25

THE SPIKE PROTEIN OF TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS CONTROLS THE TROPISM OF PSEUDORECOMBINANT VIRIONS ENGINEERED USING SYNTHETIC MINIGENOMES

A. Izeta,[1] C. M. Sanchez,[1] C. Smerdou,[1] A. Mendez,[1] S. Alonso,[1]
M. Balasch,[2] J. Plana-Durán,[2] and L. Enjuanes[1]

[1]Department of Molecular and Cell Biology
Centro Nacional de Biotecnología, CSIC
Campus Universidad Autónoma, Canto Blanco
28049 Madrid, Spain
[2]Fort Dodge Veterinaria
Vall de Bianya
17813 Girona, Spain

1. ABSTRACT

The minimum sequence required for the replication and packaging of transmissible gastroenteritis virus (TGEV)-derived minigenomes has been determined. To this end, cDNAs encoding defective RNAs have been cloned and used to express heterologous spike proteins, to determine the influence of the peplomer protein in the control of TGEV tropism.

A TGEV defective interfering RNA of 9.7 kb (DI-C) was isolated, and a cDNA complementary to DI-C RNA was cloned under the control of T7 promoter. *In vitro* transcribed DI-C RNA was replicated *in trans* upon transfection of helper virus-infected cells. A collection of DI-C deletion mutants (TGEV minigenomes) was generated and tested for their ability to be replicated and packaged. The size of the smallest minigenome replicated *in trans* was 3.3 kb. The rescue system was used to express the spike protein of an enteric TGEV isolate (C11) using as helper virus a TGEV strain (C8) that replicates very little in the gut. A mixture of two pseudorecombinant viruses containing either the helper virus genome or the minigenome was obtained. These pseudorecombinants display in the sur-

Coronaviruses and Arteriviruses, edited by Enjuanes *et al.*
Plenum Press, New York, 1998

face the S proteins from the enteric and the attenuated virus, and showed 10^4-fold increase in their gut replication levels as compared to the helper isolate (C8). In addition, the pseudorecombinant virus increased its enteric pathogenicity as compared to the C8 isolate.

2. INTRODUCTION

TGEV is a member of the *Coronaviridae* family (Lai and Cavanagh, 1997; Enjuanes and Van der Zeijst, 1995) with a plus-stranded, polyadenylated RNA genome of 28.5 kb (Eleouet *et al.*, 1995). Defective RNAs, i.e. deletion mutants maintaining the *cis*-signals required for replication and packaging but dependent on other viral functions that can be supplied *in trans* by a helper virus, may arise in coronavirus replication cycle. To study the molecular basis of coronavirus replication, evolution and tropism, it would be convenient to have a full-length infectious cDNA clone. Since this is not available, an alternative approach is the cloning of subgenomic-sized defective RNAs to manipulate the coronavirus genome. Recently, cDNAs encoding small coronavirus defective RNAs have been successfully used to express heterologous genes (Zhang *et al.*, 1997; Liao *et al.*, 1995; Liao and Lai, 1994; Lin and Lai, 1993).

In our laboratory, three TGEV defective interfering RNAs of 22, 10.6 and 9.7 kb (DI-A, DI-B and DI-C, respectively) were isolated (Mendez *et al.*, 1996). This chapter reports the construction of a full-length cDNA clone from the smallest TGEV DI RNA, DI-C, which is replicated and packaged upon transfection of helper virus-infected cells. The construction and rescue of a collection of TGEV minigenomes has provided a TGEV-derived expression system, in which several heterologous genes have been expressed.

In order to identify the molecular bases of TGEV enteropathogenicity, the S gene of the virulent strain C11 of PUR46 virus, which grew to high levels in the gut, was cloned into a minigenome and it was rescued (i.e. replicated and packaged *in trans*) using an attenuated strain, clone C8 of PUR46, which grew poorly in the enteric tract. Pseudorecombinant viruses derived from the attenuated C8 clone, carrying the S protein from the enteric strain C11, increased 10^4-fold their gut replication levels, confirming data from our laboratory (Ballesteros *et al.*, 1997; Sanchez *et al.*, 1992) that demonstrated that the tropism of TGEV depends on the origin of the S protein. To our knowledge, this constitutes the first report of a tropism change being engineered in a coronavirus.

3. MATERIALS AND METHODS

3.1. Cells and Viruses

Viruses were grown in swine testis (ST) cells (McClurkin and Norman, 1966). TGEV PUR46MAD (Sanchez *et al.*, 1990) and PUR46 C8 and C11 strains (Sanchez *et al.*, 1997) have been described.

3.2. Construction of cDNAs Encoding RNA Minigenomes

(i) pDI-C. Four overlapping cDNA fragments of DI-C RNA obtained by RT-PCR amplification (Mendez *et al.*, 1996) were corrected for point mutations introduced by RT-PCR procedure and they were assembled into plasmid pSL1190 (Pharmacia), under the control of T7 promoter. A cDNA encoding HDV ribozyme and T7 terminator was cloned by PCR from

transcription vector 2,0, kindly provided by A. Ball, University of Alabama (Pattnaik *et al.*, 1992), and placed immediately downstream of the cDNA complementary to DI-C RNA plus a synthetic poly(A) tract. (ii) Plasmid pDI-C-derived deletion mutants. Sequence analysis of pDI-C was performed and several DI-C internal deletion mutants, i.e. M54, were made by deleting the sequences between two restriction endonuclease sites using standard procedures (Sambrook *et al.*, 1989). (iii) Plasmid M54-S_{C11}. Five RT-PCR overlapping fragments of strain C11 S gene were assembled into pGEM-T (Promega), and sequenced with an Applied Biosystems 373A automated DNA sequencer. The complete S gene, including its intergenic sequences, was then inserted into pDI-C deletion mutant M54 in ORF1b gene.

3.3. *In Vitro* Transcription

In vitro transcription of linearized DNA templates was performed with T7 RNA Polymerase (Promega), according to the manufacturer instructions.

3.4. Electroporation of Helper Virus-Infected ST Cells

ST cells were grown to confluence and infected with helper virus with a m.o.i. of 10 PFU/cell. At 4–6 h p.i., cells were trypsinized, collected and resuspended in ice-cold PBS. The cells were then electroporated with *in vitro* transcribed RNA in a Gene Pulser apparatus (BioRad). The electroporated cells were incubated at 37ºC for 12h. Supernatants of these cultures were used to infect fresh ST cell monolayers, and at least six passages were performed to amplify the RNA.

3.5. RNA Analysis by Northern Hybridization and Northern Blot

Cytoplasmic RNA was extracted from helper virus infected and RNA transfected ST cells at different passages, as described previously (Mendez *et al.*, 1996). Northern hybridization was performed as described elsewhere (Penzes *et al.*, 1996), using a leader-specific oligonucleotide complementary to nt 66 to 91 of TGEV genome. Northern Blot analysis was performed using a 3'-UTR specific DNA probe, complementary to nt 28299 to 28543, and following standard procedures (Sambrook *et al.*, 1989).

3.6. Virus Tropism Analysis

The *in vivo* growth of the pseudorecombinant virus was determined after oro-nasal and intragastric inoculation of two to three days-old NIH miniswine (Lunney *et al.*, 1986; Sachs *et al.*, 1976) with doses of 5×10^9 PFU. Piglets were sacrificed at days 1, 2, 3, and 4 post-inoculation, and the virus present in jejunum, ileum, and intestinal content was determined. Tissues were processed as previously described (Ballesteros *et al.*, 1997).

4. RESULTS

4.1. Replication and Packaging of Synthetic DI-C RNA in TGEV-Infected ST Cells

A cDNA complementary to DI-C RNA was cloned under the control of T7 promoter. Four overlapping fragments comprising the full length DI-C RNA where corrected to have

the consensus virus sequence, and assembled. DI-C cDNA was cloned immediately downstream of T7 promoter with only two extra bases of non viral origin (GG) between the promoter and DI-C cDNA (Figure 1A and 1B). The 3' end of the poly(A) tail (25A) was self-processed to give perfect 3' ends, by inserting after the poly(A) a cDNA encoding the hepatitis delta virus (HDV) ribozyme and a T7 transcription terminator (results not shown).

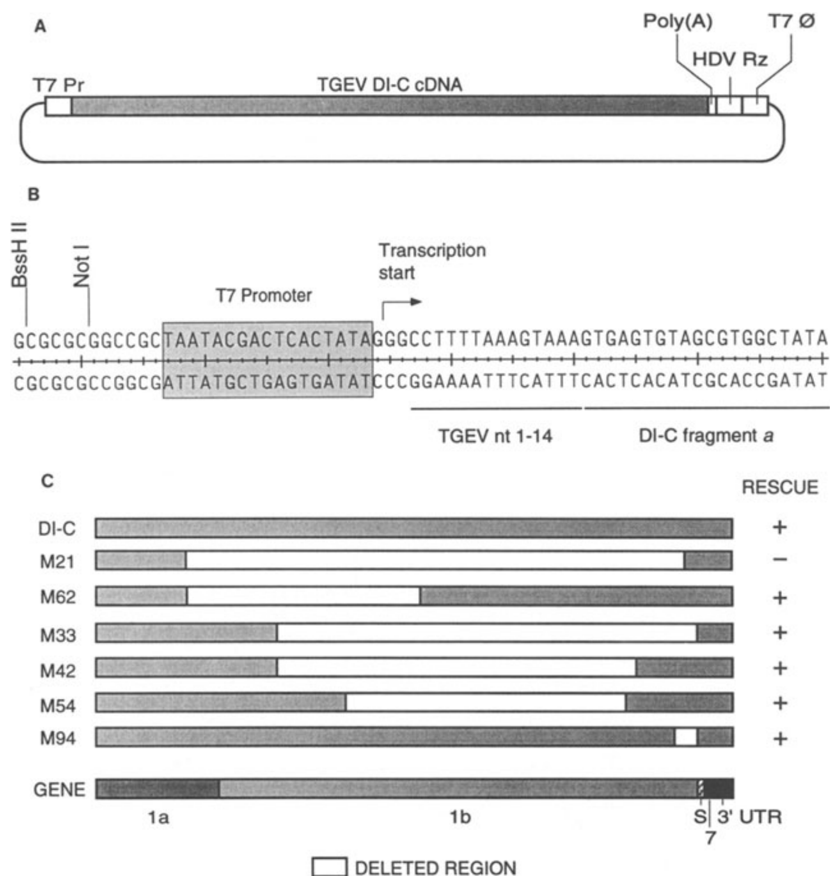


Figure 1. Rescue of TGEV minigenomes. (A) General structure of the plasmid-encoding DI-C RNA. A cDNA complementary to DI-C RNA was assembled downstream of T7 promoter. At DI-C 3' end, a synthetic poly(A) tract was added. HDV Rz, Hepatitis delta virus ribozyme; T7Ø, T7 transcription terminator. (B) Sequence of DI-C cDNA 5' end. Two restriction endonuclease sites, T7 Promoter and TGEV nucleotides 1–14 were placed upstream of DI-C fragment *a*, which started at TGEV nt 15 (Mendez *et al.*, 1996). TGEV nt 1–14 and DI-C fragment *a* 20-first nucleotides are underlined. (C) The structure of DI-C deletion mutants and their ability to be rescued by PUR46MAD helper virus is shown. Cytoplasmic RNAs were analyzed by Northern hybridization using a oligonucleotide complementary to nt 66 to 91 of TGEV genome. The presence of bands with the expected size for each of the minigenomes is indicated (+), while the absence of the band was interpreted as the absence of rescue by the helper virus (-). Grey boxes, sequence of the minigenomes. Empty boxes, deleted fragments.

TGEV PUR46MAD-infected ST cells were electroporated with *in vitro* transcribed RNA from linearized pDI-C. Supernatants of these cultures were passed six times onto fresh ST cell monolayers and total intracellular RNA was extracted. In Northern hybridization analysis, synthetic DI-C RNA was clearly detected and remained stable in RNA transfected cultures at least from passage 3 to 6, but not in the non-transfected ones (results not shown).

4.2. Effects of Internal Deletions on the Replication and Packaging of DI-C

A collection of TGEV minigenomes was generated and their ability to be replicated and packaged using PUR46MAD as helper virus was tested (Figure 1C). Minigenomes are named by a number that indicates their size in hundreds of nucleotides. All minigenomes but one were rescued, some of them to higher levels than the naturally selected DI-C RNA. The non-rescued RNA (M21) of 2.1 kb, was the smallest RNA generated. The smallest that was rescued (M33) had 3.3 kb. By comparing the structure of the non-rescued RNA with those of the rescued minigenomes, it was concluded that at least one of the two ORF1 fragments of 4.1 and 1.3 kb present in M62 and M33 but not in M21 was required for rescue, but none of these fragments was essential. Whether these fragments are required for replication or packaging is being investigated.

4.3. Expression of S_{C11} Heterologous Protein in M54 TGEV Minigenome

The rescue system was used to generate pseudorecombinant viruses expressing the spike protein from two viruses, with low and high replication level in the enteric tract (clones C8 and C11 of TGEV, respectively). Using clone C8 as helper virus, a recombinant M54 minigenome expressing the spike gene of C11 strain was rescued (Figure 2A). Northern blot analysis of cytoplasmic RNA of helper virus infected ST cells showed the standard set of viral mRNAs (Figure 2B, left panel). Analysis of the RNAs from ST cells infected with the helper virus and transfected with M54 RNA (with no heterologous insert) showed the standard RNA pattern plus an RNA with the size expected for the minigenome, which was rescued to high levels. Finally, when RNAs from cells infected with the helper virus and transfected with the M54-S_{C11} minigenome were analyzed, the mRNA with the expected size for the RNA encoding the C11 spike protein was observed. Interestingly, the C11 spike gene was transcribed to levels similar to those of the mRNA encoding the S protein of the helper virus.

4.4. Pseudorecombinant Virus Tropism

Three groups of 4 piglets were infected either with TGEV strains C8, C11, or with the pseudorecombinant virus (Figure 2B, right panel). One piglet of each group was sacrificed at different days post-inoculation, and the titer of the virus recovered from intestinal homogenates was determined. The pseudorecombinant virus grew up to titers of 10^6 PFU/g of tissue, in contrast to the C8 isolate, that grew to a limited extent (10^2 PFU/g of tissue). In addition, all the animals infected with the pseudorecombinant virus presented enteritis 2 days post-infection, a pathology not observed in the animals infected with strain C8. These results indicate that the pseudorecombinant virus, carrying the spike proteins from clones C8 and C11, increased 10.000-fold its replication level in the gut.

5. DISCUSSION

The rescue of synthetic TGEV derived minigenomes has been shown. The minimum size of the minigenomes that have been rescued is 3.3 kb. This minigenome requires for replication and packaging a 1.3 kb fragment, not present in a 2.1 kb minigenome that is not rescued with the same helper virus (M21). Alternatively it may require a 4.1 kb fragment present in M62 but not in M21. Both fragments are required for rescue, although none of these fragments is essential, since defective minigenomes with either one or the other fragment could be replicated and passaged at least six times in ST cells. Whether these fragments are required for replication or packaging is not known at present.

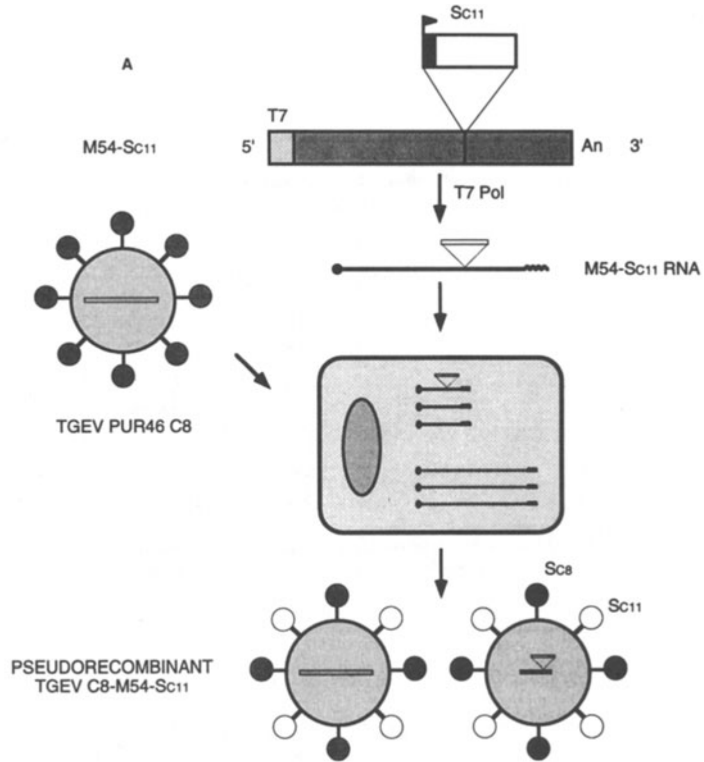
The synthetic minigenomes with the helper virus were useful to express a heterologous antigen in large amounts. In fact, pseudorecombinant viruses expressing the S protein from the helper virus plus the S protein of a virus clone that replicates to high levels in the enteric tract increased ten thousand-fold their replication in the enteric tissues. This result strongly suggests two important conclusions: (i) The S protein of the enteric virus provided *in trans* was sufficient to increase the enteric tropism of the helper virus, and (ii) the expression levels of the enteric virus spike protein were probably as high as those coming from the helper virus S protein expression, in order to form pseudorecombinants with a significantly increased enteric tropism. This conclusion is also supported by the high levels of the mRNA corresponding to the S protein of the enteric virus, encoded by the minigenome.

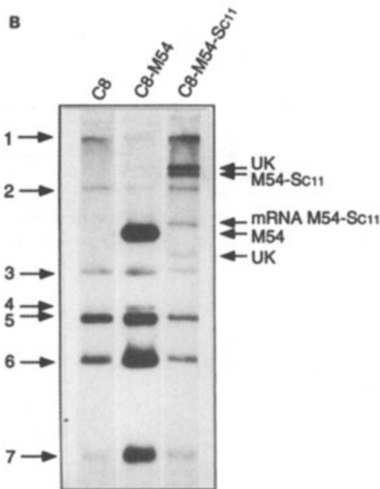
The possibility of modifying the proteins assembled into coronaviruses by engineering coronavirus derived minigenomes opens important avenues to study the role of the different structural proteins of this virus family.

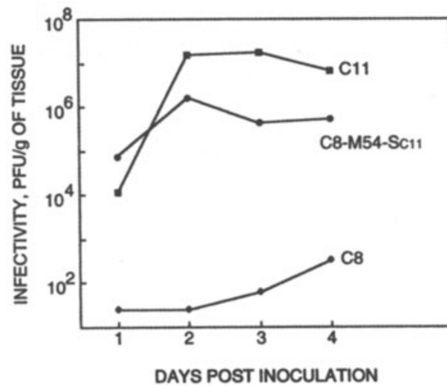
ACKNOWLEDGMENTS

This work has been supported by grants from the Consejo Superior de Investigaciones Científicas, the Comisión Interministerial de Ciencia y Tecnología (CICYT), La Consejería de Educación y Cultura de la Comunidad de Madrid, and Fort Dodge Veterinaria from Spain, and the European Communities (Projects Biotechnology and FAIR). AI and SA received fellowships from the Department of Education, University and Research of the Gobierno Vasco.

Figure 2. Generation of pseudorecombinant viruses with increased enteric tropism. (A). Scheme of pseudorecombinant virus generation. The role of S protein in tropism was studied by constructing pseudorecombinant viruses based on the helper virus C8, with a reduced enteric tropism, carrying the S protein from the highly enteric isolate C11. The S_{C11} gene was cloned into M54 minigenome. *In vitro* transcription with T7 RNA polymerase led to the synthesis of a minigenome RNA that was transfected into ST cells previously infected with helper virus C8. A mixture of pseudorecombinant viruses containing either the helper virus genome or the minigenome was obtained. Dark and empty peplomers represent C8 and C11 spike proteins, respectively. (B). Analysis of the pseudorecombinant virus RNA replication and infectivity in the enteric tract. Left panel: supernatants from ST cells infected with isolate C8 and transfected with different minigenome RNAs were amplified by virus passage on ST cell monolayers. Cytoplasmic RNAs were analyzed by Northern Blot using an anti-3'UTR ^{32}P-labeled probe. Numbers on the left indicate the viral mRNAs. Arrows on the right show the position of the transfected M54-S_{C11} and M54 RNAs, as well as the mRNA transcribed from M54-S_{C11}. UK, unknown RNAs. Right panel: three groups of 4 piglets each were infected with PUR46 strains C8, C11 and with the indicated pseudorecombinant virus. One piglet of each group was sacrificed at days 1, 2, 3, and 4 post-inoculation, and the titer of the virus recovered from intestinal homogenates (jejunum, ileum, and intestinal content) was determined.


A
Sc11
T7
M54-Sc11
5'
An
3'
T7 Pol
M54-Sc11 RNA
TGEV PUR46 C8
Sc8
Sc11
PSEUDORECOMBINANT
TGEV C8-M54-Sc11


B
C8
C8-M54
C8-M54-Sc11
1
2
3
4
5
6
7
UK
M54-Sc11
mRNA M54-Sc11
M54
UK


INFECTIVITY, PFU/g OF TISSUE
10^8
10^6
10^4
10^2
C11
C8-M54-Sc11
C8
1
2
3
4
DAYS POST INOCULATION

REFERENCES

Ballesteros, M. L., Sanchez, C. M., and Enjuanes, L., 1997, Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism, *Virology* **227:** 378–388.

Eleouet, J. F., Rasschaert, D., Lambert, P., Levy, L., Vende, P., and Laude, H., 1995, Complete sequence (20 kilobases) of the polyprotein-encoding gene 1 of transmissible gastroenteritis virus, *Virology* **206:** 817–822.

Enjuanes, L., and Van der Zeijst, B. A. M., 1995, Molecular basis of transmissible gastroenteritis coronavirus epidemiology, in: *The Coronaviridae* (S. G. Siddell, Ed.), Plenum Press, New York, pp. 337–376.

Lai, M. M. C., and Cavanagh, D., 1997, The molecular biology of coronaviruses, *Adv. Vir. Res.* **48:** 1–100.

Liao, C.-L., and Lai, M. M. C., 1994, Requirement of 5'-end genomic sequence as an upstream *cis*-acting element for coronavirus subgenomic mRNA transcription, *J. Virol.* **68**(8): 4727–4737.

Liao, C.-L., Zhang, X., and Lai, M. M. C., 1995, Coronavirus defective-interfering RNA as an expression vector: the generation of a pseudorecombinant mouse hepatitis virus expressing hemagglutinin-esterase, *Virology* **208:** 319–327.

Lin, Y. J., and Lai, M. M. C., 1993, Deletion mapping of a mouse hepatitis virus defective interfering RNA reveals the requirement of an internal and discontiguous sequence for replication, *J. Virol.* **67:** 6110–6118.

Lunney, J. K., Pescovitz, M. D., and Sachs, D. H., 1986, The swine major histocompatibility complex: its structure and function, in: *Swine in biomedical research* (M. E. Tumbleson, Ed.), Plenum Press, New York, pp. 1821–1836.

McClurkin, A. W., and Norman, J. O., 1966, Studies on transmissible gastroenteritis of swine. II. Selected characteristics of a cytopathogenic virus common to five isolates from transmissible gastroenteritis, *Can. J. Comp. Vet. Sci.* **30:** 190–198.

Mendez, A., Smerdou, C., Izeta, A., Gebauer, F., and Enjuanes, L., 1996, Molecular characterization of transmissible gastroenteritis coronavirus defective interfering genomes: Packaging and heterogeneity, *Virology* **217:** 495–507.

Pattnaik, A. K., Ball, L. A., LeGrone, A. W., and Wertz, G.W., 1992, Infectious defective interfering particles of VSV from transcripts of a cDNA clone, *Cell* **69:** 1011–1020.

Penzes, Z., Wroe, C., Brown, T. D. K., Britton, P., and Cavanagh, D., 1996, Replication and packaging of coronavirus infectious bronchitis virus defective RNAs lacking a long open reading frame, *J. Virol.* **70:** 8660–8668.

Sachs, D., Leight, G., Cone, J., Schwarz, S., Stuart, L., and Rosemberg, S., 1976, Transplantation in miniature swine. I. Fixation of the major histocompatibility complex, *Transplantation* **22:** 559–567.

Sambrook, J., Fritsch, E. F., and Maniatis, T., 1989, *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanchez, C. M., Jiménez, G., Laviada, M. D., Correa, I., Suñé, C., Bullido, M. J., Gebauer, F., Smerdou, C., Callebaut, P., Escribano, J. M., and Enjuanes, L., 1990, Antigenic homology among coronaviruses related to transmissible gastroenteritis virus, *Virology* **174:** 410–417.

Sanchez, C. M., Gebauer, F., Suñé, C., Mendez, A., Dopazo, J., and Enjuanes, L., 1992, Genetic evolution and tropism of transmissible gastroenteritis coronaviruses, *Virology* **190:** 92–105.

Sanchez, C. M., Ballesteros, M. L., and Enjuanes, L., 1997, Tropism, virulence and primary genome structure in a cluster of transmissible gastroenteritis coronavirus derived from the Purdue isolate, Submitted for publication.

Zhang, X., Hinton, D. R., Cua, D. J., Stohlman, S. A., and Lai, M. M. C., 1997, Expression of interferon-γ by a coronavirus defective-interfering RNA vector and its effect on viral replication, spread, and pathogenicity, *Virology* **233:** 327–338.