

# Two Amino Acid Changes at the N-Terminus of Transmissible Gastroenteritis Coronavirus

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To study the molecular basis of TGEV tropism, a collection of recombinants between the PUR46-MAD strain of transmissible gastroenteritis coronavirus (TGEV) infecting the enteric and respiratory tracts and the PTV strain, which only infects the respiratory tract, was generated. The recombinant isolation frequency was about  $10^{-9}$  recombinants per nucleotide and was 3.7-fold higher at the 5'-end of the S gene than in other areas of the genome. Thirty recombinants were plaque purified and characterized phenotypically and genetically. All recombinant viruses had a single crossover and had inherited the 5'- and 3'-halves of their genome from the enteric and respiratory parents, respectively. Recombinant viruses were classified into three groups, named 1 to 3, according to the location of the crossover. Group 1 recombinants had the crossover in the S gene, while in Groups 2 and 3 the crossovers were located in ORF1b and ORF1a, respectively. The tropism of the recombinants was studied. Recombinants of Group 1 had enteric and respiratory tropism, while Group 2 recombinants infected the respiratory, but not the enteric, tract. Viruses of both groups differed by two nucleotide changes at positions 214 and 655. Both changes may be in principle responsible for the loss of enteric tropism but only the change in nucleotide 655 was specifically found in the respiratory isolates and most likely this single nucleotide change, which leads to a substitution in amino acid 219 of the S protein, was responsible for the loss of enteric tropism in the closely related PUR-46 isolates. The available data indicate that in order to infect enteric tract cells with TGEV, two different domains of the S protein, mapping between amino acids 522 and 744 and around amino acid 219, respectively, are involved. The first domain binds to porcine aminopeptidase N, the cellular receptor for TGEV. In the other domain maps a second factor of undefined nature but which may be the binding site for a coreceptor essential for the enteric tropism of TGEV. © 1997 Academic Press

## INTRODUCTION

Transmissible gastroenteritis virus (TGEV) is a member of the *Coronaviridae* family (Cavanagh *et al.*, 1994; Enjuanes and Van der Zeijst, 1995; Siddell, 1995). TGEV replicates in both the villus epithelial cells of the small intestine and in lung cells of newborn piglets, resulting in a mortality of nearly 100% (Saif and Wesley, 1992). Frequently these TGEV strains are referred to as enteric, as opposed to the respiratory strains which do not infect the enteric tract. Coronaviruses attach to host cells through the spike (S) glycoprotein (Cavanagh *et al.*, 1986; Holmes *et al.*, 1989; Sturman and Holmes, 1983; Suñé *et al.*, 1990), and TGEV entry into swine testis (ST) cells is mediated through interactions between the virus S glycoprotein and the porcine aminopeptidase N (pAPN) which serves as the cellular receptor (Delmas *et al.*, 1992). The S glycoprotein domain recognized by the cellular receptor on ST cells is thought to be located spatially close to the antigenic sites A and D (Suñé *et al.*, 1990). In fact, recent studies (Godet *et al.*, 1994) showed that baculovirus-expressed polypeptides corresponding

to amino acids 522 to 744 of the spike protein were able to efficiently recognize the pAPN.

Since the S protein is responsible for the virus binding to the cell, it is expected that this protein would play an essential role in the control of the dominant tropism of TGEV. Accordingly, there are data suggesting a correlation between tropism and S protein structure. Porcine respiratory coronaviruses (PRCVs) have been originated independently in Europe (Callebaut *et al.*, 1988; Pensaert *et al.*, 1986; Sánchez *et al.*, 1992) and in North America (Vaughn *et al.*, 1994; Wesley *et al.*, 1991, 1990b) from enteric isolates (Enjuanes and Van der Zeijst, 1995; Sánchez *et al.*, 1992). PRCVs replicate to high titers only in the respiratory tract (Cox *et al.*, 1990) and have a large deletion at the 5' end of the spike gene, in positions ranging from nucleotides (nt) 45 to 745 (Enjuanes and Van der Zeijst, 1995; Sánchez *et al.*, 1992; Vaughn *et al.*, 1994; Wesley *et al.*, 1991). Since this deletion is present in all independently derived PRCVs it may be responsible for their loss of enteric tropism.

However, it can not be excluded that other viral genes, apart from the S gene, could be involved in the determination of the tropism of TGEV. In fact, changes in ORF3a have been associated with the loss of enteric tropism. In PRCVs, the ORF3a intergenic region has lost the ca-

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nonical sequence CUA AAC required for the leader primed transcription by the introduction of deletions and point mutations. These mutations lead to the lack of ORF3a expression (Britton *et al.*, 1990; Enjuanes and Van der Zeijst, 1995; Laude *et al.*, 1993; Rasschaert *et al.*, 1990; Wesley *et al.*, 1990a).

Interestingly, the Purdue-type virus (PTV), which displays respiratory tropism, has an S gene with an identical size to that of the enteric isolates. The PTV S gene was sequenced and compared with the homologous sequence of several enteric isolates. Only three nucleotide differences, not observed in enteric isolates, were noted and all introduced amino acid substitutions. Two of these changes were located at nucleotides 214 and 655 within the area deleted in PRCVs, while the other was outside, at nucleotide 2098 (Sánchez *et al.*, 1992). The nucleotide change at position 214 was also present in several enteric isolates. These data lead us to propose that alterations in the S gene around residue 655 could affect enteric tropism (Sánchez *et al.*, 1992).

In order to analyze the role of different viral genes in tropism, we have isolated recombinant TGEVs by crossing the enteric PUR46 strain and the respiratory strain PTV-*ts-dmar*, a temperature-sensitive mutant (*ts*) resistant to neutralization by monoclonal antibodies specific for two different antigenic subsites (*dmar*). Analysis of the tropism of the recombinant isolates demonstrates that two changes at nucleotides 214 and 655 of the spike gene, leading to aspartic acid to asparagine and to alanine to serine amino acid changes, respectively, were associated to the loss of enteric tropism in the PTV isolate.

## MATERIALS AND METHODS

### Cells and viruses

Viruses were grown in swine testis (ST) cells (McClurkin and Norman, 1966). The virus strain PUR46-MAD-CC120 has been described (Sánchez *et al.*, 1992). The Purdue virus strain PTV was previously named NEB72 (Sánchez *et al.*, 1992); however, due to sequence similarity to the PUR46 strain, its name was changed to PTV (Purdue-type virus). A *ts* mutant derived from PTV was kindly provided by M. Welter (Ambico). This *ts* mutant was obtained after 5-fluorouridine mutagenesis using a previously described procedure (Robb *et al.*, 1979) and three cycles of plaque purification. The *ts* mutant growth was reduced  $>10^3$ -fold at the restrictive temperature (39°) and showed a reduced capacity for RNA synthesis. Two neutralizing monoclonal antibodies (MAbs), 1C.C12 and 1D.E7, that are specific for the antigenic subsites Aa and Ab, respectively (Correa *et al.*, 1988), were used to select the neutralization escape mutant (double MAb-resistant mutant, *dmar*) using the PTV-*ts* strain. The procedure used to obtain the *dmar* mutant was identical to the one previously described (Correa *et al.*, 1988; Geb-

auer *et al.*, 1991; Jiménez *et al.*, 1986). The clone obtained was named PTV-*ts-dmar*1C.C12-1D.E7. The PTV-*ts* and PTV-*ts-dmar* virus strains were grown at the permissive temperature (34°).

### Virus neutralization, temperature inactivation, and purification

The procedure for virus neutralization has been described (Correa *et al.*, 1988; Suñé *et al.*, 1990). The neutralization index (NI) was defined as the log of the ratio of the plaque-forming units (PFU) after incubating the virus in the presence of medium or the indicated MAb. To analyze virus inactivation by temperature, viruses were grown at both the permissive (34°) and nonpermissive (39°) temperatures. The temperature inactivation index (TII) was calculated as the log of the ratio of the PFU after growing the virus at 34° or 39°.

To partially purify TGEV, ST cells were grown in 500-cm<sup>2</sup> roller bottles and infected with a multiplicity of infection (m.o.i.) of 10 PFU/cell. Supernatant was harvested 48 hr postinfection (h.p.i.) and clarified by centrifugation in a Sorvall GSA rotor for 20 min at 6000 rpm. Virions were concentrated by centrifugation at 25000 rpm at 4° in a Kontron TST28.18 rotor for 2 hr through a 31% (w/v) sucrose cushion. To clear the virus from the remaining sucrose, the pellet was resuspended in TEN (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 M NaCl) and sedimented by centrifugation under the same conditions. The viral pellet was resuspended in 500  $\mu$ l of TNE (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA).

### Antigenic characterization of virus by radioimmunoassay (RIA)

The binding of a large panel of MAbs to purified virus was performed by RIA as previously (Correa *et al.*, 1988; Sánchez *et al.*, 1990) using optimum amounts of virus (0.5  $\mu$ g of protein per well).

### RNA isolation

Genomic RNA was extracted from partially purified virus as described previously (Mendez *et al.*, 1996). Briefly, ST cells from 10 roller bottles (500 cm<sup>2</sup>) were infected with a m.o.i. of 5. Medium was harvested at 22 h.p.i. Virions were partially purified as described above. The viral pellet was dissociated by resuspending in 500  $\mu$ l of TNE containing 2% SDS and digested with 50 ng of proteinase K (Boehringer Mannheim) for 30 min at room temperature. RNA was extracted twice with phenol-chloroform and precipitated with ethanol.

RNA from TGEV-infected ST cells was obtained as described previously (McKittrick *et al.*, 1993). Briefly, ST cells, grown in 8-cm<sup>2</sup> wells, were infected with TGEV at a m.o.i. of 5. At 22 h.p.i., the cells were resuspended in 400  $\mu$ l of phosphate-buffered saline (PBS) and were incubated for 10 min on ice with 40  $\mu$ l of 2 mM Vanadyl

ribonucleoside complexes (New England BioLabs), 20  $\mu$ l of tRNA (15 mg/ml), 40  $\mu$ l of 2 M sodium acetate, pH 4, and 400  $\mu$ l of phenol. The mixture was then centrifuged for 15 min at room temperature at 13000 *g*. RNA was precipitated with ethanol.

### Recombinant virus isolation

ST cells were coinfecting with the enteric PUR46-MAD and the respiratory PTV-*ts-dmar* strains (m.o.i. of 20 for each parental virus). Cultures were first incubated at 34° for 2.5 hr and then the temperature was shifted to 39°. At 8.5 h.p.i. the supernatant was collected and the titer was determined. To minimize recombinant virus neutralization due to formation of chimeras with spike proteins from both parental viruses, the supernatant from the coinfection was used to infect ST cells at a m.o.i.  $\leq$  0.5 in the absence of neutralizing MAbs. A significant proportion of virions resulting from this infection should have only the spike protein encoded by one virus genome. The supernatant was harvested at 12 h.p.i., and neutralization with MAbs 1C.C12 and 1D.E7 was performed to select recombinant viruses. Potential recombinants were plaque isolated at restrictive temperature in the presence of the two neutralizing MAbs used in the selection. The surviving isolates were phenotypically characterized by calculating their neutralization and temperature inactivation indices. In parallel, independent ST cell cultures were infected with each of the two parental viruses and the same recombinant selection procedure was attempted.

### Cloning and sequencing analysis

To identify nucleotide differences between the two parental viruses, cDNA fragments covering different regions of the genome were synthesized by RT-PCR. These regions included the first 1 kb from the 5'-end of the genome, ORF1 nucleotides 12208 to 20363, the first 5' 2.3 kb of the S gene, and the most 4.3 kb 3' end of PTV-*ts-dmar*. These cDNAs were subcloned into pBluescript (SK<sup>-</sup>) (Stratagene) or pGEM-T (Promega) vectors. Plasmid DNA was purified using the FlexiPrep kit (Pharmacia) and sequenced with Sequenase 2.0 (USB). Sequence data were compiled using the University of Wisconsin Genetic Computer Group (UWGCG) sequence analysis software package and compared to previously published PUR46 virus strains (Eleuet *et al.*, 1995; Kapke and Brian, 1986; Mendez *et al.*, 1996; Rasschaert *et al.*, 1987; Sánchez *et al.*, 1992). Mutations were confirmed by sequencing the viral RNA of the two parental viruses PUR46-MAD and PTV-*ts-dmar*.

RNA was directly sequenced by oligodeoxynucleotide primer extension and dideoxynucleotide chain termination procedures (Sanger *et al.*, 1977), using a modified protocol previously described (Fichot and Girard, 1990). Nucleotide differences between the parental viruses were used as genetic markers and were named M1 to

TABLE 1

Oligonucleotides Used to Sequence the Nucleotide Differences between Parental Viruses

Oligonucleotide <sup>a</sup>	Genetic marker <sup>b</sup>	Primer sequence <sup>c</sup>
5'-1216	M1	5' ACACCTCAACATCACCAG 3'
5'-13326	M2	5' TTGAAATGGTAACCTGCAG 3'
S-250	M3	5' TAATCCCAATACAATGCTTT 3'
S-752	M4	5' ACTAGCACATTGATCAGTGC 3'
S-1980	M5	5' GTACACCCACTATGTTGTCT 3'
S-2250	M6	5' TGTTAATGGAAGTGATAGGC 3'
3a-409	M7	5' TGCTGTTGACTATAACACG 3'
3a-409	M8	5' TGCTGTTGACTATAACACG 3'
3b-112	M9	5' TTAACATACCAAAGTATGC 3'

<sup>a</sup> The name of each oligonucleotide is indicated by name of the ORF and the position of the 5' nucleotide in that ORF.

<sup>b</sup> The differences (genetic markers) found between the two parental viruses are named M1 to M9.

<sup>c</sup> All oligonucleotides have reverse sense polarity.

M9. The origin of the nucleotides in the recombinant viruses, in the positions indicated by these molecular markers, were determined by sequencing RT-PCR-derived cDNA fragments using the fmol DNA Sequencing System (Promega). The primers used to sequence the genetic markers M1–M9 are described in Table 1.

### Virus tropism analysis

Viral tropism was determined in NIH miniswine (Lunney *et al.*, 1986; Sachs *et al.*, 1976) or in piglets derived from crossing Belgium Landrace and Large White swine. Two- to three-day-old conventional (i.e., non-colostrum-deprived) piglets were used. Piglets were obtained from sows that were seronegative for TGEV neutralizing antibodies by RIA. Inbred and outbred animals were orally and intragastrically inoculated with doses of  $5 \times 10^7$  and  $5 \times 10^8$  PFU, respectively, in a final volume of 2 ml of PBS supplemented with 2% of fetal calf serum. Groups of piglets inoculated with the same virus were grouped and housed in isolation chambers located in a P3 level containment facility at 18° to 20°. Animals were fed three times per day with 30 ml of milk formula for newborns (Nidina 1-Nestlé). Virus titers at 1, 2, 3, and 4 days postinoculation were determined in tissue extracts from jejunum and ileum and lungs. Tissues were homogenized at 4° using a tissue homogenizer Pro-200 (Pro-Scientific). Lungs and jejunum and ileum extracts were obtained by homogenizing the whole organs in order to obtain representative samples.

## RESULTS

### Generation and characterization of TGEV mutant PTV-*ts-dmar*1C.C12,1D.E7

To generate a panel of recombinants between enteric and respiratory strains of TGEV, parental viruses that

facilitate the selection of recombinants were generated and characterized. A double *mar* mutant virus (PTV-*ts-dmar*1C.C12-1D.E7) derived from the respiratory PTV-*ts* strain of TGEV was isolated. The growth of PTV-*ts* and PTV-*ts-dmar* viruses in ST cells at 39° was at least 10<sup>3</sup>-fold lower than at 34°, while the parental PUR46 virus strain replicated similarly well at both temperatures (data not shown). The antigenic characterization of the mutant PTV-*ts-dmar* and its ancestor PTV-*ts* by RIA and neutralization using MAbs showed (Fig. 1) that the PTV-*ts-dmar* mutant lacked the S protein antigenic subsite Aa completely, and subsite Ab partially, since some MAbs specific for these subsites did not bind or neutralized this isolate. In addition, the S protein of the escaping mutant was not bound by MAbs 8B.F3 and 9F.C11, suggesting that the epitopes recognized by these MAbs are located in subsites Aa or Ab or in close association with these subsites. Sequencing of the S gene in the PTV-*ts-dmar* mutant showed that the loss of antigenic subsites Aa and Ab was due to a single point mutation at nucleotide 1756, resulting in a change of aspartic acid to tyrosine at position 586.

### Recombinant virus isolation

In order to study the molecular basis of TGEV tropism recombinant viruses were obtained by coinfecting ST cells with the enteric PUR46-MAD and respiratory PTV-*ts-dmar* strains (Fig. 2). ST cell monolayers were infected in parallel, with either PUR46-MAD or PTV-*ts-dmar* strains. To isolate recombinants, selective pressure based on virus inactivation at high temperature and MAb neutralization was used. The virus titer in the ST cell culture coinfecting with the two parental viruses was 1.9 × 10<sup>3</sup> PFU/ml, while cells infected with the respiratory or enteric parental viruses contained 35 and less than 10 PFU/ml, respectively, indicating that recombinant viruses resistant to the selective pressure were likely generated.

The supernatant from the ST cell culture coinfecting with the enteric and respiratory strains was used to plaque purify 34 putative recombinant clones and the progeny of the coinfection was phenotypically characterized under restrictive conditions. Thirty of the 34 clones analyzed showed the recombinant phenotype (Table 2). Most of the progeny isolates (53%) showed the expected selectable recombinant phenotype (SR), being resistant to both MAb neutralization and temperature inactivation. Among the recombinant viruses, 35% were *dmar* mutants which were partially sensitive to the restrictive temperature (*ts* intermediate phenotype recombinants, IPR). No virus was isolated with the phenotypic characteristics against which the selection had been performed (nonselectable recombinants, NSR) (Table 2).

### Genotypic characterization of the recombinant isolates

The identification of genetic markers was required to map the recombination sites. In order to identify such

markers, the first 1 kb from the 5' end of the genome, ORF1 nucleotides 12208 to 20363, the first 5' end 2.3 kb of the S gene, and the most 3' end 4.3 kb were sequenced in the PTV-*ts-dmar* isolate. These sequences were compared to the PUR46-PAR strain (Eleouet *et al.*, 1995; Rasschaert *et al.*, 1987) and to PUR46-MAD (Mendez *et al.*, 1996; Sánchez *et al.*, 1992). Nine nucleotide differences between the two parental viruses were identified (Fig. 3A). These markers were named M1 to M9 in order from the 5'-end of the genome. The PTV-*ts-dmar* virus was derived from a PTV-*ts* isolate which originated from PTV. These three related isolates all display respiratory tropism. All of them differ with the enteric PUR46-MAD by three nucleotide substitutions in the S gene (M3, M4, and M6). In addition, PTV-*ts-dmar* differs by a fourth nucleotide change (M5), which is responsible for the *dmar* mutation. Two of the nine nucleotide differences (M8 and M9) did not result in an amino acid change. M8 was located in the intergenic region between ORF3a and ORF3b, and M9 in ORF3b. Neither of these two areas are expressed in PTV or PUR46-MAD strains.

Analysis of the recombinant genome sequences showed that the recombinants originated by fusing 5' sequences of the enteric parental virus to 3' sequences of the respiratory parental virus. Using these molecular markers, the 30 recombinants were classified into three different groups according to the position of the crossover (Fig. 3). Group 1 recombinants contained 8 clones and their crossover was located within the 1102 nucleotides spanning the genetic markers M4 and M5 (Fig. 3). Group 2 recombinants comprised 15 isolates that had the crossover located between M2 and M3 markers. The viruses included in Groups 1 and 2 had the same sequence except for nucleotides 214 and 655 of the S protein gene (genetic markers M3 and M4) that were derived from the enteric parent in Group 1 isolates, and from the respiratory parent in Group 2 isolates. Group 3 recombinants included 7 isolates that had recombined between genetic markers M1 and M2.

Molecular marker M1 was sequenced in all recombinant viruses, since it was the nucleotide difference located closest to the 5' end. All recombinants inherited this marker from the PUR46-MAD strain, indicating that there was most likely only one crossover at the 5'-half of the genome. Three nucleotide differences were observed from gene 3 up to the 3' end of the genome (Fig. 3A). Only one of these differences (genetic marker M7, located in ORF3) led to an amino acid change. However, this nucleotide difference was not present in the respiratory PTV strain, strongly suggesting that it was not involved in the control of TGEV tropism. The possibility of a second crossover at the 3' half of the genome was not analyzed, because if a second crossover had taken place, it would have replaced a fragment by another one with an equivalent sequence.

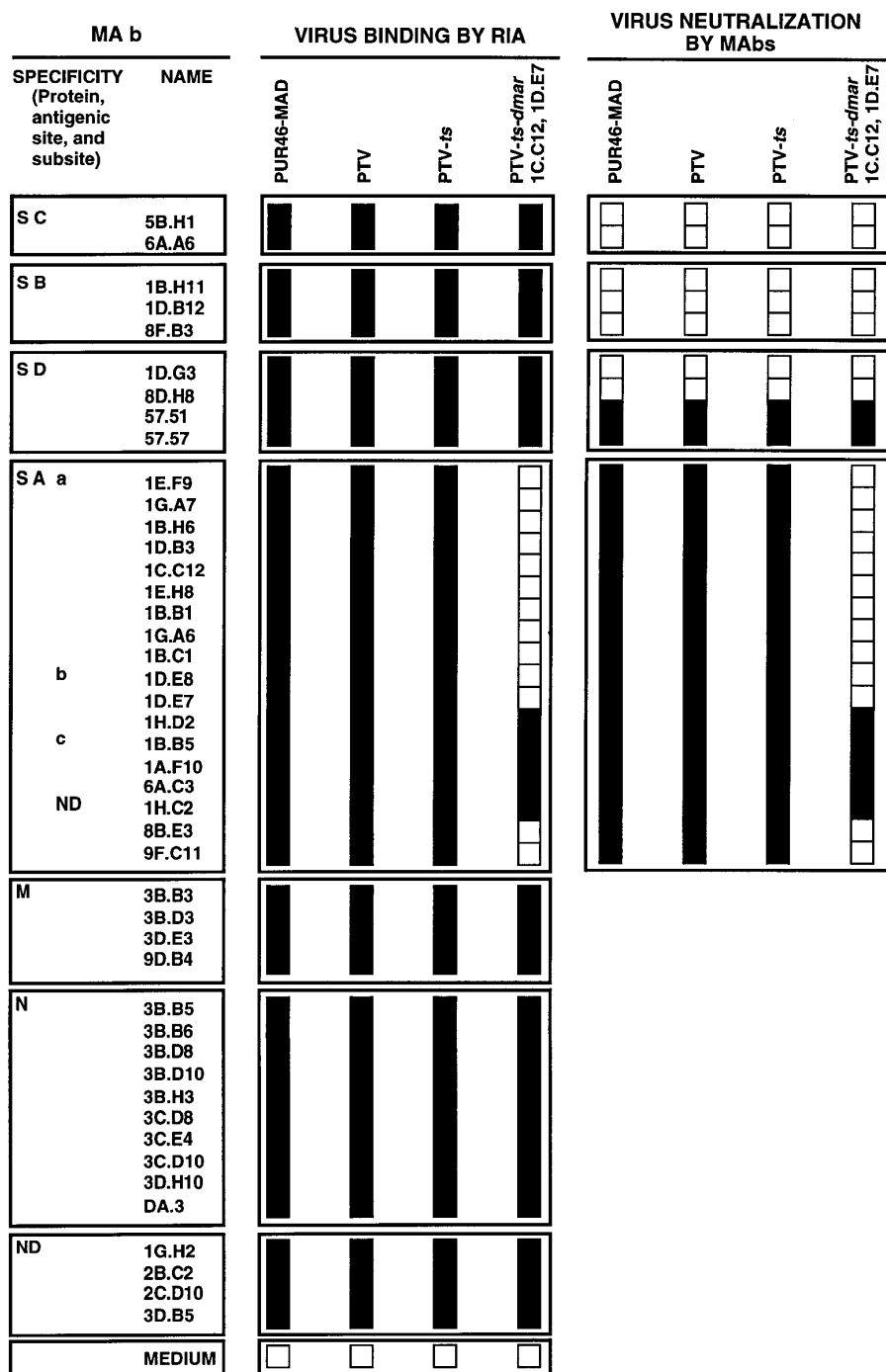


FIG. 1. Antigenic characterization of TGEV isolates. The antigenic pattern of TGEV strains PUR46-MAD, PTV (Purdue-type virus), a PTV temperature-sensitive (*ts*) mutant, and a double MAb-resistant (*dmar*) mutant of PTV-*ts*, PTV-*ts-dmar*1C.C12,1D.E7 was determined by RIA and neutralization assays (see Materials and Methods). In the first column, the specificity of MAbs is named according to Correa *et al.* (1988). The first letter indicates the name of the virus protein (S, M, N); ND, not determined. The second and the third letters of this column indicate the antigenic site (C, B, D, and A) and subsites within the site (a, b, and c), respectively. The value of MAb binding to PUR46-MAD determined by RIA was used as the reference value (100). The neutralization index (NI) was defined as the log of the ratio of the PFU after incubating the virus in the presence of either medium or the indicated MAb. Symbols: (□) binding 0 to 30, or NI < 0.8; (■) binding 51 to 100, or NI > 1.5.

### Virus tropism

The tropism of the three groups of recombinants was next studied. Two isolates from Group 1 and two from Group 2 were evaluated. The results obtained were simi-

lar for the two viruses from each group. In addition, one isolate from Group 3 recombinants was studied. Tropism was studied in parallel in the parental viruses PUR46, PTV-*ts-dmar*, and the PTV strain. Each isolate was tested

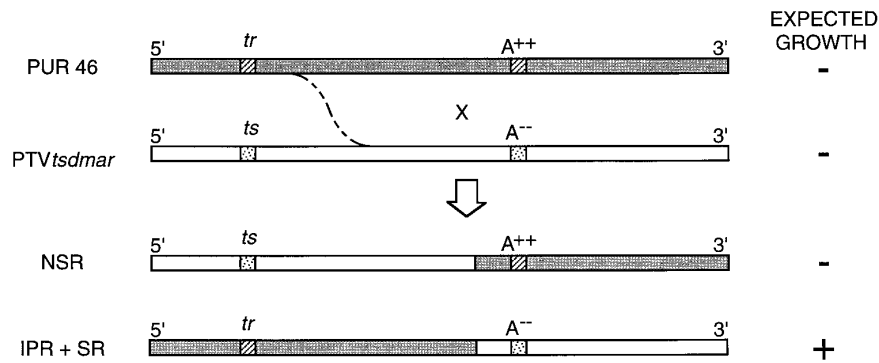


FIG. 2. Recombinant virus isolation. Two TGEV strains, PUR46-MAD and PTV-*ts-dmar* (see Fig. 1) with enteric and respiratory tropism, respectively, were used to coinfect ST cells at a m.o.i. of 20 for each virus. As a first step, selective pressure based only on restrictive temperature was used. To minimize virus neutralization due to the formation of chimeras containing spike proteins from both parental viruses, the supernatant from ST cells coinfecting with the two viruses was used to infect a new ST cell monolayer with a m.o.i.  $\leq 0.5$ . This supernatant was collected at 12 h.p.i., neutralized with the MAbs 1C.C12 and 1D.E7, and then used to plaque isolate recombinant viruses at restrictive temperature. Horizontal bars represent virus genomes. The position of mutations used in the selection of recombinant viruses is indicated; *tr* or *ts* represent temperature resistance or sensitivity, respectively, A<sup>++</sup> and A<sup>--</sup> represent the presence or the absence, respectively, of the antigenic subsites Aa, and part of Ab of S protein. Coinfection of the ST cell culture with the two parental viruses generated three kinds of recombinant viruses. The first had inherited the *ts* mutation from the PTV-*ts-dmar* strain and the position A<sup>++</sup> from the PUR46-MAD strain. These viruses could not grow under selective conditions and are indicated as nonselectable recombinants (NSR). The two other recombinants types, with intermediate *ts* phenotype (IPR) and the selectable recombinants (SR), were grown under the selective conditions and were further characterized.

at least three times. All recombinants and the parental viruses were isolated from the lungs, but only the PUR46 strain and the recombinants of Group 1 could be isolated from the small intestine (Fig. 4). Maximum virus production in the lungs varied from  $10^4$  to  $10^6$  PFU/g tissue (Fig. 4). PTV-*ts-dmar* produced less infectious virus than the other strains. Maximum PTV-*ts-dmar* production was about  $10^4$  PFU/g of lung tissue, while its ancestor PTV-*wt* could replicate to higher titers ( $10^6$  PFU/g tissue).

### Recombinant isolation frequency

The procedure used to isolate recombinant viruses favored the selection of viruses which had recombined between the two markers used in the selection, the *ts* and the *dmar* mutations. Preliminary results (data not

shown) indicated that the *ts* mutation mapped at the ORF1a of the respiratory isolate, between genetic markers M1 and M2 (i.e., from nt 955 to nt 13272 of ORF1). The *dmar* mutation was localized at position 1756 of S gene (genetic marker M5). Thus, the minimum distance for recombination with the selective pressure used (high temperature and neutralization by MAbs) was the interval between markers M2 and M5. The recombinant isolation frequency was calculated as the ratio between the progeny virus titer with a recombinant genotype ( $1.8 \times 10^3$  PFU) and the titer ( $8 \times 10^7$  PFU) of the parental viruses grown in parallel in the absence of selective pressure, divided by the distance between M2 and M5. This frequency was  $\leq 2.3 \times 10^{-9}$  recombinants per nucleotide for this interval. The recombinant frequency was also calculated for recombinants of Groups 1, 2, and 3 and

TABLE 2

Phenotypic Characterization of Potential Recombinants between Enteric PUR46 and Respiratory PTV-*ts-dmar*

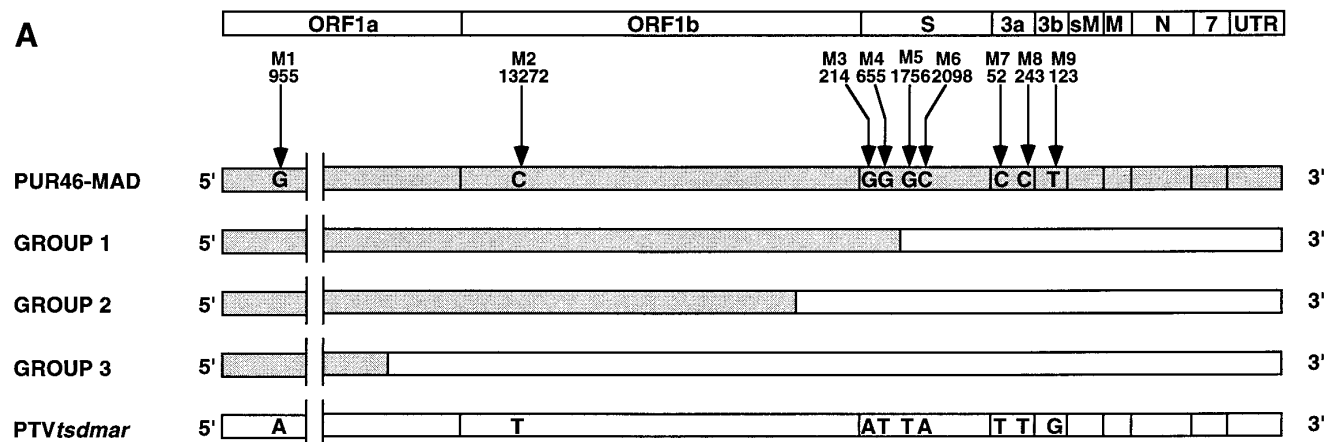
Phenotype	Neutralization index <sup>a</sup>	Inactivation index <sup>b</sup>	Recombinants	
			Total number	Percentage
PUR46- <i>wt</i>	$3.7 \pm 0.3^c$	$0.5 \pm 0.3^d$	1	3.0
PTV- <i>ts-dmar</i>	$0.5 \pm 0.3$	$3.0 \pm 0.2$	3	9.0
Nonselectable recombinants (NSR)	$3.7 \pm 0.3$	$3.0 \pm 0.2$	0	—
Selectable recombinants (SR)	$0.5 \pm 0.3$	$0.5 \pm 0.3$	18	53.0
Intermediate <i>ts</i> phenotype recombinants (IPR)	$0.5 \pm 0.3$	$2.4 \geq \text{TII} \geq 0.5$	12	35.0

<sup>a</sup> The neutralization index (NI) was defined as the log of the ratio of the PFU after incubating the virus in the presence of medium or MAbs 1C.C12 and 1D.E7.

<sup>b</sup> The temperature inactivation index (TII) was defined as the log of the ratio of the PFU after growing the virus at permissive or nonpermissive temperatures.

<sup>c</sup> Mean and standard error of the NI.

<sup>d</sup> The mean and standard error of the TII.



**B**

RECOMBINANT GROUP	CROSSOVER LOCATED BETWEEN MARKERS	DISTANCE (nt) BETWEEN CROSSOVER MARKERS	RECOMBINANTS Number	%	RECOMBINANT ISOLATION FREQUENCY PER NUCLEOTIDE
1	M4-M5	1102	8	27	$5.5 \times 10^{-9}$
2	M2-M3	7307	15	50	$1.5 \times 10^{-9}$
3	M1-M2	12318	7	23	$4.1 \times 10^{-10}$

**FIG. 3.** Genotypic characterization of recombinant isolates. (A) Schematic representation of the TGEV genome structure. In the top bar the approximate locations of TGEV open reading frames (ORFs) are indicated. Below this bar, the location of nine nucleotide differences (genetic markers M1 to M9) between the two parental viruses is indicated. Recombinants were classified into three groups (named 1 to 3), and the origin of their genomes, whether derived from the enteric parental (dark bars) or from the respiratory parental (white bars) is indicated. In the bars corresponding to the parental viruses PUR46-MAD and PTV-*ts-dmar*, the individual nucleotide differences are indicated. (B) Summary of the genetic characterization of the groups of recombinant viruses. The two markers flanking each crossover and the distance expressed in nucleotides between the two markers are indicated in columns 2 and 3, respectively. Since the exact location of the *ts* mutation is not known, the crossover in Group 3 recombinants is indicated as a maximum distance. The number of recombinants included in each group and the percentage in relationship to the total recombinant virus population are shown in column 4. The last column shows the frequency of recombinants isolated in each group, as the ratio of the number of isolates in the group relative to the number of nucleotides between the molecular markers flanking the crossover site.

were  $5.5 \times 10^{-9}$ ,  $1.5 \times 10^{-9}$ ,  $4.1 \times 10^{-10}$ , respectively (Fig. 3B). Since the exact location of the *ts* mutation is not known, the interval in which the crossover takes place in Group 3 recombinants can not be precisely defined and the frequency provided for this group is the minimum. The calculated data indicate that the frequency of recombinant isolation at the 5' of the S gene was 3.7-fold higher than that of Group 2 recombinants.

## DISCUSSION

In order to determine the role of different viral genes in TGEV tropism, a collection of 30 recombinants was generated by coinfecting ST cells with enteric (PUR46-MAD) and respiratory (PTV-*ts-dmar*) strains of TGEV. Phenotypic, genotypic, and biological characterizations of the recombinants showed that two nucleotide changes at

residues 214 and 655 of the spike gene were responsible for the loss of enteric tropism.

## Recombination among isolates of the TGEV cluster

Initial attempts to isolate TGEV recombinant viruses using selective pressure with neutralizing MAbs specific for a single epitope lead to the isolation of neutralization escape mutants instead of recombinants (data not shown). To diminish the frequency of escape mutants, selective pressure with two MAbs specific for different epitopes of antigenic subsites Aa and Ab (Gebauer *et al.*, 1991) were used. This strategy, in fact, decreased the frequency of neutralization escape mutants, although the selected *dmar* mutant used as a parental virus in the recombination (PTV-*ts-dmar*) had only a single nucleotide change, instead of two nucleotide changes that might

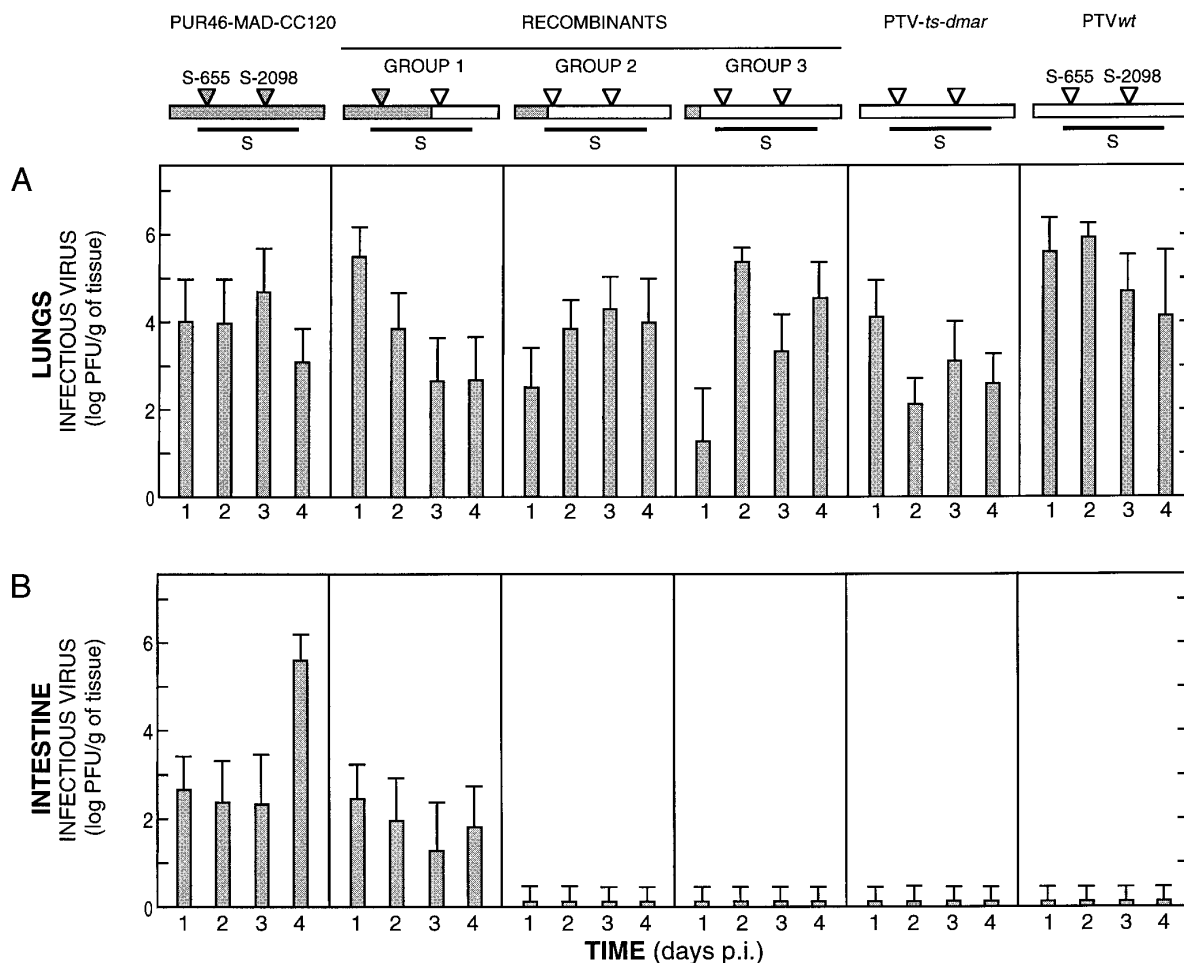


FIG. 4. Virus tropism. Growth of parental and recombinant viruses, either in lungs (A) or in the intestine (B) is shown. To study TGEV tropism, 2- to 3-day-old non-colostrum-deprived piglets were individually inoculated with two isolates from Group 1, with two isolates from Group 2, and with 1 isolate from Group 3 recombinants. Recombinants were isolated by crossing the enteric strain PUR46 and the respiratory strain PTV-*ts-dmar*. Top horizontal thick bars indicate the genome and the origin of each recombinant, whether enteric (dark bar) or respiratory (white bar). The thin horizontal bar indicates the S gene. Triangles indicate the positions of nucleotides 655 and 2098 of the S gene and the origin of these nucleotides, whether enteric (dark triangle) or respiratory (white triangle). Diagram is not at scale and the size of the S gene has been magnified. The recovery of infectious virus was determined in PFU per gram of tissue at the indicated time in h.p.i. Each virus was tested at least three times. Vertical thin bars indicate standard error of the mean.

have been expected for a *mar* mutant escaping to the simultaneous neutralization by two different MAbs.

Isolation of TGEV recombinants required the use of selection pressure. Using this procedure the frequency of recombination was estimated at  $\leq 2.3 \times 10^{-9}$  for TGEV. In contrast, the isolation of the MHV recombinant does not require the use of selection pressure (Makino *et al.*, 1986). The recombination frequencies estimated for both coronaviruses are not directly comparable since the selection strategies were very different; nevertheless, from reported data it seems that the recombinant isolation frequency is higher for MHV than for TGEV. Both recombination and the generation of defective interfering (DI) genomes occurs at a lower frequency in TGEV (Mendez *et al.*, 1996) than in MHV (Lai, 1990), possibly due to a higher accuracy in the replication of TGEV RNA.

Group 1 recombinants were isolated at a frequency

3.7-fold higher than that of Group 2 recombinants. This could be due to a selective advantage in their growth on cell cultures or to a higher recombination frequency at the 5'-end of S gene, between nucleotides 655 and 1756. In fact, extensive sequence variability has been observed in this region. During the isolation of TGEV-defective interfering viruses, a deletion is introduced at the beginning of the S gene, starting from nucleotides 6 to 74 and ending at ORF7 (Mendez *et al.*, 1996). In addition, during the generation of both European and American PRCVs in field conditions, four different deletions at the beginning of the S gene have been identified in positions ranging from nucleotides 45 to 745 (Sanchez *et al.*, 1992; Vaughn *et al.*, 1994; Wesley *et al.*, 1991). These data suggest that the 5'-half of the S gene is an area with an intrinsically high recombination frequency. Although a selective advantage for the recombinants could not be



excluded, it seems unlikely because Group 1 and 2 recombinants differ only in two nucleotide positions located at the 5'-half of the S gene (nucleotides 214 and 655), and recombinants which had the same 5'-half S gene as Group 2 recombinants grew as efficiently as PUR46. An increased recombination frequency in the S gene of MHV has also been described (Fu and Baric, 1994).

### Molecular basis of TGEV tropism.

Only nine nucleotide differences were found between the enteric PUR46-MAD and the respiratory PTV-*ts-dmar* strains of TGEV. Four of them mapped in the S protein gene at nucleotides 214, 655, 1756, and 2098. The nucleotide change at position 1756 of S protein gene, which is responsible for the neutralization escape phenotype, is not responsible of the loss of enteric tropism since it was not present in the respiratory isolate PTV which lacks enteric tropism.

In order to analyze which of the other three nucleotide changes located in the S protein gene, at positions 214, 655, and 2098, were involved in the control of the enteric tropism, recombinant viruses containing one or the three nucleotide differences from the respiratory isolate were selected. These recombinants belong to Groups 1 and 2, respectively. Group 2 recombinants only infected lungs, while Group 1 replicated in the epithelial cells of both the enteric and respiratory tracts. The two nucleotide changes between the enteric recombinants (Group 1) and the respiratory ones (Group 2) were at nucleotides 214 and 655 of the S protein gene, which caused amino acid changes from aspartic acid to asparagine at residue 72 and from alanine to serine at residue 219. These results demonstrate that two amino acid changes at the N-terminus of the viral spike protein were associated to the loss of enteric tropism in the TGEV cluster of viruses. The possibility that the loss of enteric tropism was a consequence of the addition of a nucleotide change at position 655 of S gene to a preexisting change at nt 214 cannot be completely ruled out. Nevertheless, this possibility seems unlikely because most enteric viruses have the same nucleotide as PTV at position 214 (Sanchez *et al.*, 1992), indicating that most likely a single nucleotide change at position 655 was responsible for the loss of enteric tropism. Nucleotides 214 and 655 are located within the area of the S gene which is deleted in PRCVs, strongly suggesting that this deletion was responsible for the loss of enteric tropism in PRCVs. In human immunodeficiency and other virus systems it has also been shown that a single point mutation can alter tropism (Takeuchi *et al.*, 1991).

An intragastric inoculation route was employed to assure that the inoculum of each isolate was introduced into the stomach, independently of their tropism. While viruses with enteric tropism have been found in the intes-

tine until the fourth day postinoculation, virus was never detected in the enteric tract in any of the more than 60 piglets inoculated with a respiratory isolate. This indicates that the virus detected in the enteric tract was not due to residual virus from the inoculation, nor swallowed virus originating in the respiratory tract, but was the result of local virus replication in the intestine.

All the isolated recombinants, including the ones lacking enteric tropism, were temperature resistant, indicating that the *ts* mutation was not responsible for the loss of enteric tropism.

Studies on PUR46-PAR *mar* mutants also showed a correlation between the N-terminal half of the S protein and viral pathogenesis (Bernard and Laude, 1995). Nevertheless, these results did not differentiate between virus tropism and virulence, since only parameters such as death, or weight loss, caused by the virus mutants were studied, but not virus replication in enteric or respiratory tissues.

Coronavirus spike protein is involved in virus attachment to cells (Cavanagh *et al.*, 1986; Holmes *et al.*, 1989; Sturman and Holmes, 1983; Suñé *et al.*, 1990). Studies on the inhibition of virus binding to cells indicated that the receptor binding site for TGEV had to be located between antigenic sites D and A of the spike protein (Suñé *et al.*, 1990), mapping between amino acids 385 and 631. In agreement with these data, it was shown that porcine APN, the receptor for TGEV (Delmas *et al.*, 1992), binds to S protein residues between aminoacids 522 and 744 (Godet *et al.*, 1994). These sequences map to a distal area in relationship to amino acid 219 of S protein, which, as shown in this paper, influences TGEV enteric tropism. Since pAPN is a protein present in lung epithelium and in enterocytes (Kenny and Maroux, 1982; Norén *et al.*, 1986; Semenza, 1986), and the respiratory PTV isolate conserves the pAPN binding site previously described, the loss of enteric tropism in the PTV isolate should not be due to a failure in pAPN attachment. Furthermore, it has been demonstrated that PRCV isolates attach to pAPN (Delmas *et al.*, 1992), although they cannot infect the enteric tract. This apparent discrepancy could be explained if an interaction between pAPN and two domains of S protein located at both areas (amino acids near residue 219 and amino acids 522 to 744) are required to infect the enteric tract. Alternatively, a putative second factor, such as coreceptor, mapping around amino acid 219 of the spike protein could be specifically required to infect the enteric tract and responsible for the loss of enteric tropism in PTV and PRCV isolates. Other explanations are also possible and the loss on enteric tropism could also be due to: (i) a decrease in the pH stability required to allow the virus passage through the stomach, (ii) a decrease in virion resistance to bile salts and proteolytic enzymes in gut, and (iii) an alteration in the strength or affinity of the S/receptor interaction. Recent studies also located the receptor binding

site of the JHM strain of MHV in rats on the S1 subunit of the spike protein (Suzuki and Taguchi, 1996), and in MHV it was suggested that a second cellular factor, apart from the cellular receptor which interacts with the S protein, is involved in virus entry (Yokomori *et al.*, 1993). The requirement of a coreceptor to infect cells has been described in human immunodeficiency virus and in poliovirus (Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996; Shepley and Racaniello, 1994).

ORF3a is not expressed in PRCVs isolates, while it is expressed in enteric strains. Thus, it has been proposed that ORF3a plays an essential role in the control of virus enteropathogenicity (Britton *et al.*, 1991; Laude *et al.*, 1993; Wesley *et al.*, 1991). RNA sequence comparison of the 3'-half of the respiratory virus PTV-*ts-dmar*, from ORF3a to 3'-UTR, with that of the enteric isolate PUR46-MAD sequence revealed three nucleotide differences (genetic markers M7, M8, and M9). Only one of them, M7, lead to an amino acid change at position 52 of the gene 3a. This nucleotide difference was not present in the parental PTV genome, so it is likely not responsible for the loss of enteric tropism, since PTV is already a respiratory strain. Furthermore, sequences located between the S gene and the 3'-end of the genome are identical in Groups 1 and 2 recombinants which have enteric and respiratory tropism, respectively. These data indicate that the enteric tropism of TGEV related viruses can be lost without changes downstream of the S gene.

The generation and analysis of TGEV recombinants has been very informative in the study of viral functions such as tropism. A better understanding of the molecular basis of virus tropism may help us to target expression vectors based on defective coronavirus genomes to respiratory or enteric areas in order to effectively induce mucosal immune responses.

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