detected a protein corresponding to the predicted size of the SH protein. However, the same antiserum failed to detect the SH protein in immunofluorescence assays, thus preventing studies to determine its subcellular localization. New antibodies targeting different sequence regions or epitope tagging strategies will be required for further investigation.

Western blot analyses, immunofluorescence and radiolabelling strategies will be required for further investigation.

These studies were aimed at characterizing the expression of the JPV SH, TM and G genes at both the transcriptional and translational levels. Northern blot analyses indicate that the putative transcriptional initiation and termination signals flanking each of the SH and TM genes are functional, supporting their allocation as discrete genes on the basis of nucleotide sequence data. Also consistent with predictions based on the JPV genome sequence, probes specific to ORF-G and ORF-X both identified an mRNA molecule corresponding in size to monocistronic G species.
gene mRNA, while no evidence was found for the existence of an mRNA molecule specific to ORF-X alone. The TM protein was identified in JPV-infected cells, and data from two different experimental approaches supported predictions that it is an integral membrane protein orientated with its C terminus at the cell surface. The SH protein was also identified in JPV-infected cells, although no indication as to its specific cellular localization was obtained. In contrast to the positive identification of proteins translated from the TM and SH genes, no evidence was found for the translation of ORF-X in JPV-infected cells.

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REFERENCES


