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Two Amino Acid Changes at the N-Terminus of Transmissible Gastroenteritis Coronavirus Spike Protein Result in the Loss of Enteric Tropism

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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 $10^{10}$ 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.
nonical sequence CUAAAC 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., 1990).

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 subtypes (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°C) and showed a reduced capacity for RNA synthesis. Two neutralizing monoclonal antibodies (MAbs), 1C.C12 and 1D.E7, that are specific for the antigenic subtypes 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; Gebauer et al., 1991; Jiménez et al., 1986). The clone obtained was named PTV-ts-dmar1C.C12-1D.E7. The PTV-ts and PTV-ts-dmar virus strains were grown at the permissive temperature (34°C).

Virus neutralization, temperature inactivation, and purification

The procedure for virus neutralization has been described (Correa et al., 1988; Suh 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°C) and nonpermissive (39°C) temperatures. The temperature inactivation index (TII) was calculated as the log of the ratio of the PFU after growing the virus at 34°C or 39°C.

To partially purify TGEV, ST cells were grown in 500-cm² roller bottles and infected with a multiplicity of infection (m.o.i.) of 10 PFU/cell. Supernatant was harvested at 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°C 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 μl 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 μg 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 cm²) 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 μl 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-cm² wells, were infected with TGEV at a m.o.i. of 5. At 22 h.p.i., the cells were resuspended in 400 μl of phosphate-buffered saline (PBS) and were incubated for 10 min on ice with 40 μl of 2 mM Vanadyl
ribonucleoside complexes (New England BioLabs), 20 μl of tRNA (15 mg/ml), 40 μl of 2 M sodium acetate, pH 4, and 400 μl 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 coinfected 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 coinfected was used to infect ST cells at a m.o.i. < 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 IC.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 3’ end of PTV-ts-dmar. These cDNAs were subcloned into pBluescript (SK-) (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 PRRSV virus strains (Eleouet 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 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.

### RESULTS

**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 oronasally and intragastrically inoculated with doses of 5 × 10⁷ and 5 × 10⁸ 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 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.

**TABLE 1**

| Oligonucleotide | Genetic marker | Primer sequence |
|-----------------|----------------|-----------------|
| 5’-1216         | M1             | 5’ ACACCTCAACATACGCCG 3’ |
| 5’-13326        | M2             | 5’ TGAAAGTACATGCG 3’ |
| 5’-250          | M3             | 5’ TAATCCCAATACGCTTT 3’ |
| 5’-750          | M4             | 5’ ACTACGAATGATACGTCG 3’ |
| 5’-1980         | M5             | 5’ TGACACCCATATGGTCT 3’ |
| 5’-2250         | M6             | 5’ TGTTAATGAGGTCGACGC 3’ |
| 3a-409          | M7             | 5’ TGGCGATGCTAACAACCG 3’ |
| 3a-409          | M8             | 5’ TGGCGATGCTAACAACCG 3’ |
| 3b-112          | M9             | 5’ CAACCTCAACATACGCCG 3’ |

* The name of each oligonucleotide is indicated by name of the ORF and the position of the 5’ nucleotide in that ORF.

* The differences (genetic markers) found between the two parental viruses are named M1 to M9.

* All oligonucleotides have reverse sense polarity.
facilitate the selection of recombinants were generated and characterized. A double mar mutant virus (PTV-ts-dmar1C.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°C was at least 10³-fold lower than at 34°C, 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 coinfected with the two parental viruses was 1.9 × 10³ 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 coinfected 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 (non-selectable 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; Sanchez 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-dmar1C.C12,1D.E7 was determined by RIA and neutralization assays (see Materials and Methods). In the first column, the specificity of MAb 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 similar 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 coinfected 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, $A^{++}$ and $A^{-}$ represent the presence or the absence, respectively, of the antigenic subites $Aa$, 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 $A^{+}$ 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.

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 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 $10^6$ PFU/g of lung tissue, while its ancestor PTV-wt could replicate to higher titers ($10^8$ PFU/g tissue).

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**TABLE 2**

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

| Phenotype                                | Neutralization index | Inactivation index | Recombinants |
|------------------------------------------|----------------------|--------------------|--------------|
|                                           |                      |                    | Total number | Percentage |
| PUR46-wt                                  | $3.7 \pm 0.3^c$       | $0.5 \pm 0.3^d$    | 1            | 3.0         |
| PTV-ts-dmar                               | $0.5 \pm 0.3$         | $3.0 \pm 0.2$      | 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 $ts$ phenotype recombinants (IPR) | $0.5 \pm 0.3$     | $2.4 \gg \text{TII} \gg 0.5$ | 12           | 35.0        |

$^a$ 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.

$^b$ 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.

$^c$ Mean and standard error of the NI.

$^d$ The mean and standard error of the TII.
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.

| RECOMBINANT GROUP | CROSOVER LOCATED BETWEEN MARKERS | DISTANCE (nt) BETWEEN CROSSOVER MARKERS | RECOMBINANTS NUMBER | RECOMBINANT ISOLATION FREQUENCY PER NUCLEOTIDE |
|-------------------|----------------------------------|------------------------------------------|-------------------|-----------------------------------------------|
| 1                 | M4-M5                            | 1102                                     | 8                 | 5.5 x 10^{-9}                                 |
| 2                 | M2-M3                            | 7307                                     | 15                | 1.5 x 10^{-9}                                 |
| 3                 | M1-M2                            | 12318                                    | 7                 | 4.1 x 10^{-10}                                |

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 have been 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 cannot 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

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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 have been 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 cannot 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.
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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 $\approx 2.3 \times 10^{-9}$ 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 (Sánchez 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 5ꞌ-half of the S gene (nucleotides 214 and 655), and recombinants which had the same 5ꞌ-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 (Sánchez 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 intesti-
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 ORF3a from 3′-half of the respiratory virus PTV-ts-dmar, 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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REFERENCES

Bernard, S., and Laude, H. (1995). Site-specific alteration of transmissible gastroenteritis virus spike protein results in markedly reduced pathogenicity. J. Gen. Virol. 76, 2235–2241.

Britton, P., Mawditt, K. L., and Page, K. W. (1991). The cloning and sequencing of the virion protein genes from a British isolate of porcine respiratory coronaviruses: Comparison with transmissible gastroenteritis virus genes. Virus Res. 21, 181–198.

Britton, P., Page, K. W., Mawditt, K., and Pocock, D. H. (1990). Sequence comparison of porcine transmissible gastroenteritis virus (TGEV) with porcine respiratory coronavirus. VIIIth International Congress of Virology, pp. 6–018. IUMS, Berlin.

Callebaut, P., Correa, I., Pensaert, M., Jiménez, G., and Enjuanes, L. (1988). Antigenic differentiation between transmissible gastroenteritis virus of swine and a related porcine respiratory coronavirus. J. Gen. Virol. 69, 1725–1730.

Cavanagh, D., Brian, D. A., Enjuanes, L., Holmes, K. V., Lai, M. M. C., Laude, H., Siddell, S. G., Spaan, W., Taguchi, F., and Talbot, P. (1994). Revision of the taxonomy of the Coronavirus, Torovirus, and Arterivirus genera. Arch. Virol. 135, 227–237.

Cavanagh, D., Davis, P. J., Derbyshire, J. H., and Peters, R. W. (1986). Coronavirus IBV: virus retaining spike glycoprotein S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J. Gen. Virol. 67, 1435–1442.

Correa, I., Jiménez, G., Suñé, C., Bullido, M. J., and Enjuanes, L. (1988). Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus. Virus Res. 10, 77–94.

Cox, E., Hooyberghs, J., and Pensaert, M. B. (1990). Sites of replication of a porcine respiratory coronavirus related to transmissible gastroenteritis virus. Res. Vet. Sci. 48, 165–169.

Delmas, B., Gelfi, J., L’Haridon, R., Vogel, L. K., Norén, O., and Laude, H. (1992). Aminoepitidase N is a major receptor for the enteropathogenic coronavirus TGEV. Nature 357, 417–420.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, D., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. Nature 381, 661–666.

Dragic, T., Littwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A. (1996). HIV-1 entry into CD4 cells is mediated by the chemokine receptor CC-CX3R. Nature 381, 667–673.

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 (TGEV) epidemiology. In “The Coronavirusidae” (S. G. Siddell, Ed.), pp. 337–376. Plenum, New York.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272, 872–877.

Fichot, O., and Girard, M. (1990). An improved method for sequencing of RNA templates. Nucleic Acids Res. 18, 6162.

Fu, K., and Banic, R. S. (1994). Map locations of mouse hepatitis virus temperature-sensitive mutants: confirmation of variable rates of recombination. J. Virol. 68, 7458–7466.

Gebauer, F., Posthumus, W. A. P., Correa, I., Suñé, C., Sánchez, C. M., Smerdou, C., Lenstra, J. A., Melen, R., and Enjuanes, L. (1991). Residues involved in the formation of the antigenic sites of the S protein of transmissible gastroenteritis coronavirus. Virology 183, 225–238.

Godet, M., Grosclaude, J., Delmas, B., and Laude, H. (1994). Major receptor-binding and neutralization determinants are located within the same domain of the transmissible gastroenteritis virus (coronavirus) spike protein. J. Virol. 68, 8008–8016.

Holmes, K. V., Williams, R. K., Stephensen, C. B., Compton, L. R., Cardellichio, C. B., Hay, C. M., Knobler, R. L., Weismiller, D. G., and Boyle, J. F. (1989). Coronavirus receptors. In “Cell Biology of Virus Entry, Replication, and Pathogenesis” (R. W. Combs, A. Helenius, and M. B. A. Oldstone, Eds.), pp. 85–94. A. R. Liss, New York.

Jiménez, G., Correa, I., Melgosa, M. P., Bullido, M. J., and Enjuanes, L. (1986). Critical epitopes in transmissible gastroenteritis virus neutralization. J. Virol. 60, 131–139.

Kapke, P. A., and Brian, D. A. (1986). Sequence analysis of the porcine spike protein from transmissible gastroenteritis coronavirus. Virus Res. 181–198.
transmissible gastroenteritis coronavirus nucleocapsid protein gene. Virology 151, 41–49.

Kenny, A. J., and Maroux, S. (1982). Topology of microvillar membrane hydrodases of kidney and intestine. Physiol. Rev. 62, 91–128.

Lai, M. M. C. (1990). Coronavirus-organization, replication and expression of genome. Annu. Rev. Microbiol. 44, 303–333.

Laude, H., Vanreeth, K., and Pensael, M. (1993). Porcine respiratory coronavirus-molecular features and virus host interactions. Vet. Res. 24, 125–150.

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.), pp. 1821–1836. Plenum, New York.

Makino, S., Keck, J. G., Stohlman, S. A., and Lai, M. M. C. (1986). High-frequency RNA recombination of murine coronaviruses. J. Virol. 57, 729–737.

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.

McKitterick, S. P., Georgalis, A. M., and Atchison, B. A. (1993). Efficient extraction of viral RNA for PCR amplification. BioTechniques 15, 802–805.

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.

Nórén, O., Sjöström, H., Danielsen, E. M., Cowell, G. M., and Skovbjerg, H. (1986). "The Enzymes of the Enterocyte Plasma Membrane." Elsevier/North-Holland, Amsterdam.

Pensael, M., Callebaut, P., and Hooijberghs, J. (1986). Transmissible gastroenteritis virus in swine: Old and news. 9th Int. Pig Vet. Soc. Cong. 1986, pp. 40–45. Barcelona, Spain.

Rasschaert, D., Duarte, M., and Laude, H. (1990). Porcine respiratory coronavirus differs from transmissible gastroenteritis virus by a few genomic deletions. J. Gen. Virol. 71, 2599–2607.

Rasschaert, D., Gelfi, J., and Laude, H. (1987). Enteric coronavirus TGEV: partial sequence of the genomic RNA, its organization and expression. Biochimie 69, 591–600.

Robb, J. A., Bond, C. W., and Leibowitz, J. L. (1979). Pathogenic murine coronaviruses. III. Biological and biochemical characterization of temperature-sensitive mutants of JHM V. Virology 94, 385–399.

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.

Saif, L. J., and Wesley, R. D. (1992). Transmissible Gastroenteritis. In "Diseases of Swine" (A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor, Eds.), pp. 362–386. Wolfe, Ames, Iowa.

Sánchez, C. M., Gebauer, F., Suhé, C., Méndez, A., Dopazo, J., and Enjuanes, L. (1992). Genetic evolution and tropism of transmissible gastroenteritis coronaviruses. Virology 190, 92–106.

Sánchez, C. M., Jiménez, G., Laviada, M. D., Correa, I., Suhé, 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.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Semenza, G. (1986). Anchoring and biosynthesis of stalked brush border membrane proteins: glycosidases and peptidases of enterocytes and renal tubuli. Annu. Rev. Cell Biol. 2, 225–313.

Shepley, M. P., and Racaniello, V. R. (1994). A monoclonal antibody that blocks poliovirus attachment recognizes the lymphocyte homing receptor CD44. J. Virol. 68, 1301–1308.

Siddell, S. G. (1995). "The Coronaviridae." Plenum, New York.

Sturman, L. S., and Holmes, K. V. (1983). The molecular biology of coronaviruses. Adv. Virus Res. 28, 36–112.

Suhé, C., Jiménez, G., Correa, I., Bullido, M. J., Gebauer, F., Smerdou, C., and Enjuanes, L. (1990). Mechanisms of transmissible gastroenteritis coronavirus neutralization. Virology 177, 559–569.

Suzuki, H., and Taguchi, F. (1996). Analysis of the receptor binding site of murine coronavirus spike protein. J. Virol. 70, 2632–2636.

Takeuchi, Y., Akutsu, M., Murayama, K., Shimizu, N., and Hoshino, H. (1991). Host range mutant of human immunodeficiency virus type 1: modification of cell tropism by a single point mutation at the neutralization epitope in the env gene. J. Virol. 65, 1710–1718.

Vaughn, E. M., Halbur, P. G., and Paul, P. S. (1994). Three new isolates of porcine respiratory coronavirus with various pathogenicities and spike (S) gene deletions. J. Clin. Microbiol. 32, 1809–1812.

Wesley, R. D., Woods, R. D., and Cheung, A. K. (1990a). Genetic basis for the pathogenesis of transmissible gastroenteritis virus. J. Virol. 64, 4761–4766.

Wesley, R. D., Woods, R. D., and Cheung, A. K. (1991). Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus. J. Virol. 65, 3369–3373.

Wesley, R. D., Woods, R. D., Hill, H. T., and Biwer, J. D. (1990b). Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus, in the United States. J. Vet. Diagn. Invest. 2, 312–317.

Yokomori, K., Asanaka, M., Stohlman, S. A., and Lai, M. M. C. (1993). A spike protein-dependent cellular factor other than the viral receptor is required for mouse hepatitis virus entry. Virology 196, 45–56.