

In vitro and *in vivo* expression of foreign genes by transmissible gastroenteritis coronavirus-derived minigenomes

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A helper-dependent expression system based on transmissible gastroenteritis coronavirus (TGEV) has been developed using a minigenome of 3.9 kb (M39). Expression of the reporter gene β -glucuronidase (GUS) (2–8 μ g per 10^6 cells) and the porcine respiratory and reproductive syndrome virus (PRRSV) ORF5 (1–2 μ g per 10^6 cells) has been shown using a TGEV-derived minigenome. GUS expression levels increased about eightfold with the m.o.i. and were maintained for more than eight passages in cell culture. Nevertheless, instability of the GUS and ORF5 subgenomic mRNAs was observed from passages five and four, respectively. About a quarter of the cells in culture expressing the helper virus also produced the reporter gene as determined by studying GUS mRNA production by *in situ* hybridization or immunodetection to visualize the protein synthesized. Expression of GUS was detected in the lungs, but not in the gut, of swine immunized with the virus vector. Around a quarter of lung cells showing replication of the helper virus were also positive for the reporter gene. Interestingly, strong humoral immune responses to both GUS and PRRSV ORF5 were induced in swine with this virus vector. The large cloning capacity and the tissue specificity of the TGEV-derived minigenomes suggest that these virus vectors are very promising for vaccine development.

Introduction

Transmissible gastroenteritis virus (TGEV) is a member of the *Coronaviridae* family that, with the *Arteriviridae*, forms the *Nidovirales* order (Enjuanes *et al.*, 2000b). The TGEV RNA genome has a length of 28.5 kb (Penzes *et al.*, 2001). About two-thirds of the entire RNA comprise the ORF1ab encoding the replicase gene. The 3' one-third of the genome comprises the genes encoding the structural and non-structural proteins. Coronaviruses include a large family of viruses that infect a broad range of vertebrates, from mammalian to avian species (Lai & Cavanagh, 1997; Siddell, 1995). Coronaviruses are mainly associated with respiratory, enteric, hepatic and central

nervous system diseases. Nevertheless, organs such as kidney, heart and eye can also be affected. In humans and fowl, coronaviruses primarily cause upper respiratory tract infections, while porcine and bovine coronaviruses establish enteric infections that result in severe economic loss (Caul & Egglestone, 1982; Denison, 1999; Enjuanes *et al.*, 2001).

Coronaviruses have several advantages as vectors over other virus expression systems. They are single-stranded (ss) RNA viruses that essentially replicate within the cytoplasm without a DNA intermediary, making integration of the virus genome into a host cell chromosome unlikely (Lai & Cavanagh, 1997). Coronaviruses have the largest RNA virus genome and, in principle, have room for the insertion of large foreign genes (Enjuanes *et al.*, 2000a; Masters, 1999). Since they usually infect the mucosal surfaces, both respiratory and enteric, they may be used to target the antigen to the enteric and respiratory areas to induce a strong secretory immune response

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This paper is dedicated to the memory of Professor Helmut Wege.

(Ballesteros *et al.*, 1997; Enjuanes & Van der Zeijst, 1995; Kuo *et al.*, 2000; Leparco-Goffart *et al.*, 1998; Sánchez *et al.*, 1999).

Two types of expression systems have been developed based on coronavirus genomes (Enjuanes *et al.*, 2001; Liao *et al.*, 1995; Zhang *et al.*, 1997). One type, the helper-dependent expression system, requires two components, and the other requires a single genome that is modified either by targeted recombination (Masters, 1999) or by engineering a cDNA encoding an infectious RNA (Almazán *et al.*, 2000; Thiel *et al.*, 2001; Yount *et al.*, 2000). The first attempt to use a coronavirus for heterologous gene expression was based on mouse hepatitis virus (MHV) by using a helper-dependent expression system (Liao *et al.*, 1995; Zhang *et al.*, 1997). Expression with MHV has been based on the use of either internal ribosome entry sites or transcription regulatory sequences (TRSs) present within the viral genes (Liao *et al.*, 1995; Lin & Lai, 1993; Zhang *et al.*, 1997). More recently, helper-dependent expression systems based on infectious bronchitis virus (IBV) (Stirrup *et al.*, 2000), human coronavirus HCoV-229E (Thiel *et al.*, 1998) and bovine coronavirus (Krishnan *et al.*, 1996) have also been developed.

Helper-dependent expression systems have been designed based on TGEV-derived minigenomes (Alonso *et al.*, 2002; Izeta *et al.*, 1999). The expression of the reporter gene β -glucuronidase (GUS) under the control of optimized TRSs has been shown (Alonso *et al.*, 2002). An improvement introduced in these systems was a two step amplification system based on expression of the viral minigenome under the control of the cytomegalovirus (CMV) early promoter within the nucleus, coupled to a second amplification step of minigenome RNAs translocated to the cytoplasm by the viral polymerase (Izeta *et al.*, 1999), as previously described for other positive-stranded RNA genomes (Dubensky *et al.*, 1996).

In this report, the expression of GUS and the ORF5 involved in the protection against the porcine reproductive and respiratory syndrome virus (PRRSV), a virus with a high impact on animal health (Pirzadeh & Dea, 1998; Plana-Durán *et al.*, 1997a, b), has been studied both in tissue culture and in swine. The protein expression levels, the stability of the vector, the tissue distribution and the humoral immune response elicited against the heterologous gene have been analysed. It has been shown that the TGEV-derived virus vector achieved high foreign gene expression levels, which led to the induction of significant immune responses in swine. The stability of TGEV-derived minigenomes was highly dependent on the heterologous gene and was significantly increased over previous systems.

Methods

■ **Cells and viruses.** The TGEV PUR46-MAD strain (Sánchez *et al.*, 1990) was grown and titrated as described (Jiménez *et al.*, 1986). Viruses were grown in swine testis (ST) cells (McClurkin & Norman, 1966). PRRSV Olot91 strain (Plana-Durán *et al.*, 1992) was grown in African green monkey kidney (MA-104) cells (Meulenberg *et al.*, 1998).

■ **Construction of cDNAs encoding RNA minigenomes.** The construction of DI-C-derived cDNA encoding RNA minigenome M39 was previously described (Izeta *et al.*, 1999). To increase minigenome RNA expression levels, the cDNAs were preceded by the CMV promoter (Dubensky *et al.*, 1996; Penzes *et al.*, 1998). The minigenome was flanked at the 3' end by the hepatitis delta virus ribozyme and the bovine growth hormone polyadenylation and termination sequences (Penzes *et al.*, 1998).

To evaluate expression levels using the minigenomes, *E. coli* K12 GUS was used as a reporter gene (Jefferson *et al.*, 1986; Schlaman *et al.*, 1994). The GUS gene was amplified by PCR from plasmid pGUS1 (Plant Genetic Systems) using a forward 40-mer oligonucleotide (5' **GCGGC-CGCAGGCCTGTCGACGACCATG**ATGGTCCGTCCTGTAG 3') which included *NofI*, *StuI* and *SalI* restriction endonuclease sites (bold nucleotides). The GUS initiation codon is underlined. Nucleotides shown in italics were included to fit the consensus motif of the ribosome scanning model (Kozak, 1991a, b). The reverse primer was 41 nt long (5' **GGTACCGCGCGCCTGGGCTAGCGGATCATAGGCGTCTCGC** 3') and included *KpnI*, *BssHII* and *NheI* restriction sites (bold nucleotides). PRRSV Olot91 strain ORF5 (nt 1763–2365 of sequence deposited in EMBL, accession no. X92942) was amplified by PCR from plasmid pMTL25-PRRSV-ORF5 using a forward 33-mer oligonucleotide (5' **GGTCGACGACCATGAGATGTTCTACAAATTGG** 3') and a 29-mer reverse primer (5' **GGTAGCCTAGGCTTCCCATTGCTCA-GCC** 3') that included the restriction endonuclease sites *SalI* and *NheI* (bold nucleotides), respectively. The consensus motif of the ribosome scanning model is shown in italics and the translation initiation codon is underlined.

The expression cassettes encoding the GUS gene and the PRRSV ORF5 were cloned at position 3337 from the 5' end of minigenome M39 as described by Alonso *et al.* (2002), generating minigenomes M39-GUS and M39-ORF5, respectively. To ensure that the expected plasmids were generated, the constructs were sequenced at the cloning junctions using an Applied Biosystems 373 DNA sequencer.

■ **Rescue of minigenomes encoding the expression cassettes.** ST cells grown to 50% confluence in 35 mm dishes were transfected with 10 μ g of plasmid DNA encoding CMV-driven minigenomes and 15 μ l of Lipofectin reagent in Optimem medium (Gibco-BRL), according to the manufacturer's instructions. The transfected cells were infected with TGEV PUR46-MAD (m.o.i. 5) at 4 h post-transfection. Supernatants obtained from these cultures at 22–24 h post-infection (p.i.) were used to infect fresh ST cell monolayers. The indicated number of passages were performed to amplify the helper virus and minigenome-derived RNAs.

■ **RNA analysis by Northern blotting.** Total intracellular RNA was extracted at 16 h p.i. from DNA-transfected and helper virus-infected ST cells at different passages using the Ultraspec RNA isolation system (Biotecx), following the manufacturer's instructions. RNAs were separated in denaturing 1% agarose, 2.2 M formaldehyde gels. Following electrophoresis, RNAs were irradiated for 0.2 min using a UVP cross-linker (CL-1000) and blotted onto nylon membranes (Duralon-UV, Stratagene) using a Vacugene pump (Pharmacia). The nylon membranes were irradiated with two pulses of 70 mJ/cm² and hybridized with [α -³²P]dATP-labelled ssDNA probes following standard procedures (Sambrook *et al.*, 1989). The 3' UTR-specific ssDNA probe was complementary to nt 28300–28544 of the TGEV PUR46-MAD strain genome (Penzes *et al.*, 2001). The GUS- and ORF5-specific probes were complementary to the first 296 and 276 nt of these genes, respectively. The membrane was exposed to an X-OMAT Kodak Scientific Imaging film for 8 h at –70 °C.

■ **Western blot and immunoprecipitation analysis.** GUS expression in cells transfected with cDNA encoding a minigenome and infected with helper virus was analysed at passage four by Western blot as described previously (Alonso *et al.*, 2002). Purified GUS protein (Sigma) was used as a positive control. A GUS-specific polyclonal rabbit antibody (5 Prime-3 Prime) diluted 1:200 in TBS buffer (Tris-HCl 20 mM pH 7.5, NaCl 500 mM) was used as the primary antibody to detect GUS protein. Rabbit-specific goat-antibody conjugated to peroxidase, diluted 1:8000 in TTBS buffer (TBS with Tween-20, 0.1%), was used as secondary antibody.

Immunoprecipitation of PRRSV ORF5 was performed as described (Torres *et al.*, 1995). Briefly, ST cell monolayers grown in 35 mm dishes were infected with a mixture containing the helper virus (TGEV PUR46-MAD) and the minigenome encoding PRRSV ORF5 (M39-ORF5). MA-104 cell monolayers grown in 35 mm dishes infected with PRRSV were used as a positive control. After 1 h of virus adsorption at 37 °C, fresh medium was added and cells were incubated for 4 h at 37 °C. Cells were washed with starvation medium methionine- and cysteine-free, overlaid with this medium containing 2% foetal calf serum, and incubated for 1 h at 37 °C. The medium was then replaced by *in vitro* labelling mix containing 60 µCi of L-[³⁵S]methionine/cysteine (Amersham Pharmacia Biotech) and incubated for 13 h at 37 °C. The cells were detached from the dish with a rubber policeman, washed with PBS by centrifugation at 3000 r.p.m. for 15 min at 4 °C and lysed in RIPA buffer (Torres *et al.*, 1995). Antisera used for preclearing and for immunoprecipitation were bound to protein A-Sepharose beads by overnight incubation at 4 °C. Cell extracts were precleared by incubation with a preimmune rabbit antiserum bound to protein A-Sepharose beads for 3 h at 4 °C. Supernatants were next immunoprecipitated by overnight incubation at 4 °C with a rabbit antiserum specific for PRRSV Olot91 strain ORF5, obtained by immunization with an ORF5-derived synthetic 18 amino acid peptide (NH₂-142-TNFIVDDRGRHRWKSPI-159-COOH), bound to protein A-Sepharose beads. After four washes in RIPA buffer containing 0.2% SDS, the pelleted beads were resuspended in SDS sample buffer containing 2.5% β-mercaptoethanol, boiled for 3 min and centrifuged at low speed to sediment the beads. The immunoprecipitated proteins were resolved in an SDS/5–20% polyacrylamide gel. The gel was fixed (10% acetic acid, 35% ethanol) before incubation with 14% (w/w) sodium salicylate (Merck) for 30 min at room temperature and finally dried at 80 °C for 1 h and exposed to an X-OMAT Kodak Scientific Imaging film.

■ **ELISA.** Antibodies generated against GUS and PRRSV ORF5 were detected by ELISA as described (Ausubel, 1987). ELISA was performed using as antigen purified TGEV (0.2 µg per well), partially purified PRRSV (1:100 dilution of partially purified PRRSV with 3.2 × 10⁴ TCID₅₀/ml), purified GUS protein (Sigma, 0.5 µg per well) or the KLH-conjugated ORF5 peptide (0.5 µg per well). ORF5 peptide was conjugated to KLH using the Imject Immunogen EDC conjugation kit with mKLH (Pierce) following the manufacturer's instructions. To perform the ELISA, antigens were bound to 96-well microplates as previously described (Correa *et al.*, 1988), saturated with 5% BSA in PBS for 2 h at 37 °C and incubated with the serum sample diluted 1:4 in PBS-0.1% BSA for 3 h at room temperature. Microplates were washed six times with 0.1% BSA and 0.1% Tween-20 in PBS and sequentially incubated with peroxidase-conjugated protein A diluted 1:2000 in PBS with 0.1% BSA. Microplates were washed six times before incubation with the peroxidase substrate phenylenediamine dihydrochloride (Sigma FAST) for 15 min at room temperature. Reactions were stopped with 1.5 M H₂SO₄ and the absorbance was read at 492 nm.

■ **GUS chemiluminescent detection in cell extracts.** GUS expression in cell extracts was detected by a chemiluminescent assay

(GUS-Light kit, Tropix), according to the manufacturer's instructions (Bronstein *et al.*, 1994). Cells transfected with GUS-encoding minigenome, or mock-transfected, were infected with helper virus (m.o.i. 5). The amount of protein expressed 22–24 h p.i. was estimated using standard calibration curves generated with purified GUS (Sigma) and the bicinchoninic acid protein assay (BCA, Pierce), resulting in 10⁶ relative luminometric units per 0.35 ng of GUS.

■ **Analysis of TGEV-infected newborn swine.** Conventionally raised, 2-day-old, colostrum-deprived piglets, serologically negative for TGEV were inoculated with a mixture containing 10⁸ p.f.u. of helper virus (TGEV PUR46-MAD) and the minigenome M39-GUS by both oronasal and enteric tract (using a gastric tube) routes. Three replicate experiments were performed and clinical signs were recorded during the experiments. Five piglets were sacrificed on each of the 3 days following inoculation, subjected to necropsy, and lungs, jejunum and ileum were collected. Two mock-infected piglets were also sacrificed each day.

For histopathological examination, lung tissue samples (four different locations) and samples from small and large intestine (five different locations) were either snap-frozen and stored at –70 °C until further use to prepare cryostat sections or immediately fixed in 4% neutral-buffered formalin and processed for paraffin-embedding, sectioning and haematoxylin and eosin staining.

For virus isolation, frozen tissue was thawed and homogenized on ice in PBS (1 ml/g tissue) with an Ultra-Turrax. After 12 min centrifugation at 3000 r.p.m., the supernatant was diluted 1:5 in PBS with antibiotics and left at 4 °C for 1 h. After spinning for 15 min at 12000 r.p.m., the supernatant was diluted serially in Dulbecco's modified Eagle medium with 2% foetal calf serum, including antibiotics and 40 µg/ml of DEAE-dextran. Virus was titrated on ST cell monolayers.

For immunofluorescence staining, cryostat sections of intestine and lungs were fixed with acetone (–20 °C) for 10 min and incubated for 1 h at 37 °C with FITC-labelled anti-TGEV hyperimmune serum, diluted 1:10 in 0.2 M Tris-HCl, pH 8.6 and mixed at a ratio of 3:1 with 0.005% Evan's blue solution. Sections were sealed in glycerol buffer containing 25 mg/ml of 1,4-diazabicyclo(2,2,2)octane (DABCO).

For GUS histochemistry, cryostat sections of intestine and lungs were fixed for 45 min using the fixation buffer provided with the β-glucuronidase reporter gene kit (Sigma), according to the manufacturer's recommendations. Sections were washed repeatedly with 10 mM sodium phosphate pH 7.0 and 0.2 mM EDTA and incubated overnight at 37 °C in the staining solution containing both potassium ferri- and ferrocyanide.

■ **In situ hybridization (ISH).** For preparation of non-radioactive riboprobes, *in vitro* transcription using the digoxigenin RNA labelling technique was performed as described (Zurbriggen *et al.*, 1998) with probes complementary to the 3' end of TGEV PUR46-MAD and to the GUS gene (see above). PCR-amplified DNA was cloned into the pGEM-T-Easy plasmid vector (Promega). *In vitro* transcription was performed with the RiboMAX system to yield digoxigenin-11-dUTP (Roche Molecular Biochemicals) riboprobes using sense (from SP6 promoter) and antisense (from T7 promoter) riboprimers. After shortening the probes to a length of about 150 bases they were stored in diethyl-pyrocyanate-treated water at –70 °C until further use. For ISH, tissue culture chamber slides and cryostat sections were fixed with 4% paraformaldehyde in PBS. For permeabilization and proteolytic digestion, proteinase K was applied to cells (0.1–0.5 µg/ml) and tissues (1–5 µg/ml). Hybridization was performed overnight at 50 °C using 1–2 ng/µl of the probes. To digest any unbound probe, the sections were treated with RNase T1 and DNase-free RNase (Roche). For immunological probe detection, the sections were incubated for 2 h with an alkaline phosphatase-conjugated

anti-digoxigenin antibody diluted 1:500 (Roche). Nitro blue tetrazolium and 5-bromo-4-chloro-4-indolylphosphate were used as substrates for colour reaction. Overnight development of the dark blue signal was stopped in TE buffer (pH 8.0).

Morphometric analysis of ISH signals on ST cells and lung. Morphometric analysis and quantification of TGEV-RNA- and GUS-RNA-positive cells in tissue culture and lung tissues were performed using the KS300 image analysis system (Zeiss). Calibration and threshold determination for the staining were done once for each section using the objective 20 \times . For each probe, three hybridization assays were performed in tissue culture and for each animal four locations of lung tissue were investigated and 20 randomly selected neighbouring, non-overlapping fields were measured. The evaluated parameter was TGEV-RNA- or GUS-RNA-positive area (μm^2), expressed as a percentage of the total area of cells or tissue examined.

Immunizations. Groups of 1-week-old swine (derived from crossing Belgium Landrace and Large White swine) were immunized by the oronasal and intragastric routes. Piglets were obtained from sows seronegative for TGEV and PRRSV, as determined by radioimmunoassay (Sánchez *et al.*, 1999) and immunoperoxidase monolayer assay (Harlow & Lane, 1988). One-week-old animals were immunized three times at days 7, 14 and 21 after birth, by administering each time three doses: orally (5.0×10^8 p.f.u. per pig), intranasally (5.0×10^8 p.f.u. per pig) and intragastrically (1.5×10^9 p.f.u. per pig) of the helper virus (TGEV PUR46-MAD) and the minigenome M39-GUS or M39-ORF5. Piglets inoculated with the same minigenome were taken together and housed in isolation chambers located in a P3-level containment facility at 18–20 °C. Serum was collected 7 days after the last immunization.

Results

Structure of the helper-dependent expression system and expression levels

TGEV-derived expression systems have been developed based on two components, a helper virus (the attenuated strain PUR46-MAD of TGEV) (Sánchez *et al.*, 1999) and a minigenome of 3.9 kb (M39) derived from the helper virus (Izeta *et al.*, 1999). The minigenome was cloned under the control of the CMV promoter and was amplified first in the nucleus by cell polymerase II, coupled to a second amplification step of the translocated minigenome RNAs by the viral RNA-dependent RNA-polymerase within the cytoplasm. The GUS (minigenome M39-GUS) and the PRRSV ORF5 (minigenome M39-ORF5) genes were inserted at position 3337 from the minigenome 5' end, within an expression cassette composed of a TRS including the highly conserved core sequence 5' CUA AAC 3', and 10 nt with an optimized Kozak sequence (L3, 5' GUCGACGACC 3') (Alonso *et al.*, 2002) (Fig. 1A). Expression levels were analysed by Western blot (GUS) (Fig. 1B) or by immunoprecipitation (ORF5) (Fig. 1C), using as positive controls either purified GUS or the viral proteins expressed in PRRSV-infected cells. In the immunoprecipitation of ORF5, a second band with the molecular mass expected for an ORF5 homodimer was also observed. The same type of second band was previously observed by Western blot analysis of PRRSV proteins (Meulenber *et al.*, 1995).

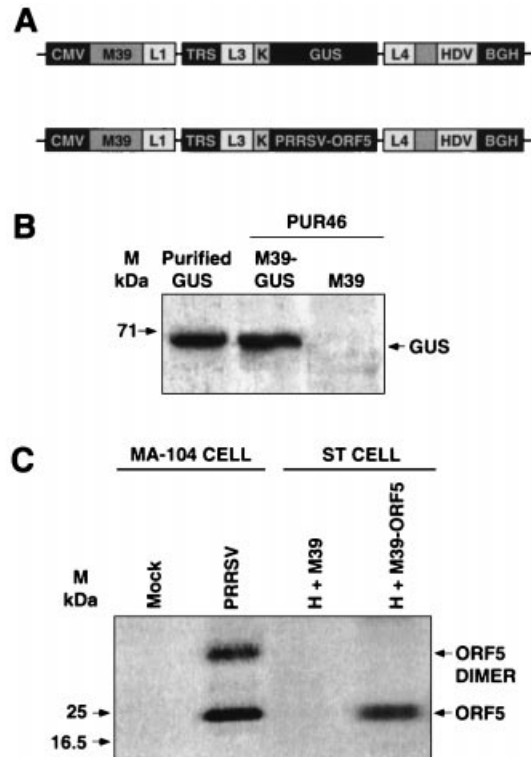


Fig. 1. Analysis of the protein expressed by TGEV-derived minigenomes. (A) Schematic structure of expression modules based on TGEV-derived minigenome M39 cloned under the control of the cytomegalovirus immediate early promoter (CMV). The expression cassette is flanked by L1 and L4 polylinkers. This cassette includes the transcription regulatory sequence (TRS), an insertion site (L3), an optimized Kozak sequence (K) and the GUS or the PRRSV ORF5 heterologous genes. HDV, Hepatitis delta virus ribozyme; BGH, bovine growth hormone termination and polyadenylation signals. (B) Western blot analysis of GUS expression using the TGEV-derived minigenome M39-GUS. Detection of the heterologous protein GUS (69 kDa) was performed under reducing conditions, using a GUS-specific polyclonal rabbit-antiserum. The molecular mass is indicated on the left. Purified GUS (Sigma) was used as the positive control. M39-GUS, cell extracts from ST cells infected with minigenome M39-GUS and helper virus (PUR46-MAD). M39, Cell extracts from ST cells infected with minigenome M39 and the helper virus. (C) Detection of PRRSV ORF5 expression using minigenome M39-ORF5 grown in the presence of [^{35}S]methionine/cysteine. Cell extracts were immunoprecipitated using rabbit antiserum specific for an ORF5 peptide (see Methods). The immunoprecipitated proteins were analysed by SDS-PAGE and autoradiography. Two bands were observed, one with the expected size for PRRSV ORF5 (25 kDa) and the other that corresponds to a dimer of this protein. Both bands are indicated by an arrow on the right side of the panel. The position of molecular mass markers (M) is indicated on the left. Mock, uninfected MA-104 cells; PRRSV, MA-104 cells infected with PRRSV; H + M39, epithelial ST cells infected with the minigenome M39 and the helper virus; H + M39-ORF5, ST cells infected with the minigenome M39-ORF5 and the helper virus; H, helper virus (PUR46-MAD).

GUS expression levels in different assays were between 2 and 8 μg per 10^6 cells. The amount of protein expressed was determined at 22–24 h p.i. using standard calibration curves generated with purified GUS (Sigma) and the dicinchoninic acid protein assay (BCA, Pierce), resulting in 10^6 relative luminometric units per 0.35 ng of GUS. The results were

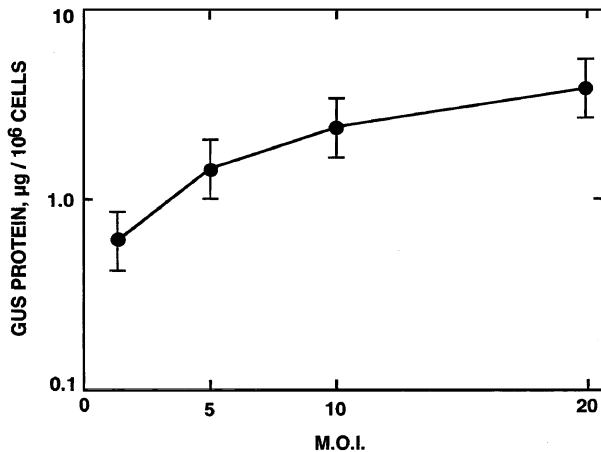


Fig. 2. Relationship between m.o.i. of the helper virus and expression levels of GUS. Confluent ST cell monolayers were inoculated at the indicated m.o.i. with supernatant from passage four containing the helper virus (TGEV PUR46-MAD) and the minigenome encoding the GUS gene (M39-GUS). Cell extracts were used to determine the amount of GUS protein per 10^6 cells expressed by the minigenome encoding the GUS gene. The data shown represent the average of at least three experiments with the standard error.

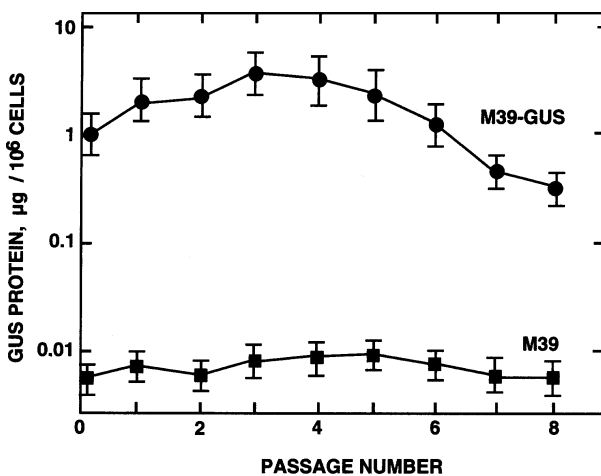


Fig. 3. Expression of GUS gene by TGEV-derived minigenomes upon passage in cell culture. GUS expression per 10^6 cells generated by minigenome M39 encoding the GUS gene under the control of an optimized TRS (M39-GUS) was followed throughout eight passages after transfection by using a luminometric assay based on the GUS enzymatic activity. Background levels are those corresponding to the minigenome M39 without insert. The data show an average of at least three experiments with similar results. Bars, standard error.

coincident with those obtained by comparing the protein band intensity obtained by Western blot analysis developed using a polyclonal rabbit antibody (5 Prime-3 Prime) and known amounts of purified GUS protein. An internal standard for ORF5 was not available but, based on band intensity, ORF5 protein expression levels were estimated between 1 and 2 μg per 10^6 cells. GUS expression levels increased more than eightfold by increasing the m.o.i. from 1 to 20 (Fig. 2), as could

be expected for the amplification of a minigenome that is dependent on helper virus replication.

Stability of TGEV-derived minigenomes encoding heterologous genes

GUS expression was studied along different passages of the helper virus with the minigenome M39-GUS by analysing GUS enzymatic activity. Expression was optimum for about five passages and then slowly declined, being about 60-fold over background at passage eight (Fig. 3). The stability of minigenome M39-GUS RNA and of the mRNA encoding GUS was evaluated at passages two and five by Northern blot using two probes, one complementary to the 3' end of the genome (Fig. 4A) and another to GUS (Fig. 4B). The 3' end probe clearly detected all viral mRNA (S, 3a, E, M, N and 7), but the probe complementary to the GUS gene was more efficient at visualizing the minigenome including the GUS gene. Interestingly, at passage two, a clean single band corresponding to minigenome M39-GUS was observed with the GUS probe, while at passage five, additional bands of smaller size appeared, probably due to the generation of deleted minigenomes. The subgenomic mRNAs (sgmRNAs) were also identified at passages two and five (Fig. 4B). Similar results were obtained by expressing GUS using a minigenome of 5.4 kb (M54) (data not shown). The heterologous GUS gene was responsible for the instability of the minigenomes, since TGEV minigenomes of 3.9 and 5.4 kb remained stable for at least 30 passages, without generating new dominant RNAs, in the absence of the heterologous gene (Izeta *et al.*, 1999) (data not shown). Increase in minigenome length from 3.9 to 5.4 kb did not improve expression levels (data not shown).

Instability in the synthesis of the minigenomes encoding the ORF5 gene was also observed (Fig. 5). Northern blot analysis indicated that at passages four and six, in addition to a minigenome with the expected size for M39-ORF5, other bands of smaller size were detected with the 3' end probe (Fig. 5A) and more clearly with the probe complementary to ORF5 (Fig. 5B). One of these RNA bands ($\Delta\text{M39-ORF5}$) was more abundant than the full-length minigenome. Interestingly, bands with the size expected for the subgenomic mRNA encoding ORF5 (sgmRNA) and for the deleted minigenome (ΔsgmRNA) were also detected. The identity of these bands was assessed by RNA extraction, amplification by RT-PCR and sequence analysis (data not shown). The relative abundance of the sgmRNAs was about 50-fold lower than that of the corresponding minigenomes.

Replication of helper virus and minigenome and expression of GUS in cell culture

The number of cells replicating the helper virus and expressing the sgmRNA encoding GUS was determined by ISH using non-radioactive labelled riboprobes complementary

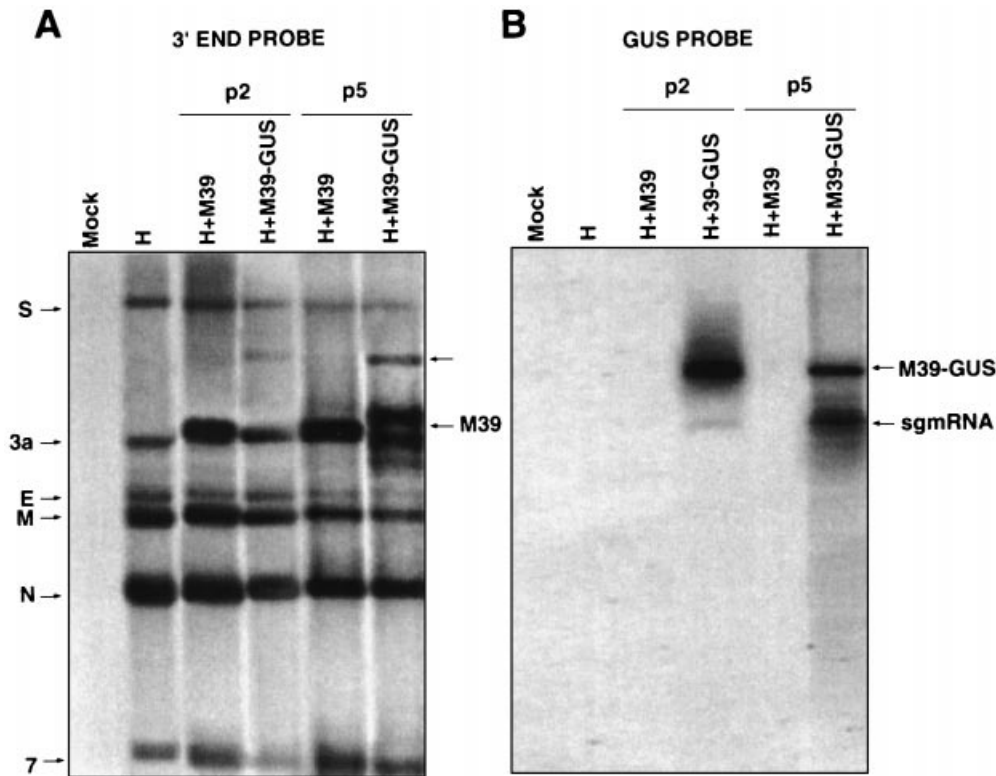


Fig. 4. Stability of TGEV-derived minigenomes encoding the GUS gene. Northern blot analysis of intracellular RNAs extracted at passages two (p2) and five (p5) from minigenome transfected and TGEV-infected cells. Hybridization was performed using probes complementary to the 3' UTR of the genome (A) and to the GUS gene (B). The positions of helper virus mRNAs (S, 3a, E, M, N, 7) are indicated on the left. The position of minigenome RNA encoding the GUS gene (M39-GUS) is indicated by an arrow on the right side of the figure. The M39 RNA overlaps with the helper virus mRNA 3a, and its position has been indicated by an arrow (M39). Mock, uninfected cells; H, helper virus (PUR46-MAD); M39, minigenome without the heterologous gene.

to the 3' end of the virus and to the GUS gene (Fig. 6). Whereas $47.1 \pm 19.2\%$ of ST cells contained TGEV-RNA (Fig. 6A), a smaller number of cells, i.e. $12.9 \pm 4.4\%$, showed hybridization signals specific for GUS minigenome RNA (Fig. 6B). The expression of viral and heterologous antigen proteins was studied by immunofluorescence and by histochemical detection using the substrate X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronid), which resulted in a Prussian blue staining of cells with GUS enzymatic activity. At 18 h p.i. TGEV antigen was demonstrated in large amounts in the cytoplasm of about 40% of ST cells (Fig. 6C), while GUS activity was detected in about 12% of the cells (Fig. 6D). The number and distribution of positive cells by ISH was very similar to results obtained by immunofluorescence and histochemistry. These results indicated that more than one-quarter of the infected cells expressed a significant amount of the GUS gene encoded within the minigenome.

In vivo replication of TGEV and expression of GUS

The helper virus carrying the minigenome replicated in both the gut and the lungs of infected piglets (Fig. 7). At different days post-infection, virus titres were 40- to 10^3 -fold

higher in the lungs than in the gut. At day 1 post-inoculation, titres in the lung and the gut were 4×10^7 and 2×10^5 p.f.u./g of tissue, respectively, and then declined, being around 10^3 p.f.u./g of tissue in both cases at day 4 p.i. (Fig. 7A, B). Immunofluorescence for TGEV correlated well with infectious virus isolation in the lungs and in the gut (Fig. 8). It was not possible to detect GUS protein in other than lung tissues of infected animals by immunofluorescence or by using the GUS-Light assay, even in the presence of 2×10^5 p.f.u. of helper virus/g of tissue, suggesting that the helper virus and the minigenome were not present within the same cell.

TGEV RNA detected by ISH was higher in tissue culture cells than *in vivo*. TGEV RNA and protein (determined by immunofluorescence) were preferentially present in bronchiolar epithelial cells and pneumocytes starting at day 1 p.i. (Fig. 8A, C) and decreased thereafter (data not shown). Lung tissue area hybridization signals specific for TGEV RNA were detectable in $4.3 \pm 2.1\%$ of the cells (Fig. 8A), whereas an overall larger number of pneumocytes ($> 20\%$) displayed TGEV antigen-specific fluorescence (Fig. 8C). The presence of a lower number of positive cells for viral RNA than for viral protein could be due to the shorter half-life of TGEV RNA in relation to that of the protein. GUS RNA was displayed in

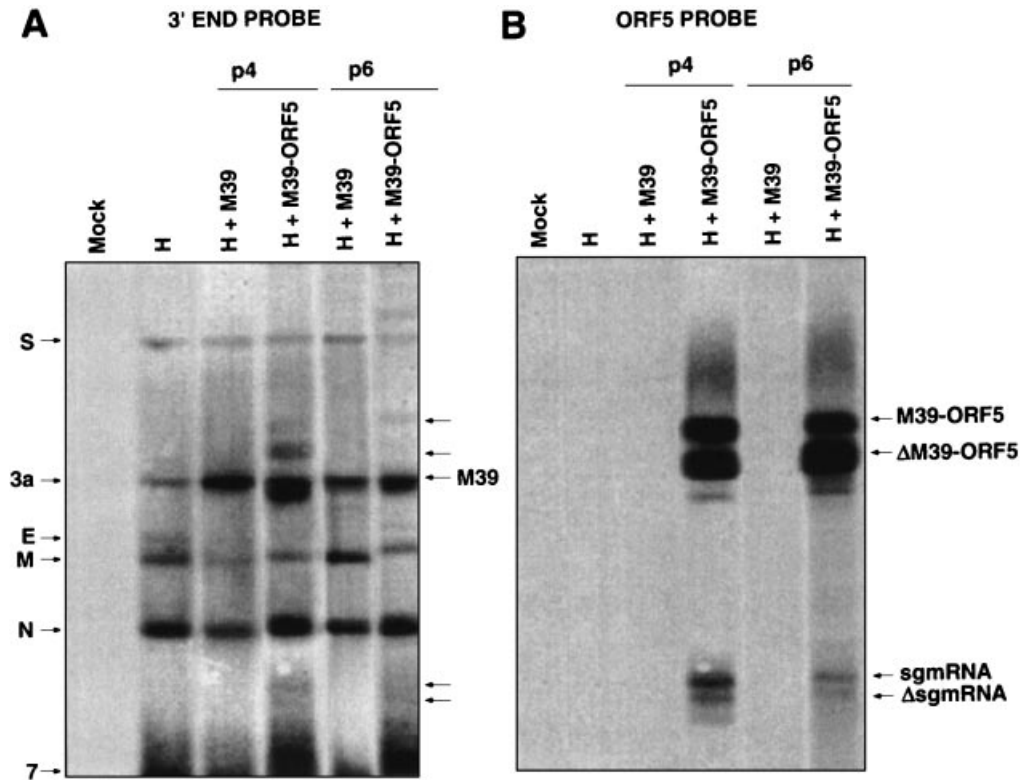


Fig. 5. Stability of TGEV-derived minigenomes encoding the PRRSV ORF5. Northern blot analysis of intracellular RNAs extracted at passages four (p4) and six (p6) from minigenome transfected and TGEV-infected ST cells. Hybridization was performed using probes complementary to the 3' UTR of the genome (A) and to the ORF5 (B). The positions of mRNAs (S, 3a, E, M, N, 7) from the helper virus are indicated on the left. The position of minigenome RNAs encoding full-length (M39-ORF5) or partially deleted (Δ M39-ORF5) PRRSV ORF5 and the sgmRNAs synthesized from them (sgmRNA and Δ sgmRNA, respectively) are indicated by arrows on the right side of the figure. The M39 RNA overlaps with the helper virus mRNA 3a. Mock, uninfected cells; H, helper virus (PUR46-MAD); M39, minigenome without the heterologous gene; its position has been indicated by an arrow to the right of (A).

$1.5 \pm 0.9\%$ of pulmonary tissue (Fig. 8B a, b and c). This relatively small number of GUS RNA-positive cells was consistent with findings obtained by histochemical staining (Fig. 8D a, b and c). More than one-quarter of the cells expressing the helper virus RNA in the lungs also expressed minigenome RNA. This fraction of GUS-positive cells in the lungs is similar to the fraction of ST cells expressing GUS in cell cultures as reported above.

Pathology and histopathology in swine administered with the helper virus and the minigenomes expressing GUS

The replication of TGEV in swine is highest in colostrum-deprived newborn piglets. This is particularly the case with attenuated viruses like the TGEV PUR46-MAD strain used in these studies. In contrast, 2-day-old conventional (non-colostrum deprived) piglets breast-fed by sows seronegative for TGEV, or for porcine respiratory coronavirus, inoculated with the attenuated strain PUR46-MAD show very mild or no obvious diarrhoea and are completely recovered by day 8 p.i. (Sánchez *et al.*, 1999).

To potentiate minigenome replication by the helper virus, colostrum-deprived newborn piglets were infected 2 days after birth. At necropsy 2 days after infection, piglets had the lungs slightly to moderately collapsed and showed consolidation and signs of bronchopneumonia especially in the cranioventral lobes. By histopathology, the lungs showed multifocal neonatal atelectasia, massive accumulation of granulocytes in the alveoli, thickened alveolar septa with slight hyperplasia of type II pneumocytes, and focal necrosis of the alveolar epithelium. Only a slight hyperaemia of small intestine vessels was occasionally seen. In the jejunum there was a slight to marked diffuse atrophy of villi together with fusion of shortened villi. Exfoliation and necrosis of surface epithelial cells, flattening of enterocytes and capillary thrombi at the tips of villi were also observed. Submucosal areas were infiltrated with granulocytes, lymphocytes and plasma cells (data not shown).

Immune response to GUS and PRRSV ORF5 elicited by the helper-dependent expression system in swine

Groups of 1-week-old conventional swine were immunized at days 7, 14 and 21 after birth with the helper virus plus the

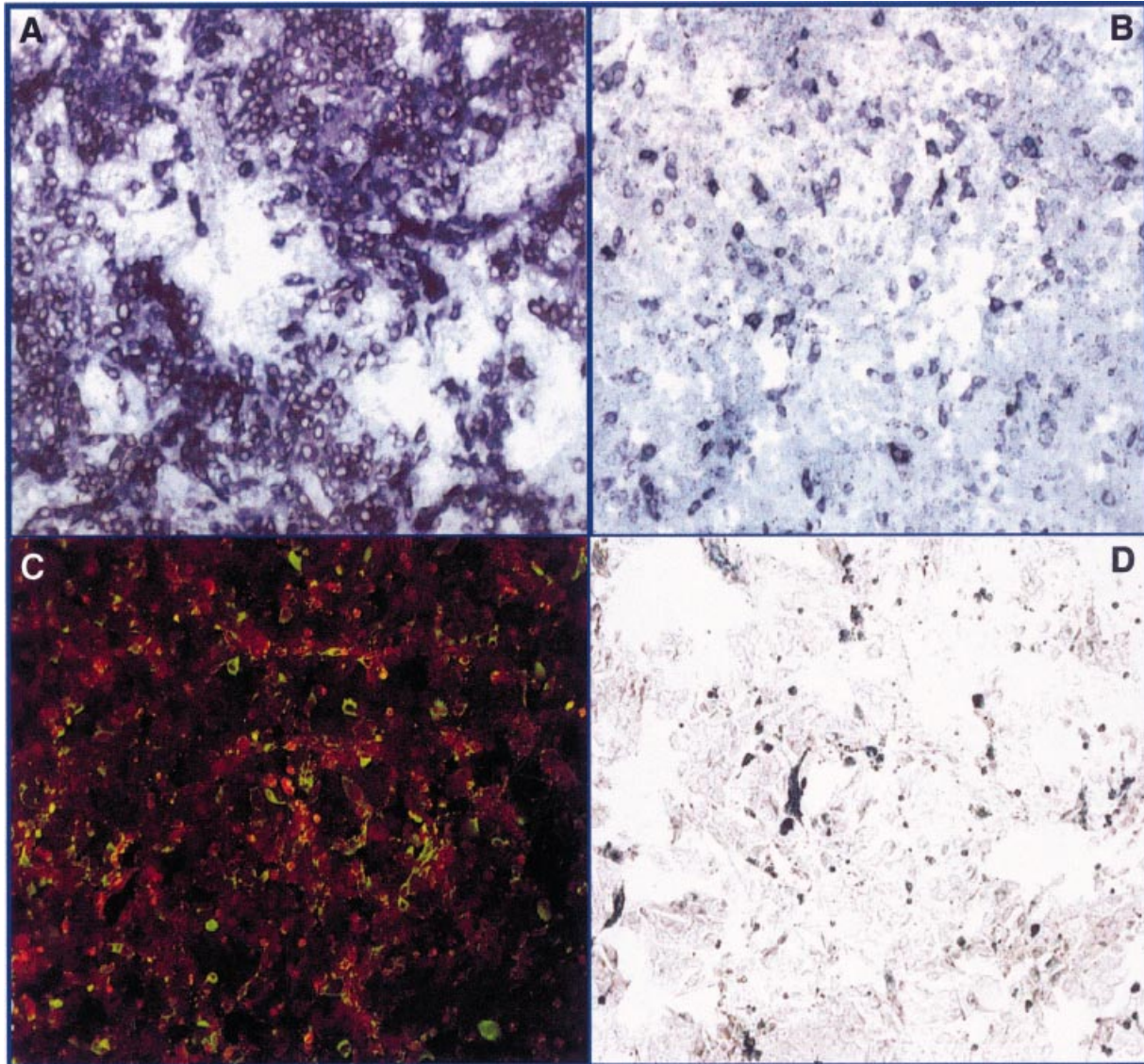


Fig. 6. Analysis of GUS gene transcription and expression from TGEV-derived minigenomes in ST cell monolayers. Confluent monolayers of ST cells were inoculated with supernatant containing the helper virus PUR46-MAD (m.o.i. 5) and the minigenome encoding the GUS gene (M39-GUS), and fixed 18 h p.i. with 4% paraformaldehyde. (A) ISH with a digoxigenin-labelled probe specific for the 3' end of TGEV RNA. (B) ISH with a probe specific for GUS RNA. (C) Direct immunofluorescence using FITC-labelled TGEV hyperimmune serum. (D) GUS enzymatic activity detected by histochemistry resulting in a Prussian blue staining of GUS-expressing cells.

minigenome expressing GUS or ORF5 by the intranasal, oral and intragastric routes. Animals were bled 7 days after the last immunization and the sera were evaluated by ELISA for the presence of antibodies to GUS or to a partially purified PRRSV, depending on the antigen used.

Immunization with the TGEV-derived vector expressing either GUS or PRRSV ORF5 elicited a significant immune response both to the helper virus and to GUS (Fig. 9A 1 and 2) or to the helper virus and ORF5 (Fig. 9B 1, 2 and 3). The antibody levels to the helper virus (TGEV) were higher than to the heterologous gene in both cases. The response to GUS and

the PRRSV ORF5 was also studied by immunizing 4-week-old newborn swine and was weaker than in 1-week-old animals (results not shown).

The immune response to ORF5 was confirmed by studying the response to a synthetic 18 amino acid peptide (NH₂-142-TNFIVDDRGRGRIHRWKSPI-159-COOH) derived from the ORF5 of the PRRSV Olot91 strain (Plana-Durán *et al.*, 1992). This peptide was selected because it was previously shown that the homologous peptide (NH₂-143-CNFIVDDRGRVHRWKSPI-160-COOH) of the PRRSV Lelystad strain was immunogenic (Meulenberg *et al.*, 1995). To evaluate the

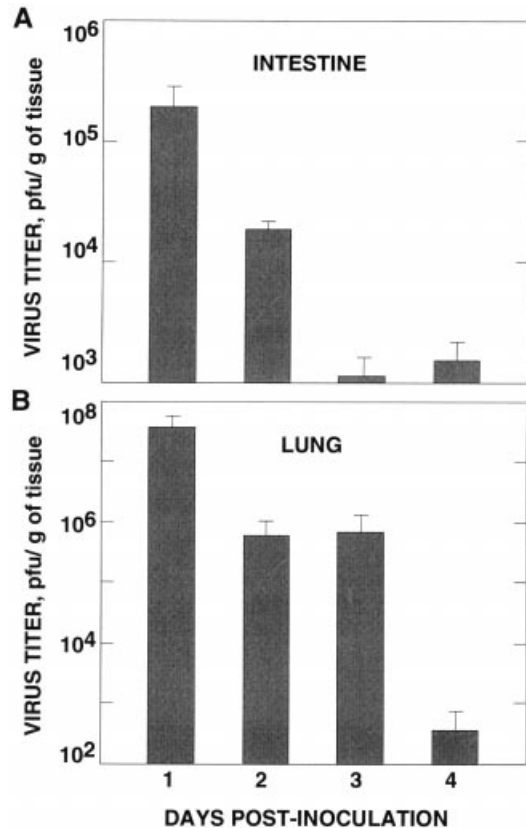


Fig. 7. Replication of the helper virus in the intestine and lung of swine. Growth kinetics of the helper virus isolated from the jejunum (A) and lungs (B) of 2-day-old colostrum-deprived piglets oronasally and intragastrically infected with a mixture containing the minigenome (M39-GUS) and 10^6 p.f.u. of the helper virus (PUR46-MAD). Animals were sacrificed on days 1, 2, 3 and 4 p.i. and subjected to necropsy. Columns indicate the mean of virus titre obtained from six piglets inoculated with the helper virus–minigenome mixture. Bars, standard error.

immune response to PRRSV, the 18-mer peptide was conjugated to KLH. A strong antibody response was detected against the peptide in the absence of a response to the carrier KLH. Interestingly, the swine antibodies raised against ORF5 neutralized the infection of MA-104 cells by 10^2 p.f.u. of PRRSV, in contrast to sera collected from the same animals before the immunization, which did not protect the cell monolayer.

Discussion

Using a TGEV-derived expression system the production of high levels of GUS and ORF5, involved in the protection against PRRSV, has been achieved in tissue culture and in swine. GUS expression lasted more than eight passages. It has been shown that the minigenome was amplified for at least five passages in cell culture and that, after passage four, deletion mutants of the minigenome were generated. In addition, the expression of GUS at cellular level in tissue culture and *in vivo* has been reported. Using the TGEV-derived virus vector, the

induction of immune responses to GUS and the PRRSV ORF5 has been demonstrated in swine.

The helper-dependent expression system showed a limited stability of the minigenome RNA during passage in cell culture. Insertion of the PRRSV ORF5 and GUS genes in the M39 minigenome led to the appearance of new minigenomes that could easily be detected at passages four and five, respectively. In contrast, the sgRNAs were detected in low amounts with the probes used in these experiments. Increase of the probe size from 300 nt to around 10^3 nt clearly revealed the presence of the sgRNAs (Alonso *et al.*, 2002). In both cases, protein expression levels were maintained for at least eight passages. The stability of the TGEV-derived minigenomes was higher than that of the MHV-based helper dependent expression system in which the expression of the foreign gene is lost within the first three passages, probably because of the lack of a packaging signal within the MHV minigenomes (Lai & Cavanagh, 1997; Liao *et al.*, 1995; Lin & Lai, 1993; Zhang *et al.*, 1997).

The stability of the expression systems is also conditioned by the type of polymerases involved in minigenome amplification and mRNA transcription (Agapov *et al.*, 1998). The expression system described in this report, based on TGEV-derived minigenomes expressed under the control of the CMV promoter, uses the eukaryotic RNA polymerase II to express the minigenome, a process that takes place with an estimated error frequency of 5×10^{-6} (de Mercoyrol *et al.*, 1992), which is lower than the mutant accumulation frequency of 10^{-4} to 10^{-5} during the *in vitro* expression of minigenome RNAs with T7 DNA-dependent RNA-polymerase (Boyer *et al.*, 1992; Sooknanan *et al.*, 1994). In addition, the eukaryotic RNA polymerase II has additional mechanisms to ensure even more accurate transcription (Thomas *et al.*, 1998). After transfection of the *in vitro*-produced RNA, synthesis of mRNA by the viral RNA-dependent RNA-polymerase should have an accumulation of mutations with a relatively higher frequency of 10^{-3} to 10^{-4} (de Mercoyrol *et al.*, 1992; Ward *et al.*, 1988). Overall, an improvement in expression stability should be observed by using expression systems initiated by DNA transfection, such as those described in this report.

Using minigenomes derived from TGEV, expression was highly dependent on the nature of the heterologous gene used. Luciferase expression with TGEV minigenomes was reduced to background levels and was lost after the fourth passage (data not shown). In contrast, the expression of GUS was higher (up to $8 \mu\text{g}$ per 10^6 cells) and was observed for at least eight passages in this and in a previous work (Alonso *et al.*, 2002). Similar results were observed using IBV minigenomes (Stirrups *et al.*, 2000).

The theoretical size of the insert accepted by the TGEV-derived minigenomes is about 24 kb, since the size of the full-length TGEV genome is 28.5 kb and the M39 has around 4 kb. This cloning capacity would be the highest among virus vectors with an RNA genome.

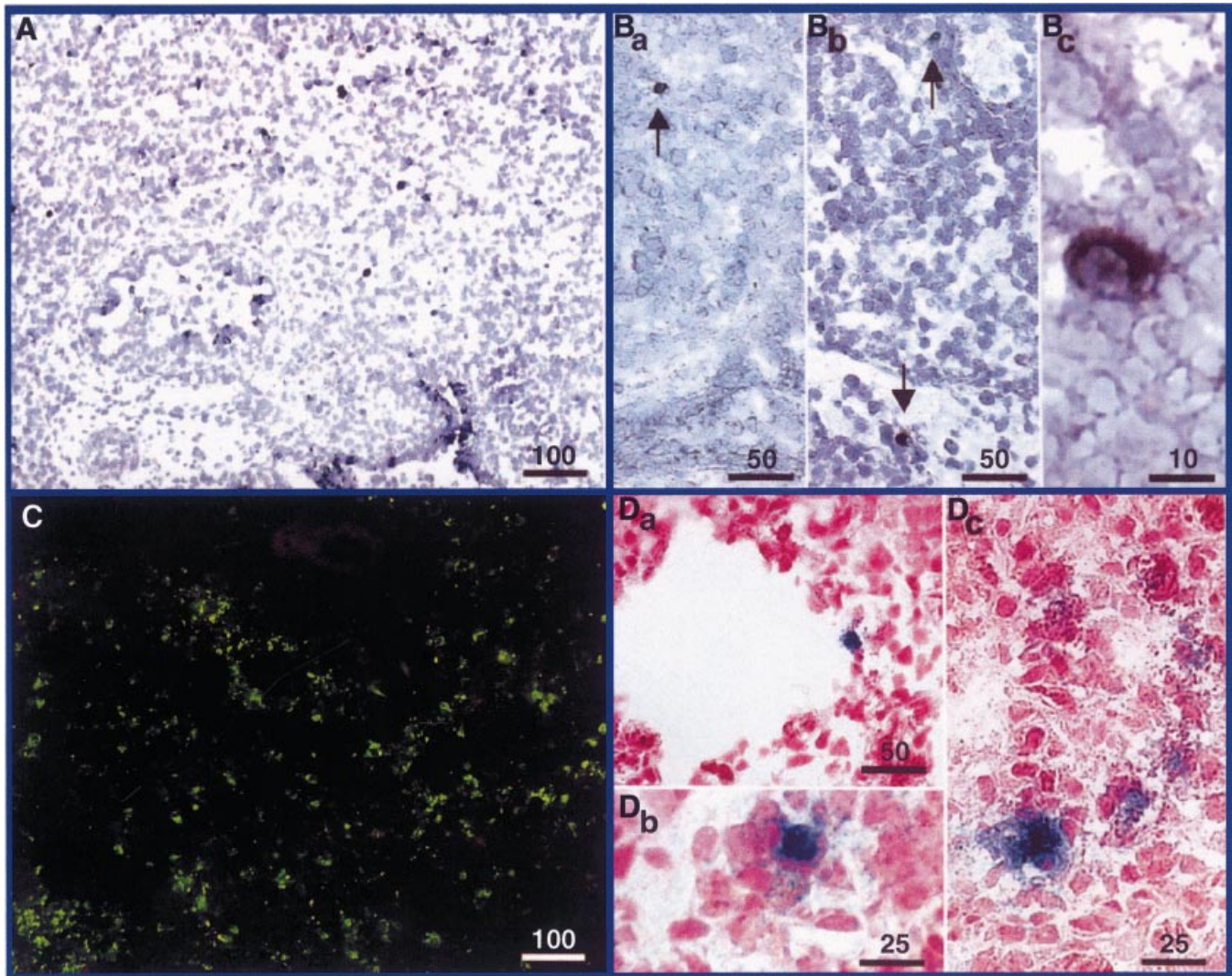
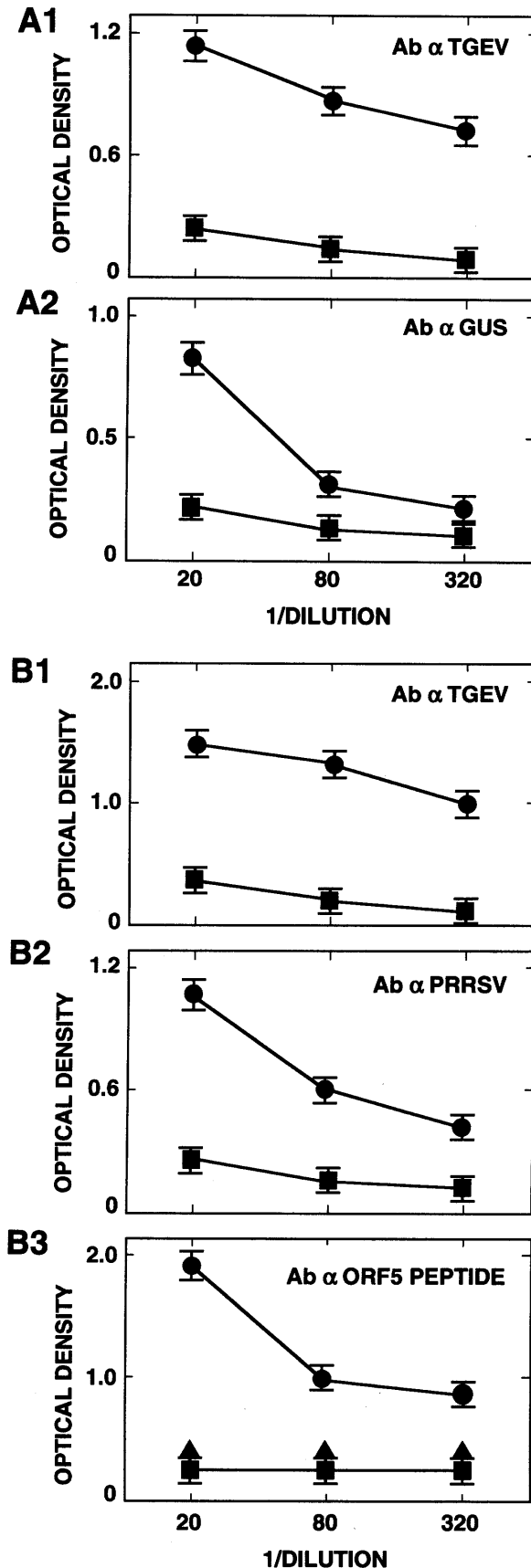


Fig. 8. Expression of the GUS gene from TGEV-derived minigenomes in swine lungs. Cryostat sections of lung tissue from 2-day-old piglets inoculated with the minigenome (M39-GUS) and 10^8 p.f.u. of helper virus (PUR46-MAD) by the oronasal route, necropsied 2 days p.i. (A) ISH in bronchiolar epithelium and scattered pneumocytes with a digoxigenin-labelled probe specific for TGEV RNA. (B) ISH, with a probe specific for GUS RNA. Pulmonary cells showing hybridization signals are indicated by arrows. (C) Immunofluorescence, using an FITC-labelled TGEV-specific antiserum. (D) GUS histochemistry, singular cells show enzymatic reactivity and Prussian blue staining. Bars indicate size (μm).

By studying the expression of GUS reporter gene in ST cells infected with an m.o.i. of 5, it was observed that when 47% of the cells expressed virus vector RNA or protein, more than one-quarter of them expressed the GUS RNA or protein. The presence of cells negative for TGEV RNA was unexpected, since the m.o.i. was 5. It is, in principle, possible that TGEV replication is dependent on the cell cycle, since cells were not synchronized.

Using TGEV-derived minigenomes, GUS was expressed in lungs but not in the enteric tract, probably because the titres of the helper virus in lungs were 40- to 10^3 -fold higher than in the gut, and in the gut the minigenome and the helper virus were not present within the same cell. The helper virus and GUS

RNAs were detected in more than 4.3% and 1.5% of the cells, respectively. Interestingly, this reduced number of cells expressing GUS (or even a lower one, since the immunized piglets were conventional animals, i.e. non-colostrum-deprived and, consequently, less susceptible to the virus than the colostrum-deprived ones in which we determined the number of infected cells) was sufficient to elicit an immune response to GUS. This immune response was stronger against the helper virus (TGEV) than against the heterologous antigens, particularly GUS, probably because the virus is a polymeric antigen that is a better immunogen than the GUS. A similar situation probably results after expression of the PRRSV ORF5. The relatively strong immune response to the ORF5 is



very promising, since this antigen is one of the major inducers of protection against PRRSV (Pirzadeh & Dea, 1998; Planadurán *et al.*, 1997a, b). Interestingly, the PRRSV ORF5 18-mer peptide selected was highly immunogenic as described for the homologous peptide from the Lelystad strain of PRRSV (Meulenberg *et al.*, 1995).

In order to study the replication of the helper virus with the minigenome, colostrum-deprived newborn animals were used to potentiate the infection by the attenuated helper virus. This resulted in an increased pathogenicity both in the lungs and in the enteric tract. These side effects should be reduced to a minimum in conventional (non-colostrum-deprived) piglets since, even when they are infected at 2 days after birth, very mild or no diarrhoea was induced after infection with the PUR46-MAD strain of TGEV (Sánchez *et al.*, 1999). These side effects are even less when older animals are infected with attenuated TGEV, such as the piglets used for the immunization that were 1-week-old. In this case, we have shown an efficient immune response to both GUS and PRRSV ORF5 in the absence of clinical symptoms. The potential side effects caused by the helper virus could be further prevented by using the PTV strain of the same Purdue cluster of TGEV (Sánchez *et al.*, 1992), since this strain is respiratory and fully attenuated (Sánchez *et al.*, 1999) and has been shown to efficiently rescue TGEV-derived minigenomes (J. M. Sánchez-Morgado, I. Sola, J. Castilla & L. Enjuanes, unpublished results).

Overall, these results showed that foreign genes were efficiently expressed in tissue culture by using TGEV-derived minigenomes. Furthermore, the expression was specifically targeted to tissues such as lung in swine, leading to the induction of an immune response against an antigen such as the PRRSV ORF5 involved in the protection against relevant virus infections of livestock.

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Fig. 9. Antibody immune response against GUS or PRRSV elicited in swine by TGEV-derived minigenomes. Swine were immunized using minigenomes with the information for the GUS gene (A1 and A2) or for the PRRSV ORF5 (B1, B2 and B3) and the helper virus (PUR46-MAD). Antibody responses against TGEV (A1 and B1), GUS protein (A2), partially purified PRRSV (B2) or a synthetic peptide from the ORF5 (see Methods) conjugated to KLH (B3) were evaluated by ELISA in the serum of immunized lactating piglets. The absorbance obtained with the serum after the third inoculation (●) or the preimmune serum (■) is shown. Binding of serum from immune animals to KLH is also shown (▲). Results show medium values from three experiments. Bars, standard deviation.

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