

Antigen selection and presentation to protect against transmissible gastroenteritis coronavirus

Luis Enjuanes, Carlos Suñé, Fátima Gebauer, Cristian Smerdou, Ana Camacho, Inés M. Antón, Silvia González, Ana Talamillo, Ana Méndez, María L. Ballesteros and Carlos Sánchez

Centro Nacional de Biotecnología and Centro de Biología Molecular, CSIC-Univ. Autónoma, Canto Blanco, Madrid, Spain

(Accepted 26 June 1992)

ABSTRACT

Enjuanes, L., Suñé, C., Gebauer, F., Smerdou, C., Camacho, A., Antón, I.M., González, S., Talamillo, A., Méndez, A., Ballesteros, M.L. and Sánchez, C., 1992. Antigen selection and presentation to protect against transmissible gastroenteritis coronavirus. *Vet. Microbiol.*, 33: 249–262.

The antigenic structure of the S glycoprotein of transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) has been determined and correlated with the physical structure. Four antigenic sites have been defined (A, B, C, and D). The sites involved in the neutralization of TGEV are: A, D, and B, sites A and D being antigenically dominant for TGEV neutralization in vitro. These two sites have specific properties of interest: site A is highly conserved and is present in coronaviruses of three animal species, and site D can be represented by synthetic peptides. Both sites might be relevant in protection in vivo. PRCV does not have sites B and C, due to a genomic deletion. Complex antigenic sites, i.e., conformation and glycosylation dependent sites, have been represented by simple mimotopes selected from a library expressing recombinant peptides with random sequences, or by anti-idiotypic internal image monoclonal antibodies. An epidemiological tree relating the TGEVs and PRCVs has been proposed. The estimated mutation fixation rate of $7 \pm 2 \times 10^{-4}$ substitutions per nucleotide and year indicates that TGEV related coronaviruses show similar variability to other RNA viruses. In order to induce secretory immunity, different segments of the S gene have been expressed using avirulent forms of *Salmonella typhimurium* and adenovirus. These vectors, with a tropism for Peyer's patches may be ideal candidates in protection against TGEV.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV), that belongs to the Coronaviridae family, causes gastroenteritis in pigs resulting in high mortality and morbidity in neonates (Spaan et al., 1988). Within this family, TGEV forms an antigenic cluster with feline infectious peritonitis virus (FIPV), feline en-

Correspondence to: L. Enjuanes, Centro Nacional de Biotecnología and Centro de Biología Molecular, CSIC-Univ. Autónoma, Canto Blanco, 28049 Madrid, Spain.

teric coronavirus (FECV), canine coronavirus (CCV), and porcine respiratory coronavirus (PRCV) (Sánchez et al., 1990). Vaccines developed against transmissible gastroenteritis (TGE) are not efficient (Moxley et al., 1989). TGEV has three structural proteins: the spike (S), the nucleoprotein (N), and the membrane (M) protein (Jiménez et al., 1986; Spaan et al., 1988). Protein S is the major inducer of TGEV neutralizing antibodies. In this protein, four antigenic sites (A, B, C, and D) have been defined by mutual competition of monoclonal antibodies (mAbs). Site A is antigenically dominant and has been divided into three antigenic subsites: Aa, Ab, and Ac (Correa et al., 1988). Three of the antigenic sites overlap with the sites described by others (Correa et al., 1990; Delmas et al., 1990). Sites A and D, and to a minor extent site B, have been involved in the neutralization of TGEV (Delmas et al., 1986; Jiménez et al., 1986), and the mechanisms of TGEV neutralization have been described (Suñé et al., 1990).

We have focused on the analysis of the different antigenic sites because of their importance in determining the molecular basis of virulence and immunoprotection against TGE. More specifically, we have studied the correlation between the antigenic and physical structure of the S glycoprotein. Since protection against TGE requires lactogenic immunity, the presentation of the selected antigens to the immune system by vectors with tropism for gut associated lymphoid tissues (GALT), such as *Salmonella typhimurium* and adenovirus, has been selected as a convenient immunization strategy.

MATERIALS AND METHODS

Cells, viruses, mAbs, and mAb resistant (mar) mutants

The characteristics of the viruses and the cells on which they were grown, and of mAbs have been reported (Jiménez et al., 1986; Sánchez et al., 1990). For simplicity, the viruses have been frequently named with three letters derived from their place of isolation and two digits which refer to the year of isolation. TGEV titration, neutralization and purification, were performed as described (Correa et al., 1988; Correa et al., 1990). *Mar* mutants were selected and characterized as described (Jiménez et al., 1986; Correa et al., 1988).

Radioimmunoassay (RIA), competitive RIA (cRIA) and epitope scanning (PEPSCAN)

The procedures for the RIA and cRIA have been described previously (Correa et al., 1988). Optimum amounts of antigen (between 0.2 and 1.0 μg of protein per well) were used in the RIA and cRIA. PEPSCAN was performed with nonapeptides as described (Posthumus et al., 1990).

DNA and RNA sequencing

DNA from Bluescript-TGEV plasmids and RNA purified from virions were sequenced by oligodeoxynucleotide primer extension and dideoxynucleotide chain termination procedure (Sanger et al., 1977; Fichot and Girard, 1990). Sequence data were assembled and analyzed using the computer programs of the Genetics Computer Group (University of Wisconsin).

Phylogenetic tree

Sequence information was analyzed following standard phylogenetic methods (Sánchez et al., 1992). The number of substitutions between each pair of nucleotide was estimated as described (Jukes and Cantor, 1969). Two gaps introduced to align the sequences were excluded from the calculations. The epidemiological tree was constructed by the neighbour-joining method as implemented in the program TREEDIST (Saituo and Nei, 1987; Sourdis and Nei, 1988), and by the least squares method as implemented in the program FITCH from the PHYLIP package, version 3.3 (Felsenstein, 1990).

Cloning and expression of TGEV S gene in S. typhimurium and adenoviruses

S gene from TGEV and from *mar* mutants was first cloned into Bluescript SK-following customer (Stratagene) and standard procedures (Gebauer et al., 1991; Sambrook et al., 1989; Ausubel et al., 1991). Expression in avirulent forms of *Salmonella typhimurium* Δ *cya*, Δ *crp*, Δ *asd* (Curtiss et al., 1990; Curtiss and Kelly, 1987; Nakayama et al., 1988) was made by cloning S gene on plasmid pYA292. The indicated nucleotide sequences of the S protein (Fig. 5) were subcloned into plasmid pYA292 and transfected into *E. coli* χ 6097F' [Δ *asd* Δ (*pro-lac*) Φ 80 *d lacZ* Δ M15]. Plasmid DNA was modified by a transforming intermediate *S. typhimurium* χ 3730 [Δ *asd hsdLT hsdSA hsdSB*] defective in restriction systems. Modified plasmids were transfected into *S. typhimurium* χ 3987 [Δ *asd* Δ *cya* Δ *crp*] where TGEV S gene fragments were constitutively expressed.

Cloning on human adenovirus 5 (Ad5) was performed using plasmids pSV2X3, pFG144K3, pAB14 and pFG173 following described procedures (Graham and Prevec, 1992). The indicated S gene fragments (Fig. 6) were flanked by the SV-40 promoter, termination, and polyadenylation signals by cloning the inserts into pSV2X3 plasmid. SV-40 expression cassettes were flanked by Ad5 sequences by subcloning them into the former locations of the deleted E3 gene of Ad5 using plasmids pFG144K3 and pAB14, which contains the 3'-end Ad5 sequences. Recombinant Ad5-TGEVs were rescued by cotransformation of human 293 cells by plasmids containing TGEV sequences and pFG173.

RESULTS

Residues involved in the antigenic sites of TGEV S glycoprotein

The residues contributing to the four antigenic sites of S glycoprotein were localized by sequence analysis of 21 mutants that escaped neutralization or binding by TGEV-specific mAbs, and by epitope scanning (Fig. 1) (Gebauer et al., 1991; Geysen et al., 1984). Site A includes amino acids 538, 591 and 543, which are essential in the formation of subsites Aa, Ab and Ac, respectively. In addition, changes in amino acid 586 have partially altered both subsite Aa and Ab, indicating that these subsites overlap in residue 586. Other amino acids contribute to site B (residues 97 and 144), site C (residues 50 and 51), and site D (residue 385). The location of site D by sequence analysis is in agreement with epitope scanning techniques. Site C can be represented by peptides 48-P-P/S-N-S-D/E-52, it is not exposed on the surface of native virus, but its accessibility can be modulated by treatment at pH > 11 (at 4°C) or temperatures > 45°C. Sites A and B are fully dependent on glycosylation for proper folding, while sites C and D are independent of glycosyla-

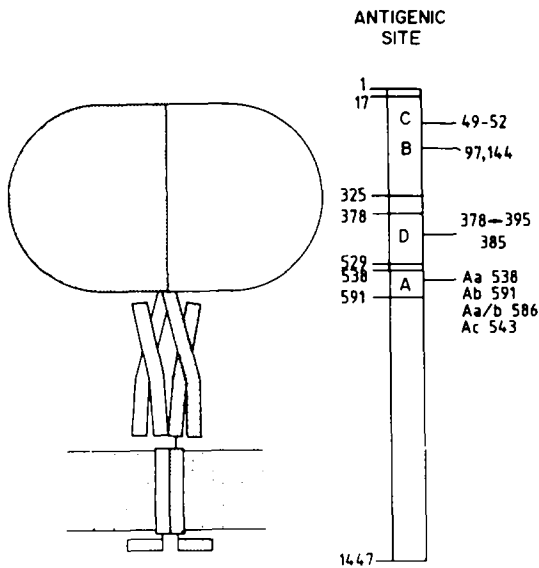


Fig. 1. Correlation between the antigenic and physical structure of the TGEV S glycoprotein. A diagram of the peplomer protein is shown (left side). The column (right side) represents the S protein (NH₂-terminus on top). Capital letters indicate the antigenic sites, small letters the antigenic subsites, and the numbers on the left of the column the segments where these sites were previously located (Correa et al., 1990). Numbers on the right side of the column show the residues involved in the different antigenic sites (Gebauer et al., 1991).

tion, respectively. Once the S glycoprotein has been assembled into the virion, the carbohydrate moiety is not essential for the antigenic sites.

Antigenic and genetic evolution of TGEV

The antigenic homology of 26 coronavirus isolates, of which 22 were related to TGEV has been determined with 42 mAbs (Sánchez et al., 1990). On the S protein, antigenic site A was highly conserved. Subsite Ac contains interspecies specific epitopes common to porcine, feline and canine coronaviruses. The other sites could be classified according to their degree of variability in the order C > B > D. Proteins N and M of these isolates shared many antigenic determinants. Binding of mAbs to different coronaviruses strongly suggests the classification of the human coronavirus 229E in a taxonomic cluster distinct from TGEV group.

Sequencing studies, in which 13 isolates of TGEV and PRCV were analyzed (Sánchez et al., 1992) showed that S proteins of the European and American PRCV isolates have deletions of 224 and 227 amino acids, starting at residues 21 and 22, respectively (Fig. 2) (Rasschaert et al., 1990; Sánchez et al., 1992; Wesley et al., 1991). The amino acid homology was higher than 98% either among the TGEV or the PRCV isolates. In contrast, the homology between TGEVs and PRCVs was around 1% lower (Sánchez et al., 1992). Although this percentage difference is small, the almost identical location of the residues changed, makes this difference significant. The same overall degree of homology was found in the globular and fibrillar areas of the S protein. Two conserved domains were identified in the globular portion of the peplomer protein of TGEVs and PRCVs, when the number of amino acid residues changed was plotted versus their position in the sequence of the S protein. The most conserved domain mapped between residues 181 and 243 of

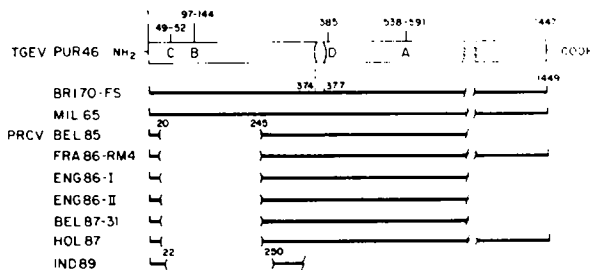


Fig. 2. Summary of the deletions present in S glycoprotein of TGEVs and PRCVs. Letters and numbers show the antigenic sites and the amino acid residues on the protein, respectively. The position of the deletions is indicated by brackets. Virus sequences were taken from different publications (Britton and Page, 1990; Gebauer et al., 1991; Rasschaert et al., 1990; DeGroot et al, 1987; Sánchez et al., 1992). The first six PRCV isolates listed were isolated in Europe, and the seventh one (IND89) in North America.

PRCV. The second most conserved area was located between amino acids 303 and 337 of PRCV (Sánchez et al., 1992).

Based on the nucleotide sequence of the globular area of the spike protein of 6 PRCVs and 6 TGEVs, which were aligned for maximum fitness, an evolutionary tree has been proposed (Fig. 3) (Sánchez et al., 1992). This epidemiological tree predicts that the PUR46 isolate was originated from a putative recent progenitor, named TGEV-1, circulating around 1941. Since then, from a main lineage, the isolates TOY56, MIL65, BRI70, and later the PRCVs were derived. At least in three cases (TOY56, MIL65, and BEL85), it can be assured with a significance of 99.9%, that for the set of viruses considered, they are lateral lineages derived from the main one. A mutation fixation rate of 0.95 ± 0.05 substitutions per year can be estimated for this evolutionary tree.

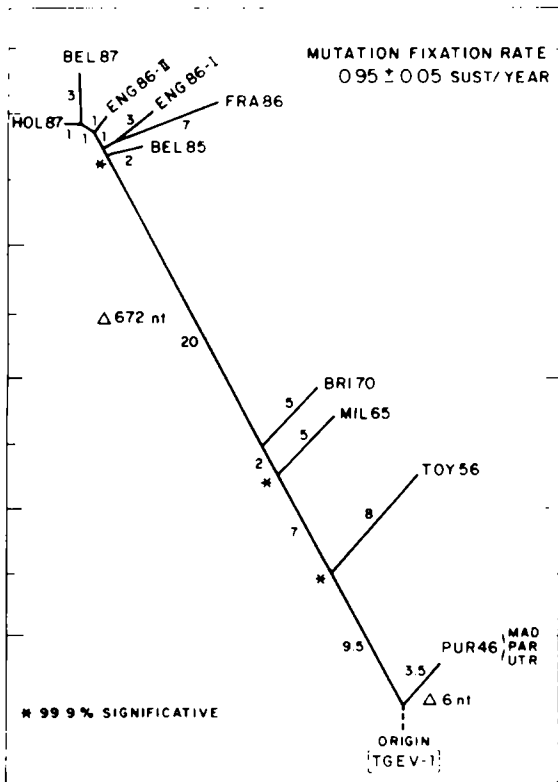


Fig. 3. Evolutionary tree of TGEV related coronaviruses. The diagram shows the derivations of secondary virus lineages from a main one. Numbers in the tree indicate residue substitutions between branching points. Δ, indicates the introduction of a deletion between branching points. *, shows the derivation of a lateral lineage with a significance of 99.9%. The origin of the sequences used is indicated in the legend of Fig. 2.

Receptor binding sites in TGEVs

TGEVs have both enteric and respiratory tropism. In contrast, respiratory viruses as the PRCVs, have an almost exclusive respiratory tropism. Even by directly infecting the intestinal tract with the PRCVs, only a few cells are infected, which are different to the epithelial cells of the villi, susceptible to the enteric viruses (Cox et al., 1990a; Cox et al., 1990b). These results imply that both types of viruses have different tropism. It could be proposed that the enteric or respiratory tropism of TGEV is conditioned by two receptor binding sites (RBS), located in the S protein (Fig. 4). The receptor binding site conferring respiratory tropism (RBS-R) should map in an area present in both TGEVs and PRCVs, while the RBS conferring enteric tropism (RBS-E) would have full activity only in TGEV isolates. Candidate domains for the location of this RBS-R could be the highly conserved areas detected between amino acids 181 to 243, or 303 to 337 (residue numbers correspond to the position that these amino acids have in the PRCV sequence) (Sánchez et al., 1992). In ST (swine testicle) cells, the RBS of TGEV probably maps within sites A and D, since mAbs specific for these sites inhibit the binding of the virus to ST cells (Suñé et al., 1990). The RBS conferring the enteric tropism is probably located between amino acids 21 to 244, or a domain structurally influenced by one of these residues, since they were absent in all PRCV isolates sequenced (Fig. 2) (Rasschaert et al., 1990; Sánchez et al., 1992; Wesley et al., 1991). Alternative explanations to understand the tropism of these viruses are also possible (see Discussion).

Representation of complex sites

Antigenic sites A and B are conformational and dependent on glycosylation (Gebauer et al., 1991). Antigenic domains of these sites may be represented by small peptides. Subsite Ac is represented, at least partially, by peptide 537-MKSGYGQPIA-547, recognized by MAb 1A.F10 in the PEPSCAN assay (Gebauer et al., 1991). This peptide might be of importance for diagnosis and protection, since epitope 1A.F10 is located in subsite Ac, which is conserved in coronaviruses of three animal species (Sánchez et al., 1990). Using

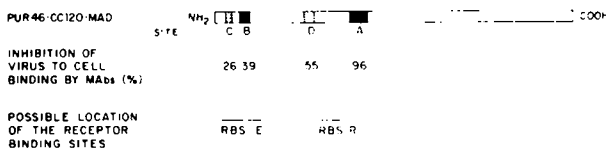


Fig. 4. Hypothetical location in the S glycoprotein of receptor binding sites conferring respiratory (RBS-R) or enteric (RBS-E) tropism. Letters below the diagram show the position of the antigenic sites (Correa et al., 1990; Gebauer et al., 1991). The inhibition (%) of the binding of TGEV, strain PUR46-CC120-MAD, to ST cells is shown in the second line. Alternative models are not excluded (see Discussion).

a recombinant DNA library expressing oligopeptides with a random sequence, we have selected hexapeptides mimicking sites B, C, and D (Lenstra et al., 1992). These synthetic peptides frequently are useful for diagnostic purposes, although their use as immunogens is less obvious.

MAb 6A.C3, specific for subsite Ac of S glycoprotein, neutralizes TGEV with high efficiency and recognizes an inter species-specific epitope. This mAb was used to induce internal image, anti-idiotypic MABs mimicking epitope 6A.C3. These β -type anti-idiotypic mAbs induced in vivo TGEV neutralizing antibodies (Suñé et al., 1991), and might be relevant in protection against TGE.

Cloning and expression of TGEV antigens in vectors to induce mucosal immunity

Secretory immunity is best induced by antigen presentation in GALT. An strategy to induce lactogenic immunity to protect against TGEV, might be the presentation of viral antigens using prokaryotic and eukaryotic vectors with tropism for Peyer's patches. We have selected *S typhimurium* and adenovirus to present TGEV antigens to the immune system.

Avirulent forms of *S. typhimurium* with potential use as vaccine carriers have been developed (Curtiss III et al., 1990; Curtiss III and Kelly, 1987; Nakayama et al., 1988). These bacteria are double mutants, Δ *cyd* and Δ *crp*, unable to produce cAMP or its receptor (CRP). These mutants induce high immunity levels in mucosae and secretions. A third mutation, Δ *asd*, introduced in *Salmonella*, affects the synthesis of diaminopimelic acid, an essential component of the bacterial wall, rendering non-viable bacteria. Cell viability can be obtained by transforming the *asd*⁻ bacteria with expression plasmids *asd*⁺, as pYA292. This plasmid codes for *lacZ* gene, under the control of *trc* promoter. Three fragments of TGEV S gene, which taken together code for the whole S protein (Fig. 5) were cloned into the 5'-end of gene *lacZ* of three pYA292 plasmids (C. Smerdou, R. Curtiss, and L. Enjuanes, unpublished data). Smooth forms of avirulent *S. typhimurium* (strain χ 3987) were independently transformed with the three plasmids, named: pYA-TS1, pYA-TS2, and pYA-TS3. In vivo protection studies using the transformed *Salmonella* are in progress.

A eukaryotic vector, Ad5, has also been used to express TGEV antigens in mammalian cells both in vitro and in vivo. This expression system might provide the glycosylation pattern needed to express properly antigens like sites A and B, which are fully dependent on glycosylation for proper folding. Four DNA segments, coding for the whole S gene and for three truncated forms of this gene have been cloned on Ad5 (Fig. 6) (C. Suñé, C.M. Sánchez, F.L. Graham, and L. Enjuanes, unpublished results). Recombinant Ad5 virus containing TGEV sequences were rescued after cotransformation of human 293 cells with two plasmids coding for the left and right portion of human

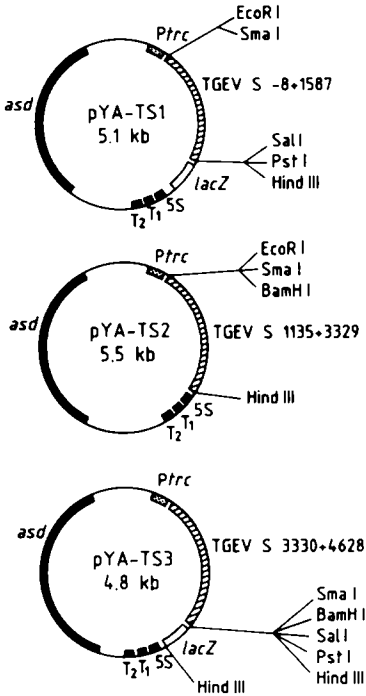


Fig. 5. Recombinant plasmids derived from plasmid pYA292 with TGEV S glycoprotein sequences. The flanking restriction endonuclease sites and nucleotides of the S gene fragment, cloned into each plasmid, are indicated next to the dashed segments. Ptrc, trc promoter, asd, sequences complementing mutant phenotypes with requirement for threonine, methionine, and diaminopimelic acid; lacZ, β -galactosidase gene; 5S, the 5S rRNA gene; T1 and T2, the rrn-BT1T2 transcription terminators (Nakayama, et al., 1988; C. Smerdou, R. Curtiss III, and L. Enjuanes, unpublished results).

adenovirus (Graham and Prevec, 1991). We have shown that porcine cell lines fully support the replication of the recombinant Ad5 with high efficiency. At present, we are evaluating the protection against TGE provided by these recombinant viruses.

DISCUSSION

The antigenic structure of the S glycoprotein of TGEV and its correlation with the physical structure has been described. In addition, the genetic homology among viruses of the TGEV antigenic cluster has been shown, and an evolutionary tree has been proposed, to establish the relationship among these viruses and their possible origin. Information on the antigenic structure and the conservation of the different sites involved in virus neutralization, allowed the selection of several antigenic determinants potentially involved in

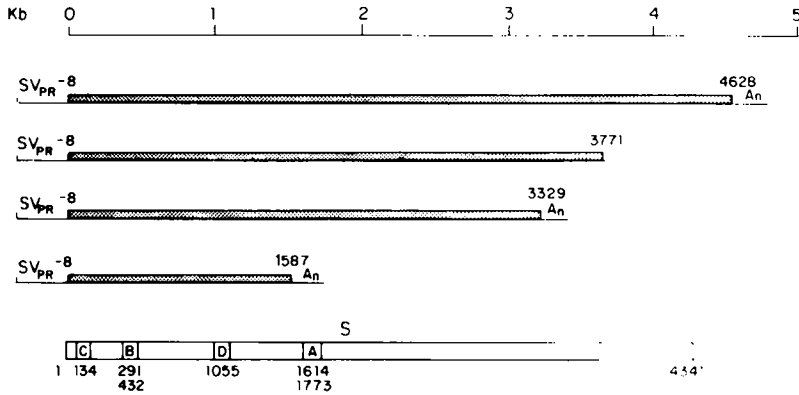


Fig. 6. S gene fragments cloned into Ad5 virus. The nucleotides flanking the sequences cloned into human Ad5 virus are indicated in both sides of the dotted bars. The S gene sequences were cloned into an expression cassette consisting of the SV-40 promoter (SV_{PR}) and polyadenylation and termination signals (A_n). Letters and numbers in the empty bar indicate the name and nucleotides included in the antigenic sites (C. Suñé, C.M. Sánchez, F.L. Graham, and L. Enjuanes, unpublished results).

protection. S gene segments coding for these sites have been cloned in vectors with tropism for Peyer's patches, such as *S. typhimurium* and adenovirus, to test their role in protection against TGEV.

The enteropathogenic TGEV was first isolated in 1946 (Doyle and Hutchings, 1946). In 1984, a non-enteropathogenic virus, the PRCV, related to TGEV appeared. In contrast to TGEV, PRCV exhibited no clinical signs of disease (Pensaert et al., 1986; Callebaut et al., 1988). The antigenic homology among isolates of TGEV and PRCV has been clearly documented (Callebaut et al., 1988; Garwes et al., 1988; Rasschaert et al., 1990; Sánchez et al., 1990; Wesley et al., 1990). The analysis of the antigenic structure of TGEV S protein has shown that the half amino terminus is highly immunogenic and that it contains the four antigenic sites defined by two groups (Delmas et al., 1986; Correa et al., 1988; Correa et al., 1990). In contrast, the PRCVs have lost sites B and C, due to a deletion of 224 or 227 amino acids in the S protein. This type of deletion has not been found in the fibrillar portion of the peplomer. Sites A and D are present in both enteric and respiratory viruses, and they are the major inducers of neutralizing antibodies. Hence, it could be expected that the variability in the globular portion of the S molecule might be higher than in the fibrillar domain. Although a few more nucleotide changes were concentrated in these sites, the overall homology in the globular and fibrillar domains was the same (Sánchez et al., 1992).

A hypothetical receptor binding site conferring tropism for respiratory tissues has been located between sites A and D, as described above. The RBS-E conferring enteric tropism has been proposed to be located in the area missing

in the PRCVs, which do not infect enteric tissues. Nevertheless, it can not be excluded that this RBS-E would be located in another domain that requires the presence of amino acids 21 to 249 to keep its functionality. An alternative explanation is the existence of a RBS which could adopt two conformations, depending on the sequence of distal domains of the S glycoprotein. These functional models assume that the ability of a virus to infect respiratory or enteric tissues, only depends on the presence of a receptor in the host cell which will recognize a RBS present on the virus. In contrast, it cannot be excluded that the expression of other genes such as those coding for the non-structural proteins, is involved in the tropism of these viruses. Hence, more information is required before a definite location of the RBS(s) is made.

Based on sequence data and evolutionary tree has been proposed relating TGEVs and PRCVs (Fig. 3) (Sánchez et al., 1992). Since we are dealing with a limited number of isolates from each continent, it might be anticipated that the inclusion of new isolates from other areas (e.g., Japan) could show that certain lateral branches would become main branches. A mutation fixation rate of 0.95 ± 0.05 nucleotide substitutions per year, approximately constant with time has been shown, suggesting the existence of a well defined molecular clock (Kimura, 1983). From the mutation fixation rate, and the number of nucleotides considered, the number of substitutions per nucleotide and year can be estimated at $7 \pm 2 \times 10^{-4}$. This rate falls into the range reported for other RNA viruses (Domingo and Holland, 1988).

Two types of PRCVs have emerged since 1984, one in Europe and the other in North America. Both have a large deletion of different size, located in a slightly different position in the NH₂-end of the S glycoprotein (Fig. 2), suggesting that they were independently derived. This data indicates that around nucleotide 60 on the S gene, there might be a site susceptible to undergoing a recombination/deletion event. Hence, two mechanisms are possibly involved in the evolution of TGEV, recombination and point mutation.

Protection against TGE requires mucosal immunity. Two vector systems have been selected to focus the antigen in GALT, *S. typhimurium* and human adenovirus. Antibodies against these vectors are not usually present in adult swine, when the immunization has to be provided to pregnant sows. The use of the corresponding porcine strains (i.e. *S. cholerae-suis* or porcine adenoviruses) might not be adequate since the immunological status of the adult animal might prevent the transitory colonization or infection of the GALT.

ACKNOWLEDGEMENTS

We are grateful to J. Palacín for his excellent technical assistance. C. Suñé, F.G., C. Smerdou and I.M.A. received fellowships from the Spanish Ministry of Education and Science. This investigation has been founded by grants from the Comisión Interministerial de Ciencia y Tecnología (Project BIO 0214),

European Community (Project BAP 0464 E), NATO (Project CRG 900430), and Fundación Ramón Areces.

REFERENCES

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore D.D., Seidman, J.G., Smith, J.A. and Struhl, K., 1991. Current protocols in molecular biology. Wiley Inc. New York.
- Britton, P. and Page, K.W., 1990. Sequence of the S-Gene from a virulent british field isolate of transmissible gastroenteritis virus. *Virus Res.* 18: 71–80.
- 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.
- Correa, I., Gebauer, F., Bullido, M.J., Suñé, C., Baay, M.F.D., Zwaagstra, K.A., Posthumus, W.P.A., Lenstra, J.A. and Enjuanes, L., 1990. Localization of antigenic sites of the E2 glycoprotein of transmissible gastroenteritis coronavirus. *J. Gen. Virol.*, 71: 271–279.
- Correa, I., Jiménez, G., Suñé, C., Bullido, M.J. and Enjuanes, L., 1988. Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus. *Vir. Res.*, 10: 77–94.
- Cos, E., Hooyberghs, J. and Pensaert, M.B., 1990a. Sites of replication of a porcine respiratory coronavirus related to transmissible gastroenteritis virus. *Res. Vet. Sci.*, 48: 165–169.
- Cos, E., Pensaert, M.B., Callebaut, P. and van Deun, K., 1990b. Intestinal replication of a porcine respiratory coronavirus closely related antigenically to the enteric transmissible gastroenteritis virus. *Vet. Microbiol.*, 23: 237–243.
- Curtiss III, R., Galan, J.E., Nakayama, K. and Kelly, S.M., 1990. Stabilization of recombinant avirulent strains in vivo. *Res. Microbiol.*, 141: 797–805.
- Curtiss III, R. and Kelly, M.S., 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infect. Immun.*, 55: 3035–3043.
- De Groot, R.J., Luytjes, W., Horzinek, M.C., Van der Zeijst, B.A.M., Spaan, W.J.M. and Lenstra, J.A., 1987. Evidence for a coiled-coil structure in the spike protein of coronaviruses. *J. Mol. Biol.*, 196: 963–966.
- Delmas, B., Gelfi, J. and Laude, H., 1986. Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. *J. Gen. Virol.*, 67: 1405–1418.
- Delmas, B., Rasschaert, D., Godet, M., Gelfi, J. and Laude, H., 1990. Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike protein. *J. Gen. Virol.*, 71: 1313–1323.
- Domingo, E. and Holland, J.J., 1988. High error rates, population equilibrium and evolution of RNA replication systems. In: E. Domingo, J.J. Holland, and P. Ahlquist, (editors), *RNA genetics*, CRC Press, Boca Ratón, Florida, pp. 3–36.
- Doyle, L.P. and Hutchings, L.M., 1946. A transmissible gastroenteritis in pigs. *J. Am. Vet. Med. Assoc.*, 108: 257–259.
- Felsenstein, J., 1990. *PHYLIP manual version 3.3*. University Herbarium University of California, Berkeley, California.
- Fichot, O. and Girard, M., 1990. An improved method for sequencing of RNA templates. *Nucleic Acids Res.*, 18: 6162.
- Garwes, D.J., Stewart, F., Cartwright, S.F. and Brown, I., 1988. Differentiation of porcine coronavirus from transmissible gastroenteritis virus. *Vet. Rec.*, 122: 86–87.
- Gebauer, F., Posthumus, W.A.P., Correa, I., Suñé, C., Sánchez, C.M., Smerdou, C., Lenstra, J.A., Meloen, 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.

- Geysen, N.M., Meloen, R.H. and Barteling, S.J., 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. Natl. Acad. Sci. USA*, 81: 3998-4002.
- Graham, F.L. and Prevec, L., 1991. Manipulation of Adenovirus vectors. In: (E.J. Murray and J.M. Walker (editors), *Methods in Molecular Biology*, vol. 7. Gene transfer and expression techniques, Humana Press. Clifton, New Jersey: pp 109-128..
- 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.
- Jukes, T.H. and Cantor, C.R., 1969. Evolution of protein molecules. In: H.N. Munro (editor), *Mammalian protein metabolism*, Academic Press, New York, pp. 21-132.
- Kimura, M., 1983. *The neutral theory of molecular evolution*. Cambridge University Press, Cambridge.
- Lenstra, J.A., Erkens, J.H.F., Zwaagstra, K.A., Posthumus, W.P.A., Meloen, R.H., Gebauer, F., Enjuanes, L. and Stanley, K.K., 1992. Selection of mimotopes from a random sequence expression library by monoclonal antibodies against transmissible gastroenteritis coronavirus. *J. Immunol. Methods*, 52: 149-157.
- Moxley, R.A., Olson, L.D. and Solorzano, R.F., 1989. Relationship among transmissible gastroenteritis virus antibody titers in serum, colostrum, and milk from vaccinated sows, and protection in their suckling pigs. *Am. J. Vet. Res.*, 50: 119-125.
- Nakayama, K., Kelly, S.M. and Curtiss III, R., 1988. Construction of an *asd*⁺ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. *Biotechnology*, 6: 693-697.
- Pensaert, M., Callebaut, P. and Vergote, J., 1986. Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis. *Vet. Q.*, 8: 257-260.
- Posthumus, W.P.A., Lenstra, J.A., Schaaper, W.M.M., van Nicuwstadt, A.P., Enjuanes, L. and Meloen, R.H., 1990. Analysis and simulation of a neutralizing epitope of transmissible gastroenteritis virus. *J. Virol.*, 64: 3304-3309.
- 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.
- Saituo, N.M. and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.
- Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Sánchez, C.M., Gebauer, F., Suñé, C., Dopazo, J. and Enjuanes, L., 1992. Genetic evolution of transmissible gastroenteritis coronaviruses. *Virology*, Vol. 190.
- Sánchez, C.M., Jiménez, G., Laviada, M.D., Gorrea, 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.
- Sanger, F., Nicklen, S. and Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, 74: 5463-5467.
- Sourdis, J. and Nei, M., 1988. Relative efficiencies of the maximum parsimony and distance-matrix methods in obtaining the correct phylogenetic tree. *Mol. Biol. Evol.*, 45: 298-311.
- Spaan, W., Cavanagh, D. and Horzinek, M.C., 1988. Coronaviruses: structure and genome expression. *J. Gen. Virol.*, 69: 2939-2952.
- Suñé, 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.
- Suñé, C., Smerdou, C., Antón, I.M., Abril, P., Plana, J. and Enjuanes, L., 1991. Anti-idiotypic monoclonal antibodies mimicking an inter species-specific epitope critical in coronavirus neutralization. *J. Virol.*, 65: 6969-6978.
- 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., 1990. Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus, in the United States. *J. Vet. Diagn. Invest.*, 2: 312–317.