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Residues Involved in the Antigenic Sites of Transmissible Gastroenteritis Coronavirus S Glycoprotein

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The S glycoprotein of transmissible gastroenteritis virus (TGEV) has been shown to contain four major antigenic sites (A, B, C, and D). Site A is the main inducer of neutralizing antibodies and has been previously subdivided into the three subsites Aa, Ab, and Ac. The residues that contribute to these sites were localized by sequence analysis of 21 mutants that escaped neutralization or binding by TGEV-specific monoclonal antibodies (MAbs), and by epitope scanning (PEPSCAN). Site A contains the residues 538, 591, and 543, which are essential in the formation of subsites Aa, Ab, and Ac, respectively. In addition, *mar* mutant 1B.H6 with residue 586 changed had partially altered both subsite Aa and Ab, indicating that these subsites overlap in residue 586; i.e. this residue also is part of site A. The peptide 537-MKSGYGQPIA-547 represents, at least partially, subsite Ac which is highly conserved among coronaviruses. This site is relevant for diagnosis and could be of interest for protection. Other residues contribute to site B (residues 97 and 144), site C (residues 50 and 51), and site D (residue 385). The location of site D is in agreement with PEPSCAN results. Site C can be represented by the peptide 48-P-P/S-N-S-D/E-52 but is not exposed on the surface of native virus. Its accessibility can be modulated by treatment at pH >11 (at 4°) and temperatures >45°. Sites A and B are fully dependent on glycosylation for proper folding, while sites C and D are fully or partially independent of glycosylation, respectively. Once the S glycoprotein has been assembled into the virion, the carbohydrate moiety is not essential for the antigenic sites. © 1991 Academic Press, Inc.

INTRODUCTION

Within the Coronaviridae family, transmissible gastroenteritis virus forms an antigenic cluster with feline infectious peritonitis virus (FIPV), feline enteric coronavirus (FECV), canine coronavirus (CCV), and porcine respiratory coronavirus (PRCV) (Sánchez *et al.*, 1990). TGEV has three structural proteins: the spike protein (S), the nucleoprotein (N), and the membrane (M) protein (Spaan *et al.*, 1988, 1990; Enjuanes and Van der Zeijst, 1991). The S protein forms the peplomers of the virus and consists of 1447 or 1449 amino acids, depending on the viral strain (Rasschaert and Laude, 1987; Jacobs *et al.*, 1987; Wesley, 1990; Britton *et al.*, 1990). Several biological activities have been associated with the S protein: (i) It is the major inducer of neutralizing antibodies and is involved in protection (Garwes *et al.*, 1978; Jiménez *et al.*, 1986; Delmas *et al.*, 1986); (ii) it includes the receptor binding site and determines viral tropism (Holmes *et al.*, 1981; Collins *et al.*, 1982; Suñé *et al.*, 1990); (iii) it is involved in pathogenicity (Taguchi and Fleming, 1989); (iv) it may induce cell fusion (Collins *et al.*, 1982; Sturman *et al.*, 1985; DeGroot *et al.*, 1989); and (v) it has hemagglutinating activity (Holmes *et al.*, 1989).

Type-, group-, and interspecies-specific antigenic determinants have been described for the S glycoprotein of TGEV (Sánchez *et al.*, 1990). In this protein, four antigenic sites (A, B, C, and D) have been defined by mutual competition of 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 sites described by others (Delmas *et al.*, 1990; Correa *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.

Previously, we have located these four sites within the 543 N-terminal amino acids of the S protein (Correa *et al.*, 1990). Site D has been analyzed by epitope scanning (PEPSCAN) (Posthumus *et al.*, 1990). In the present study we have: (i) isolated and characterized 21 TGEV mutants, each deficient in one of the antigenic sites; (ii) identified residues involved in each site by determining the nucleotide differences between the wild-type (*wt*) virus and mutant viruses; (iii) identified

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TABLE 1

NUCLEOTIDE SEQUENCE DIFFERENCES BETWEEN TGEV-*wt* AND TGEV-*mar* MUTANTS

Clone	Nucleic acid Sequenced	Antigenic subsite specificity of MAb[d]	Nucleotide sequence		Base changed	Amino acid				
			In *wt* virus	In *mar* mutant		538	543	586	591	631
BLUESCRIPT. PUR46*wt*	DNA					K	G	D	R	V
PUR46*wt*	RNA					K	G	D	R	V
PUR46.*mar* 1G.A7	RNA	Aa	AAG	AUG	1613	M				
PUR46.*mar* 1B.C1	RNA	Aa	AAG	ACG	1613	T				
PUR46.*mar* 1D.B3	RNA	Aa	AAG	ACG	1613	T				
PUR46.*mar* 1G.A6	RNA	Aa	AAG	ACG	1613	T				
PUR46.*mar* 1C.C12	RNA	Aa	AAG	ACG	1613	T				
PUR46.*mar* 1E.H8	RNA	Aa	AAG	ACG	1613	T				
PUR46.*mar* 1E.F9	RNA	Aa	AAG	AUG	1613	M				
PUR46.*mar* 1D.E7	RNA	Ab	CGA	CAA	1772				Q	
PUR46.*mar* 1H.D2	RNA	Ab	CGA	CCA	1772				P	
PUR46.*mar* 1B.H6[a]	RNA	Aa/Ab	GAC	AAC	1756			N		
PUR46.*mar* 1B.B5	RNA	Ac	GGU	GAU	1628		D			A
			GUU	GCU	1892					
BLUESCRIPT.PUR46	DNA	Ac & Aa	AAG	CAG	1612	Q	D			A
dmar 1B.B5–1B.B1[b]			GGT	GAT	1628					
			GTT	GCT	1892					
PUR46.*dmar* 1B.B5–1B.B1[c]	RNA	Ac & Aa	AAG	CAG	1612	Q	D			A
			GGU	GAU	1628					
			GUU	GCU	1892					
PUR46.*dmar* 1B.B5–1D.E7	RNA	Ac & Ab	GGT	GAT	1628		D	N		A
			GAC	AAC	1756					
			GTT	GCT	1892					

[a] The mutant *mar* 1B.H6 has altered subsites a and b. The antigenic pattern of this *mar* mutant is shown in Figs. 1 and 2.

[b] The sequence of the 5'-ends 2×10^3 nucleotides of the PUR46-CC120-MAD strain of TGE virus and of the *dmar* 1B.B5–1B.B1 mutant were obtained using cDNAs cloned in the Bluescript plasmid.

[c] RNA sequencing was performed on RNA from purified virions.

[d] The antigenic subsites were defined as described by Correa *et al.* (1988 and 1990).

sequences, by epitope scanning (Geysen *et al.*, 1984), that could represent antigenic sites A and C; and (iv) described the glycosylation dependence and surface exposure of the sites.

MATERIALS AND METHODS

Cells, viruses, and MAbs

TGEV was grown in swine testicle (ST) cells (McClurkin and Norman, 1966). The strain PUR46-CC120-MAD of TGEV (Sánchez *et al.*, 1990) was used to select virus mutants. This strain was cloned five times in our laboratory. The procedure for TGEV neutralization has been described (Correa *et al.*, 1988). The neutralization index was defined as the $\log_{10}$ of the ratio of the PFU after incubating the virus in presence of medium or the indicated MAb. The virus was purified as described (Correa *et al.*, 1988) or partially purified from supernatants of infected cultures. In the latter case, partially purified virus was collected when a CPE of 10 to 50% was observed, by clarification at 6×10^3 rpm (Sorvall GS3 rotor) for 20 min followed by sedimentation at 25×10^3 rpm (Sorvall AH627 rotor) for 1.5 hr at 4°. Mouse hepatitis virus (MHV) A59 strain (kindly provided by K. Holmes) was grown on 3T3 cells and purified as described (Sánchez *et al.*, 1990).

The characteristics of the MAbs have been previously described (Jiménez *et al.*, 1986; Correa *et al.*, 1988, 1990; Sánchez *et al.*, 1990; Suñé *et al.*, 1990).

Selection of MAb resistant (*mar*) mutants

mar mutants were selected by neutralizing MAbs as described (Jiménez *et al.*, 1986; Correa *et al.*, 1988) (Table 1). After selection, neutralization titers decreased from 4.8 to less than 0.5. With the non-neutralizing MAbs 1B.H11 and 1D.G3, *mar* mutants were selected via a procedure similar to the one described by Marlin *et al.* (1985). Briefly, 10^7 PFU of TGEV in 50 μl medium were incubated at room temperature for 30 min with 50 μl of MAb supernatant, followed by the addition of 50 μl of a 1:10 dilution of rabbit anti-mouse immunoglobulins (Cappel) in phosphate-buffered sa-

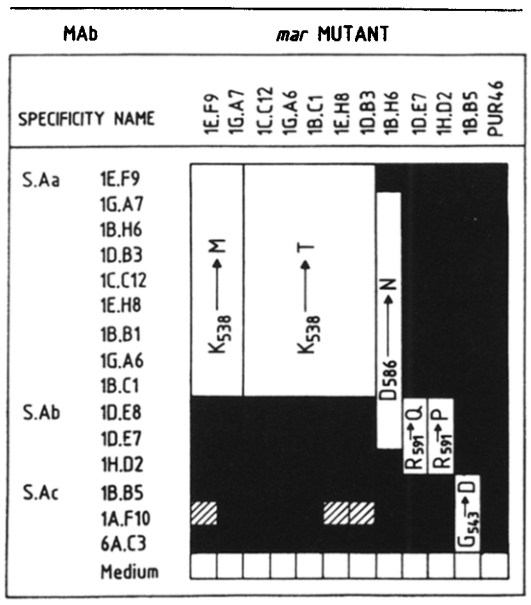

FIG. 1. Neutralization of TGEV *mar* mutants by MAbs and amino acid changes responsible for the escape of *mar* mutants. White, hatched, and black squares represent neutralization index <1, between 1 and 2, and >2, respectively. The amino acid substitutions in the white areas are indicated with the one-letter code.

line (PBS). The mixture was incubated at room temperature for 30 min and used to infect ST cells. After 2 hr of adsorption, unadsorbed virus was washed out and the cells were incubated at 37°. When CPE appeared, supernatant was collected. The whole procedure was repeated a total of five times, and virus was then plated for *in situ* immunoscreening (see below). Unstained plaques were selected, incubated in the presence of MAb and the second antibody as described above, and cloned three times.

Selection of MAb nonbinding (*manb*) mutants

Mutants that escaped binding of MAb 1D.B12 were selected by incubating 10^8 PFU of TGEV in 0.1 ml of medium with 2 μl of ascitic fluid at 37° for 2 hr. After addition of 50 μl of 20% *Staphylococcus aureus* coated with rabbit anti-mouse immunoglobulins and incubation at 4° for 1 hr, the mixture was centrifuged at 10,000 *g* for 10 min. A 50-μl sample of *S. aureus* was added, and the cycle was repeated two more times. The supernatant was used to infect ST cells, which were incubated at 37° overnight. One hundred microliters of the supernatant was collected and the whole procedure repeated four more times. Selected viruses were cloned three times and characterized by *in situ* immunoscreening (IMS) as previously described (Diez *et al.*, 1989). Briefly, the plaques were recognized by a MAb in an immunotest. A nitrocellulose filter was applied to the agar overlay of a TGEV assay to bind infectious virus from individual plaques and stored at −70°. On a second filter, which was placed directly on the cell monolayer, enough virus was bound to permit colorimetric visualization of plaques by an enzyme-linked assay using MAbs. Infectious virus was recovered by soaking the plaque containing filter in PBS with 2% fetal calf serum (FCS).

Antigenic characterization of virus by radioimmunoassay (RIA)

The procedure for the RIA has been previously described (Correa *et al.*, 1988; Sánchez *et al.*, 1990). Optimum amounts of antigen (between 0.2 and 1.0 μg of protein per well) were used in a RIA and in a competitive radioimmunoassay (cRIA).

Epitope scanning

PEPSCAN analysis was performed using all 1439 consecutive overlapping nonapeptides derived from the sequence of the S protein of TGEV (Jacobs *et al.*, 1987; Rasschaert and Laude, 1987). The peptides were synthesized on polyethylene rods and tested as described previously (Geysen *et al.*, 1984; Posthumus *et al.*, 1990). The first peptide consisted of amino acids 1 to 9, the second consisted of amino acids 2 to 10, the third consisted of amino acids 3 to 11, etc. The binding of a MAb to each peptide was tested in an enzyme-linked immunoabsorbent assay (ELISA) and expressed as the optical absorbance at 405 nm. Ascitic fluid dilutions varied from $1:10^3$ to $1:5 \times 10^3$. Binding was plotted vertically against the sequence position of the N-terminal amino acid of the peptide. Overlapping nona- to dodecapeptides from the region consisting of the residues 533 to 553 and peptides in which each amino acid of the parent sequence 537-MKRSGYGQPIA-547 was consecutively replaced by all 19 other amino acids were synthesized and tested in the same way. The amino acid replacements were plotted in alphabetical order: A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, and Y (Fig. 3B).

DNA and RNA sequencing

DNA purified from the Bluescript–TGEV plasmids and RNA purified from virions were sequenced by oligodeoxynucleotide primer extension and dideoxynucleotide chain termination procedures (Sanger *et al.*, 1977; Zimmern and Kaesberg, 1978). For RNA sequencing primers complementary to the S-gene were used. Sequence data were assembled and analyzed using the computer programs of the Genetics Computer Group (University of Wisconsin).

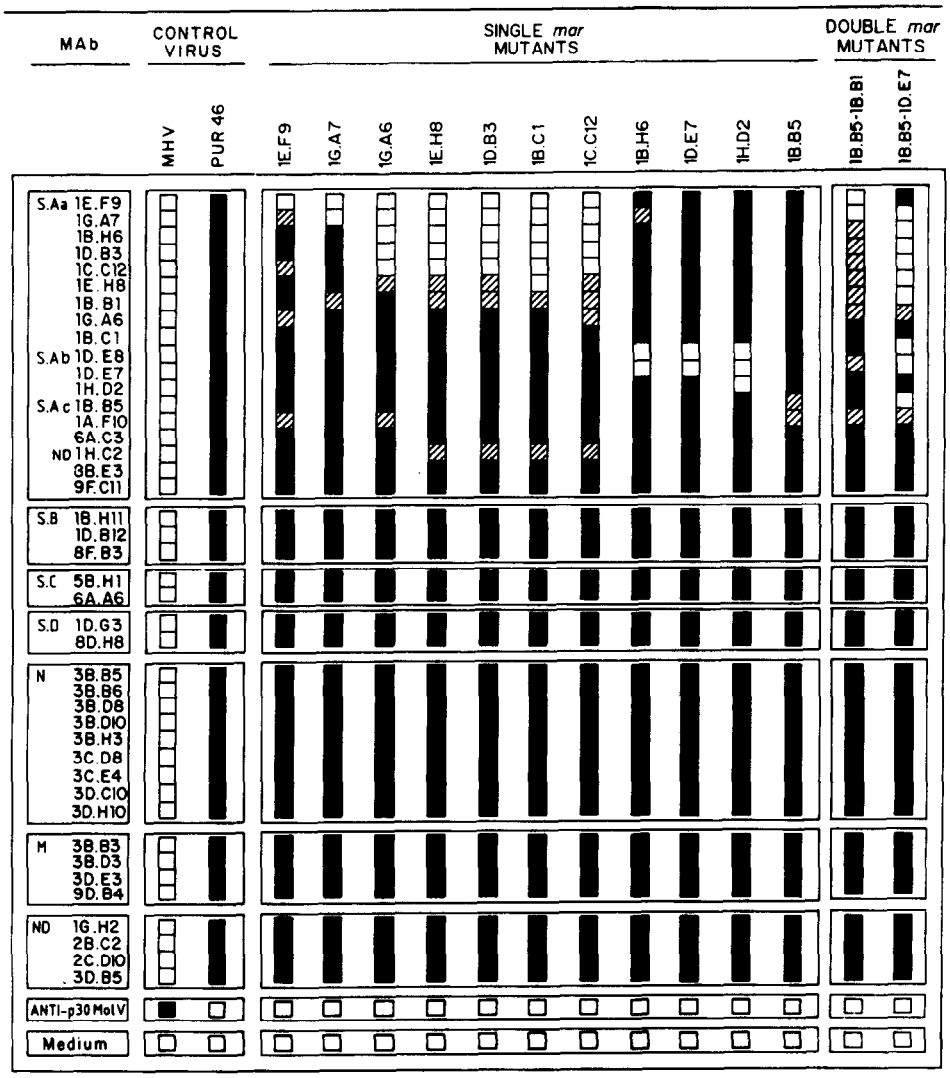

FIG. 2. Binding of MAbs to TGEV derived *mar* mutants. MAb binding was determined by RIA, with binding to strain PUR46-CC120-MAD of TGEV as reference value (100). The *mar* mutants are named according to the MAb used in their selection. The specificity of the *MAbs* is named according to Correa *et al.* (1988). White, hatched, and black squares represent relative binding between 0 to 19, 20 to 40, and 41 to 100, respectively. ND, not determined. MoIV, Moloney leukemia virus. MHV, mouse hepatitis virus.

Determination of the surface exposure of the epitopes by cRIA

The accessibility of epitopes on the virus surface was determined by cRIA. It was assumed that higher surface exposure of one epitope would provide a higher inhibition of the binding of the MAb specific for this epitope to the solid phase bound virus. TGEV antigen was plated as described above. Hybridoma supernatants of the MAbs (50 μl), at a dilution giving 90% of maximum binding, were added to TGEV coated wells and incubated at 37° for 30 min in the presence of twofold dilutions of competitor virus. To measure the binding of the MAbs to the solid-phase bound virus ^{125}I-labeled MAb (5×10^4 cpm/well; 1×10^6 cpm/μl; 1×10^7 cpm/μg protein) was added to wells and incubated at 37° for 1 hr. The radioactivity bound was determined as described for a RIA. A competition curve was generated for each virus sample. The relative exposure of an antigenic site was defined as the percentage of inhibition of binding of a MAb to virus coated wells by a fixed amount of virus in solution (5 μg per well) divided by 10. This value was extrapolated from each competition curve.

Deglycosylation and glycosylation inhibition

The S glycoprotein was purified by phase separation using Triton X-114 (Correa *et al.*, 1988). After dissolution the protein (1 μg/20 μl) in 0.1 *M* sodium acetate,

TABLE 2

FREQUENCY OF *mar* MUTANT ISOLATION

Type of mutant	Starting virus		MAb Used for the selection		Isolation frequency[b] ($-\log_{10}$)
	Name	Modified subsites[a]	Name	Subsite	
Simple	PUR 46	—	1E.F9	Aa	5.1
	PUR 46	—	1G.A7	Aa	5.4
	PUR 46	—	1B.H6	Aa	5.6
	PUR 46	—	1B.B1	Aa	5.6
	PUR 46	—	1D.E7	Ab	5.7
	PUR 46	—	1B.B5	Ac	7.3
	PUR 46	—	6A.C3	Ac	>9.0
Double	*mar* 1G.A7	Aa	1B.B5	Ac	>8.0
	mar 1D.E7	Ab	1B.B1	Aa	6.0
			1G.A6	Aa	6.4
	mar 1B.B5	Ac	1B.B1	Aa	7.2
			1D.E7	Ab	5.8
			6A.C3	Ac	0.0
	mar 1G.A6	Aa	1B.B1	Aa	0.0
Triple	*mar* 1B.B5–1B.B1	Ac/Aa	1D.E7	Ab	>7.0
	mar 1D.E7–1B.B1	Ab/Aa	1B.B5	Ac	>7.0
	mar 1D.E7–1G.A6	Ab/Aa	1B.B5	Ac	>7.0
	mar 1B.B5–1D.E7	Ac/Ab	1B.B1	Aa	0.0
			1B.C1	Aa	0.0
			1B.H6	Aa	0.0
			1G.A7	Aa	0.0

[a] The antigenic subsites were defined according to Correa *et al.* (1988).

[b] The frequency of *mar* mutant isolation was defined as previously described (Jiménez *et al.*, 1986).

pH 7, 0.5% sodium dodecyl sulfate (SDS), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.1 m*M* *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TPCK), and 1 μg/ml pepstatin was deglycosylated by incubation overnight at 37° with protein *N*-glycosidase F (0.04 U/μl; Boehringer Mannheim). The reaction was stopped by freezing. The extent of the deglycosylation was studied by Western blot analysis (Correa *et al.*, 1988) after 7.5% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS without 2-mercaptoethanol.

N-glycosylation was prevented by infecting ST cells with TGEV at a m.o.i. of 10 PFU/cell, in the presence of 5 μg of tunicamycin/ml. At the indicated times, the infected monolayers were scraped off with a rubber policeman and cells and supernatant were separated by low-speed centrifugation. The presence of S protein in these samples was determined by Western blot analysis after 0.1% SDS–PAGE in the absence of 5% 2-mercaptoethanol (unless otherwise indicated) using site-specific MAbs (Correa *et al.*, 1990).

RESULTS

Site A

A collection of 11 single MAb resistant (*mar*) mutants and 2 double mar (*dmar*) mutants has been isolated from the PUR46 strain of TGEV, using MAbs specific for three subsites (Aa, Ab, and Ac) of TGEV S glycoprotein. The nucleotide sequence differences between these mutants and parental virus have been determined by: (i) sequencing the complete S gene cDNA of the PUR46 *wt* strain and the first 2000 nucleotides of the PUR46 *dmar* 1B.B5–1B.B1 cDNA; (ii) direct RNA sequencing of nucleotides from position 1600 to 2000 of all mutants (Table 1); and (iii) direct RNA sequencing of the complete S gene of the *mar* 1D.E7 mutant. The escape of *mar* mutants from neutralization by MAbs specific for subsites Aa, Ab, and Ac was associated with amino acid changes in positions 538, 591, and 543, respectively (Table 1 and Fig. 1). Changes in amino acid 586 from Asp to Asn affected both subsites Aa and Ab. All escape mutants selected by MAb 1B.B5 had an additional amino acid change at position 631.

The *mar* mutants were characterized by neutralization (Fig. 1) and by binding of MAbs (Fig. 2). Mutants selected with site A-specific MAbs, only showed antigenic changes in site A. The mutants selected by the Aa-specific MAbs could be divided into three groups according to their binding pattern (Fig. 2): (i) *mar* 1E.F9 and 1G.A7; (ii) *mar* 1G.A6, 1E.H8, 1D.B3, 1B.C1, and 1C.C12; and (iii) *mar* 1B.H6. The binding patterns correlated well with the amino acid substitutions (Fig. 1). In *mar* 1B.H6, subsites Aa and Ab were affected. *mar* 1B.B5 escaped neutralization by MAbs specific for subsite Ac (1B.B5, 1A.F10, and 6A.C3). However, the epitope of 6A.C3 is probably only partially affected since the binding of *mar* 1B.B5 by MAb 6A.C3 was normal and MAb 6A.C3 neutralized the 1B.B5 mutant at higher concentrations (not shown). In *dmar* 1B.B5–1D.E7 mutant, introduction of changes in subsites Ab and Ac also affected subsite Aa (Fig. 2). In *dmar* 1B.B5–1B.B1 the accumulation of mutations in subsites Aa and Ac caused a minor alteration in subsite Ab (Fig. 2). The frequency of single *mar* mutant isolation in site A was generally between 10^{-5} to 10^{-6} (Table 2). When a second mutation was accumulated in another antigenic subsite, the isolation frequency was generally about 10-fold lower than for the first mutation. When the two first mutations were localized in subsites Ac/Aa or Ab/Aa, it was not possible to select triple *mar* mutants with changes accumulated in the three antigenic subsites (Table 2). In contrast, the *dmar* 1B.B5–

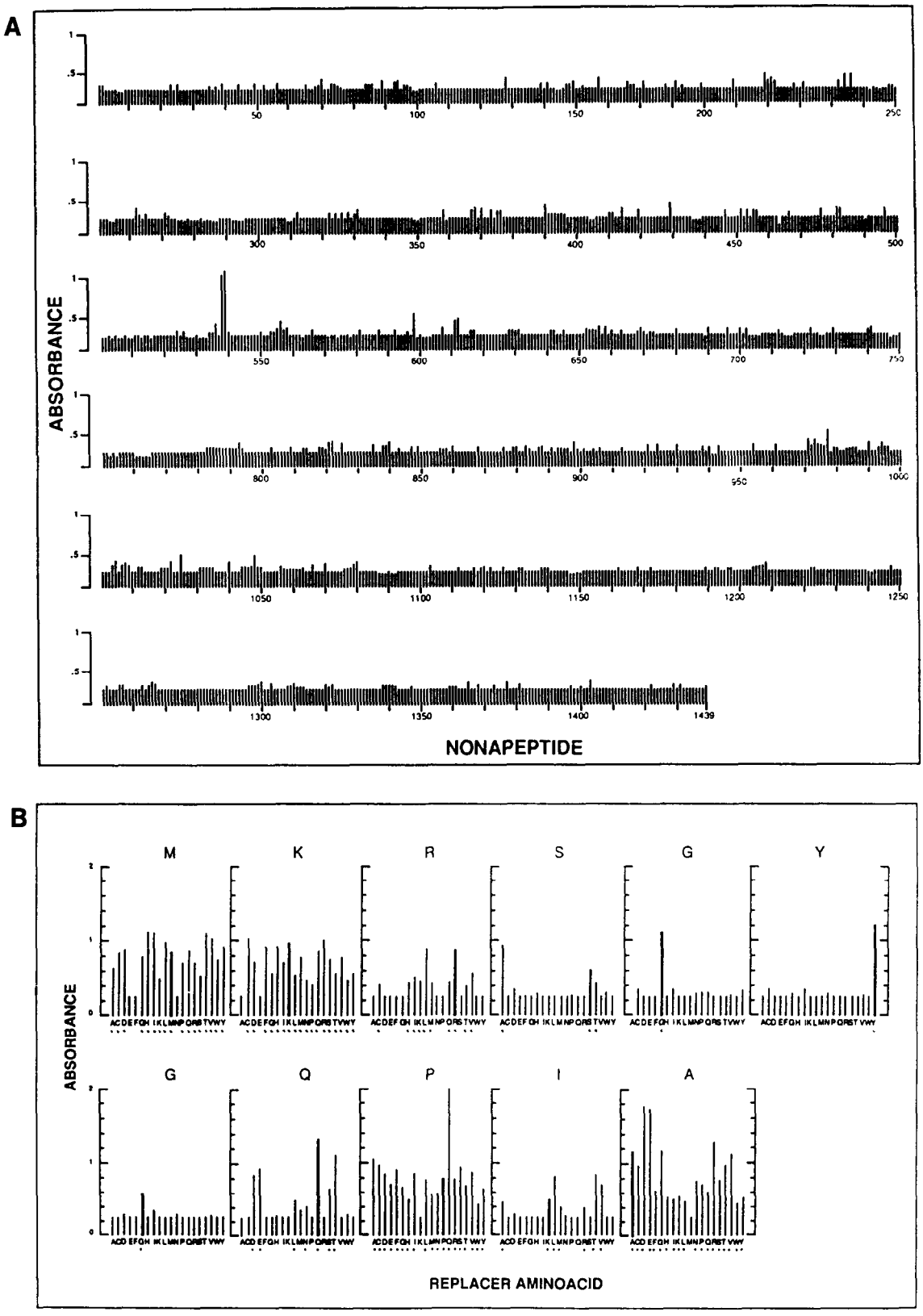

FIG. 3. PEPSCAN analysis of overlapping nonapeptides from the sequence of the TGEV spike protein. A. The binding of the MAb 1A.F10 (diluted 1:5000), specific for subsite Ac of the S protein (Correa *et al.*, 1988), was measured as the absorbance at 405 nm by ELISA, and was plotted against the sequence position of the N-terminal amino acid of the peptide (1 to 1439). B. Effect of amino acid substitutions in peptide 537-MKRSGYGQPIA-547 on the binding by MAb 1A.F10, specific for subsite Ac. Each parental residue in the sequence was consecutively

1D.E7 mutant selected with MAbs specific for subsites Ac and Ab behaved like a triple *mar* mutant in the neutralization assays performed with subsite Aa-specific MAbs (Table 2). MAb 1E.F9 neutralized *mar* 1B.H6 mutant (Fig. 1). Other site Aa-specific MAbs did not neutralize *mar* 1B.H6 mutant, indicating that the epitope recognized by MAb 1E.F9 was different to the other epitopes of subsite Aa.

As an alternative approach, epitope scanning technology was applied to study the amino acids involved in site A. Only MAb 1A.F10 (subsite Ac) showed a specific binding pattern with synthetic peptides (Fig. 3A). Two nonapeptides, which included amino acid 543, were recognized by MAb 1A.F10. To improve the observed binding, analysis of the optimum peptide length was performed using nona- to dodecapeptides. Maximum binding was obtained with the undecapeptide 537-MKRSGYGQPIA-547 (results not shown). The essential amino acids in this peptide were determined by studying the recognition of peptides with consecutive replacement of each amino acid in the native sequence by MAb 1A.F10 (Fig. 3B). The essential residues for binding were the first G (position 541), Y (position 542), and the second G (position 543). The R (position 539), S (position 540), Q (position 544), and I (position 546) could be replaced by a few amino acids, indicating that they also contribute to subsite Ac. MAbs 1B.B5, 6A.C3, and 1A.F10 recognized antigenic subsite Ac. MAb 1B.B5 and 6A.C3 did not bind to the peptides recognized by MAb 1A.F10 (data not shown), indicating that they are specific for another epitope(s).

Site B

Site B-specific MAbs have not neutralized TGEV infectivity (Suñé *et al.*, 1990). Two procedures were used to select mutants in this site. The first method was to select virus variants that did not bind MAb 1D.B12. Three mutants that did not bind MAb 1D.B12 were selected (MAb nonbinding (*manb*) mutants). Stocks of TGEV PUR46-CC120-MAD were enriched in the *manb* 1D.B12 variants by binding *wt* virions with the MAb 1D.B12 and depleting the virus–antibody complexes with *S. aureus* coated with rabbit anti-mouse immunoglobulins. The *manb* mutants were detected by an *in situ* immunoscreening procedure using nitrocellulose filter replicas of infected monolayers, on which plaques were developed (Diez *et al.*, 1989). Both PUR46 *wt* and PUR46 *manb* 1D.B12 mutants showed plaques. Only plaques caused by PUR46 *wt* were stained by MAb 1D.B12, while all plaques were positive with MAb 3B.B6 specific for the N protein (results not shown). This results confirmed that *manb* 1D.B12 mutants were not recognized by the MAb used in their selection. The second method used was to select *mar* mutants by neutralizing virus–antibody complexes with a second antibody against mouse immunoglobulins. TGEV was neutralized by site A-specific MAbs, but not by the MAbs specific for sites B, C (not shown), and D (Table 3). Infectious virus–antibody complexes, made by binding site B- and D-specific MAbs to TGEV, were neutralized up to $10^{1.4}$- and $10^{1.6}$-fold, respectively, by adding rabbit anti-mouse immunoglobulins (Table 3). Two escape mutants were isolated with MAb 1B.H11 (site B) by using this approach: *mar*1B.H11-1 and *mar*1B.H11-2 (Table 4). In contrast, site C-specific MAbs did not bind to native virus (see below) and mediated no neutralization by second antibody.

Site B was previously localized within the first 325 N-terminal residues of the S protein (Correa *et al.*, 1990). The sequence differences between the first 1600 nucleotides of the S-gene of TGEV *wt* and *manb* 1D.B12 or 1B.H11 escaping mutants were determined by direct RNA sequencing. The three *manb* and one *mar* mutant had in common a change in residue 97 from Trp to Leu or Gly (Table 4). A second mutation in residue 76 of *manb*1D.B12-1 was not observed in the other two 1D-B12 mutants and probably represented an accompanying mutation. In the second mutant selected with MAb 1B.H11 (*mar*1B.H11-2) residue 144 (Ser) was replaced by Leu. This datum indicates that this amino acid is part of site B.

The four mutants with an amino acid substitution at position 97 were recognized neither by MAbs 1D.B12 nor by MAb 1B.H11, while the *mar*1B.H11-2 mutant, with a change in residue 144, was recognized by MAb 1D.B12 but not by MAb 1B.H11 (Fig. 4). This indicates that the two MAbs see different but overlapping epitopes. The binding of MAb 8F.B3, site B-specific, to the five site B-escaping mutants was not affected by the changes in residues 97 and 144, indicating a third epitope within site B.

Site C

To determine which amino acids compose site C, consecutive overlapping nonapeptides derived from the sequence of the spike protein of TGEV (Rasschaert and Laude, 1987; Jacobs *et al.*, 1987) were tested in the PEPSCAN, as described above. The same two sets

replaced by all 20 amino acids and plotted in alphabetical order of the one-letter code. This resulted in 11 sets of 20 peptide analogues for the indicated sequence. The MAb binding (diluted 1:1000) was measured as the absorbance at 405 nm by an ELISA and was plotted vertically. The capital letters below the horizontal axis indicate the parental residues substituted. Boldface lines indicate the peptides with the native sequence. The dots indicate the values showing significative differences with the background.

TABLE 3

NEUTRALIZATION OF TGEV BY MAb ALONE OR WITH RABBIT ANTI-MOUSE IMMUNOGLOBULINS

MAb				
	Specificity		Neutralization index[a]	
Name	Protein	Antigenic site[b]	+MAb[c]	+MAb +rabbit α-mouse IgG[d]
1E.F9	S	A	2.5	3.3
1D.B12	S	B	<0.3	1.4
1B.H11	S	B	<0.3	1.0
1D.G3	S	D	<0.3	1.6
3B.B6	N	—	<0.3	<0.3

[a] Neutralization index, defined as the $\log_{10}$ of the ratio of the PFU after incubating the virus in the presence of medium or the MAb.

[b] The antigenic sites were defined by cRIA using MAbs (Correa *et al.*, 1988).

[c] Undiluted supernatants (50 μl) from hybridoma cultures were mixed with one volume of PBS containing 10^5 PFU of TGEV (PUR46) and 2% FCS. The neutralization assay was performed as described under Materials and Methods.

[d] Neutralization in the presence of a second antibody was performed by incubating the virus at 37° for 15 min in presence of the MAb, adding 0.5 vol of a dilution of 1:10 of rabbit antiserum specific for mouse immunoglobulins and incubating at 37° for 15 min.

of nonapeptides were recognized by MAbs 6A.A6 and 5B.H1 (Figs. 5A and 5B). Each nested set had a common core sequence of 49-Pro-Asn-Ser-Asp-52 and 165-Ser-Asn-Ser-Glu-168, respectively (Fig. 5C). Both sequences shared the motif Asn-Ser followed by an acidic residue (Asp or Glu). The binding of the two MAbs to nonapeptides located between positions 49 to 52 was much higher than to the peptides located between residues 165 and 168 (Figs. 5A and 5B).

In competitive RIA at physiological pH and temperature, virus in solution inhibited the binding of site A specific MAbs to virus coated wells, but did not inhibit the binding of site C specific MAbs (Fig. 6). This indicated that site C was not exposed on the surface of native TGEV. Site C becomes exposed when purified TGEV is bound to plastic plates, or after treatment with detergents, since in direct RIA, purified virus binds site C-specific MAbs (Correa *et al.*, 1988, 1990). Site C was also exposed when virions were partially denatured at pHs 11 to 13 (Fig. 6A). This treatment had almost no effect on the relative antigenicity of site A, and caused a 30-fold decrease in virus infectivity (Fig. 6A). Incubation of TGEV at temperatures ranging from 45 to 95° increased the relative exposure of site C. In contrast, the binding of site A-specific MAbs decreased after incubating at 40° and was 10-fold lower after heating the virus 5 min at 95° (Fig. 6B).

Site D

Two *mar* mutants were selected using site D-specific MAb 1D.G3, by neutralizing the virus–antibody complex with a second antibody against mouse immunoglobulins (Table 3). Direct sequencing of an RNA segment (from nucleotide 1000 to 1600) coding for the protein fragment where site D was previously located

TABLE 4

DIFFERENCES BETWEEN THE S-GENES FROM TGEV *wt* AND SITES B AND D MUTANTS

		Nucleotide sequence[a]			Amino acid change		
Clone	Antigenic site	In *wt*[b] virus	In mutant[c] virus	Base changed	Residue	From	To
manb 1D.B12-1	B	CTT	TTT	226	76	Leu	Phe
		TGG	TTG	290	97	Trp	Leu
manb 1D.B12-2	B	TGG	TTG	290	97	Trp	Leu
manb 1D.B12-3	B	TGG	TTG	290	97	Trp	Leu
mar 1B.H11-1	B	TGG	GGG	289	97	Trp	Gly
mar 1B.H11-2	B	TCT	TTT	431	144	Ser	Leu
mar 1D.G3-1	D	GGT	AGT	1153	385	Gly	Ser
mar 1D.G3-2	D	GGT	AGT	1153	385	Gly	Ser

[a] Nucleotide sequence differences were determined by direct RNA sequencing of genome segments on which antigenic sites were previously located (Correa *et al.*, 1990).

[b] All the mutants were derived from PUR46-CC120-MAD strain of TGEV (Sánchez *et al.*, 1990).

[c] The MAb nonbinding (*manb*) mutants were isolated as described in the text by depleting the parental virus stock from virions that bound MAb 1D.B12. *S. aureus* coated with rabbit anti-mouse immunoglobulins was used to remove the virus–MAb complex.

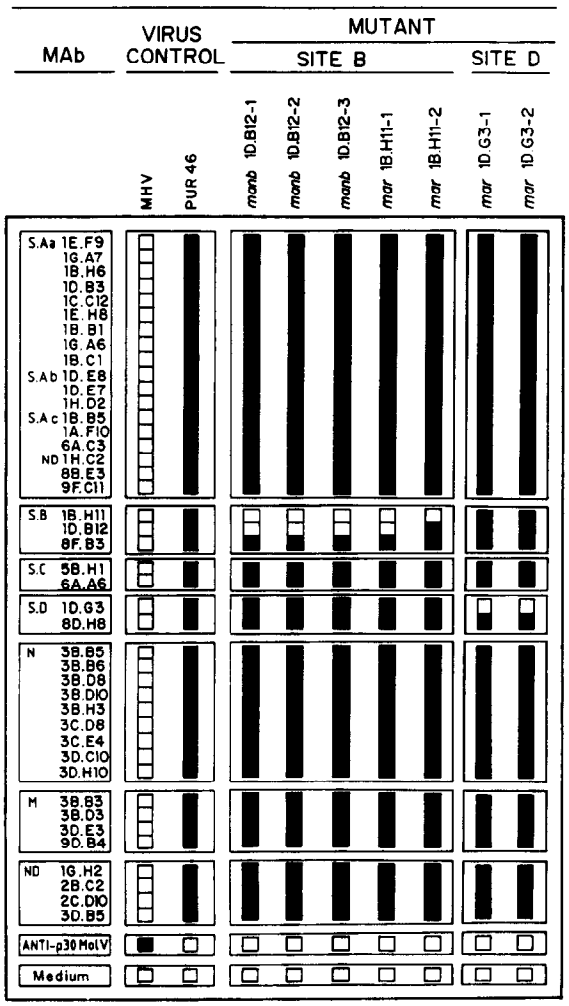

FIG. 4. Binding of MAbs to escape mutants selected from TGEV. MAb binding was determined by RIA, with the binding to strain PUR46-CC120-MAD of TGEV as reference value (100). Escape mutants were named according to the MAb used in their selection. The *manb* mutants were selected by depleting the virions recognized by MAb 1D.B12. The *mar* mutants were resistant to the neutralization of the virus-MAb complex by rabbit serum anti-mouse immunoglobulins. The specificity of the MAbs is named according to Correa *et al.* (1988). White, hatched, and black squares represent relative bindings between: 0 to 19, 20 to 40, and 41 to 100, respectively. ND, not determined. MoIV, Moloney leukemia virus. MHV, mouse hepatitis virus.

(Correa *et al.*, 1990) revealed a nucleotide difference at position 1153 between the mutants *mar* 1D.G3-1 and -2, and the *wt* virus. The nucleotide difference results in a Gly to Ser change at position 385 (Table 4). Antigenic characterization of both mutants using a collection of MAbs showed (Fig. 4) that they were recognized by all MAbs tested, with the exception of the MAb used in the selection (1D.G3). The other site D-specific MAb (8D.H8) recognized both mutants, indicating that MAb 8D.H8 is specific for an epitope distinct from the one recognized by MAb 1D.G3.

Glycosylation dependence of the formation of sites A, B, C, and D

The antigenicity of sites A, B, C, and D of the spike protein was tested after partial or complete deglycosylation of the S protein from mature TGEV with endoglycosidase H or protein *N*-glycosidase F, respectively, and after inhibiting the glycosylation during virus formation with tunicamycin. Endoglycosidase H partially removed carbohydrates on the S protein yielding a glycoprotein of about 170 kDa which was antigenic for MAbs specific for all sites (data not shown). Protein–*N*-glycosidase F removed carbohydrates from SDS-denatured S-protein down to undetectable levels, as determined by labeling the glycoprotein with [^{3}H]glucosamine and autoradiography (results not shown). The deglycosylated S protein was the same size as the apoprotein as determined by SDS–PAGE (Fig. 7A), and was recognized by MAbs specific for sites A, B, C, and D, although sites A and B were less reactive (Fig. 7A).

To study the effect of intracellular glycosylation ST cells were infected with TGEV in the presence of tunicamycin. At a tunicamycin concentration of 5 μg/ml, the glycosylation of the S protein was inhibited completely (results not shown). S protein was not detected in the culture medium. S protein isolated from the intracellular pool formed aggregates, as indicated by its low mobility during SDS–PAGE even after boiling in the presence of 2.5% SDS and 5% 2-mercaptoethanol (Fig. 7B and results not shown). This S-protein was recognized by site C-MAbs an to a lesser extent by site D-specific MAbs. These data indicate that sites C and D are completely or partially independent of glycosylation, respectively. In contrast, MAbs specific for sites A and B did not recognize unglycosilated S protein (even when the electrophoresis was performed in the presence of 0.1% SDS and in the absence of 2-mercaptoethanol) indicating that these sites are fully dependent on glycosylation for proper folding.

DISCUSSION

In this study, the analysis of TGEV mutants selected by MAbs, and PEPSCAN, have contributed to an accurate localization of the antigenic sites A, B, C, and D of the S glycoprotein. Peptides representing antigenic determinants of sites A and C have been defined. In addition, antigenic sites have been characterized in terms of surface exposure and the relative importance of glycosylation.

In order to map residues associated to different antigenic sites, the segments of the S gene coding for the protein regions, where different antigenic sites were previously localized (Correa *et al.*, 1990), have been sequenced in parental and mutant viruses. In addition,

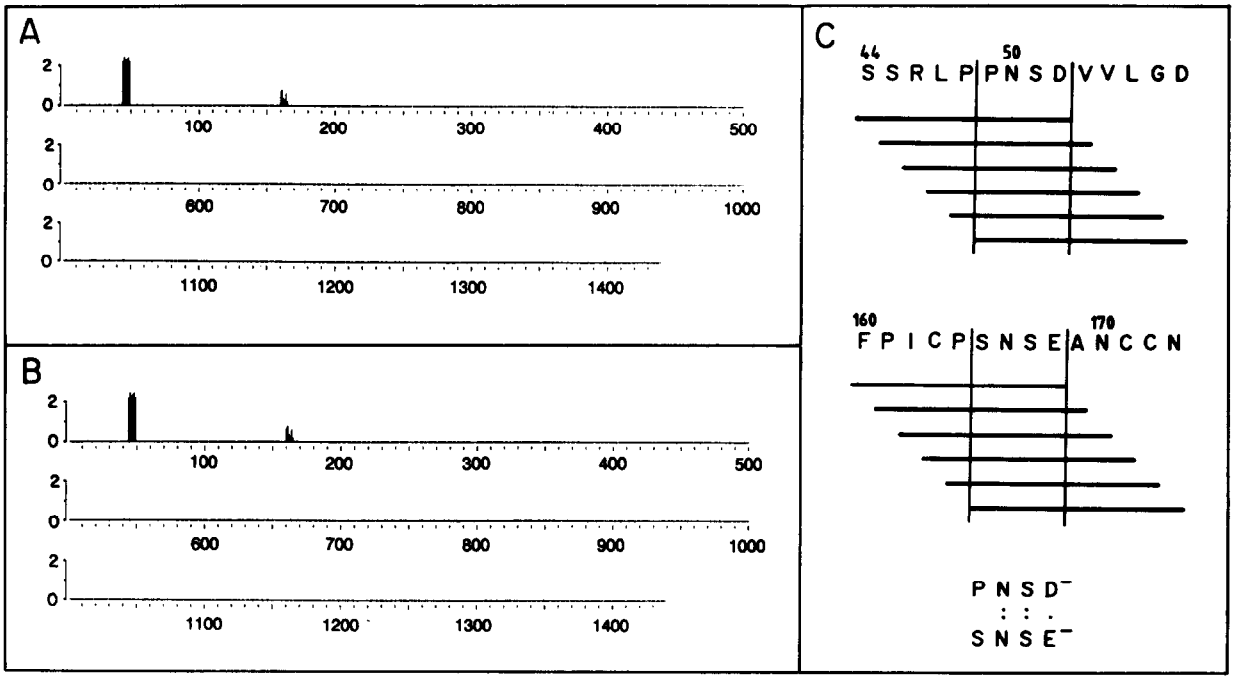

FIG. 5. PEPSCAN analysis of overlapping nonapeptides from the sequence of the TGEV spike protein. The binding of site C-specific MAbs [6A.A6 (A) and 5B.H1 (B)] was measured as the absorbance at 405 nm in an ELISA and was plotted vertically. On the horizontal axis the sequence position of the N-terminal amino acid of the peptide was plotted. Serum dilution used of both MAbs were 1:500. The position and sequence of the two sets of overlapping peptides recognized by both MAbs is indicated (C).

the complete S gene of parental virus and *mar* mutant 1D.E7 has been sequenced.

Site A

Site A is located on the surface of TGEV and is dependent on intracellular glycosylation. With one exception all mutants mapping at site A had a single nucleotide difference with the parental virus. This change was most probably responsible for the virus escaping from MAb neutralization. In other viral systems, single nucleotide differences have also been found to be responsible for escape from neutralization (Vandepol *et al.*, 1986; Taniguchi *et al.*, 1988; Wiegers *et al.*, 1990). In the *mar* 1B.B5 mutant, and in those variants derived from this virus, two nucleotide differences were detected which caused amino acid changes in residues 543 and 631 (Table 1). The change at position 543 is probably responsible for the escape from MAb 1B.B5, since MAb 1A.F10, which maps to the same antigenic subsite, binds peptides including amino acid 543 (Fig. 3 and results not shown). The change affecting residue 631 could be an accompanying mutation, although its contribution to the epitope 1B.B5 cannot be discounted. Site A contains three subsites, Aa, Ab, and Ac, which are sensitive to substitutions on the residues 538, 591, and 543, respectively. In addition, residue 586 affects both subsites Aa and Ab. These data show that site A is complex and that it is formed by residues located in distal segments of the S glycoprotein. This seems to be the most frequent situation in epitopes from other systems studied in detail (site B reported in this manuscript; DiMarchi *et al.*, 1986; Parry *et al.*, 1989; Davies *et al.*, 1988; Posthumus *et al.*, 1990; Wiegers *et al.*, 1990). Although we have defined precise amino acids contributing to the different antigenic sites, the participation of other residues to the antigenicity of the subsites is not excluded.

Peptide 537-MKRSGYGQPIA-547, recognized by MAb 1A.F10, could represent at least a portion of the antigenic subsite Ac. This peptide may be of importance for diagnosis and protection, since epitope 1A.F10 is located in subsite Ac, which is conserved in coronaviruses of three species (Sánchez *et al.*, 1990).

Our data on site A are in agreement with the data of Delmas *et al.* (1990), who found MAb-selected mutations at positions 549 and 586. A complete demarcation of the complex site A must await elucidation of the three-dimensional structure of the spike protein.

Site B

Site B is dependent on intracellular glycosylation and is complex and conformation-dependent. This site is

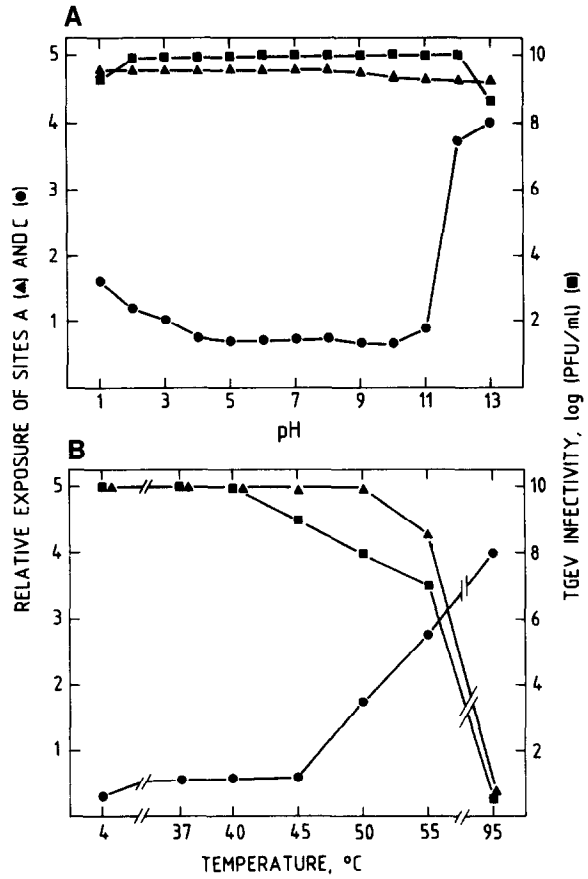

FIG. 6. Effect of the pH and temperature on the relative surface exposure of antigenic sites A and C. Purified TGEV was incubated at different pH (at 4°) (A) or temperature (B). Dilutions of the virus were used to inhibit the binding of site A- (6A.C3) and C (6A.A6)-specific MAbs to TGEV-coated plates (0.25 μg/well) in a cRIA, as described previously (Correa *et al.*, 1988). A binding inhibition curve was obtained for each viral sample after different incubations. The percentage of binding inhibition by 5 μg of virus per well was divided by ten and taken as the relative exposure of the sites. After each treatment the residual infectivity was determined in a plaque assay on ST cells.

formed by at least three epitopes. Two of these epitopes (1D.B12 and 1B.H11) are overlapping, since both include amino acid Trp at position 97 in their formation. Sequence analysis of mutants indicated that residues 97 and 144 (Table 4) are part of site B. Although site B is conformation dependent, MAbs specific for this site can bind TGEV S-glycoprotein by immunoblotting providing that the samples were not treated with 2-mercaptoethanol. Most probably, renaturation of S protein occurs during the blotting of the protein to nitrocellulose paper. Recently, we used a new approach to characterize conformation-dependent epitopes (Lenstra *et al.*, 1991). Using MAb 1D.B12, we screened a bacterial pEX expression library of hybrid proteins that consist of β-galactosidase and random hexapeptides. Sequencing of antigenic clones revealed the sequence: –ANSRPRWMKL– (bold residues were expressed by the variable insert of the plasmid). This sequence mimics the epitope of MAb 1D.B12 and may be considered a site B-mimotope (Geysen *et al.*, 1984). The three underlined residues are also found in the S protein sequence 102-RQRHNWT-96, suggesting that residues 97, 100, and 102 may be part of site B. These residues might have been displayed in a *correct* spatial conformation, which mimics the native site, by the contiguous residues present in the mimotope. The serine in position 144 also contributes to site B, since it is changed in the mar 1B.H11 mutant (Table 4). It is tempting to speculate that the serine present in the mimotope (–ANSRPRWMKL–) replaces the serine in position 144 of the parental virus.

Site C

Site C is linear and continuous since: (i) it is recognized by MAbs in Western blot analysis after treatment

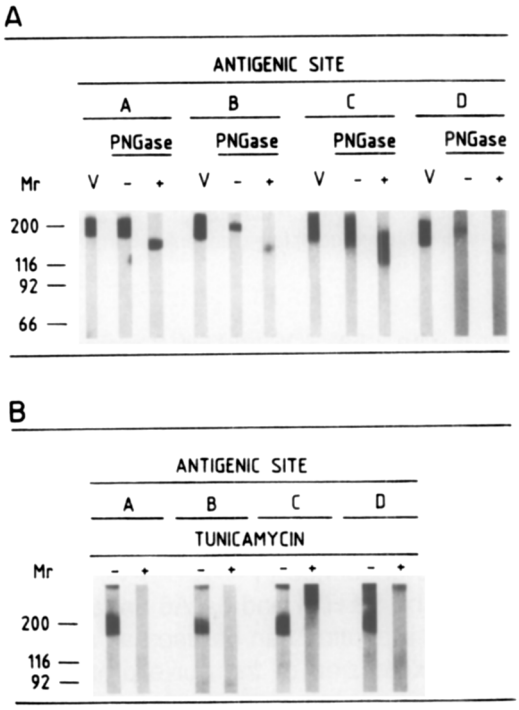

FIG. 7. Effect of deglycosylation or glycosylation inhibition on the S protein of TGEV analyzed by immunoblotting. A. The binding of MAbs specific for site A (pool of MAbs 1A.F10, 1G.A7, and 6A.C3), site B (pool of MAbs 1D.B12, 1B.H11, and 8F.B3), site C (6A.A6), and site D (1D.G3) in Western blot to the virus (V), or the S protein after incubation in the presence of protein-N-glycosydase F (+) or in its absence (–) (see Materials and Methods) is shown. B shows the binding of the MAb pools described in A, to extracts of ST cells infected with TGEV in the absence (–) or in the presence (+) of tunicamycin. M_r, position of molecular weight markers in kDa. The electrophoresis preceeding the Western blot analysis were performed in the absence of 2-mercaptoethanol to avoid the complete denaturalization of S protein.

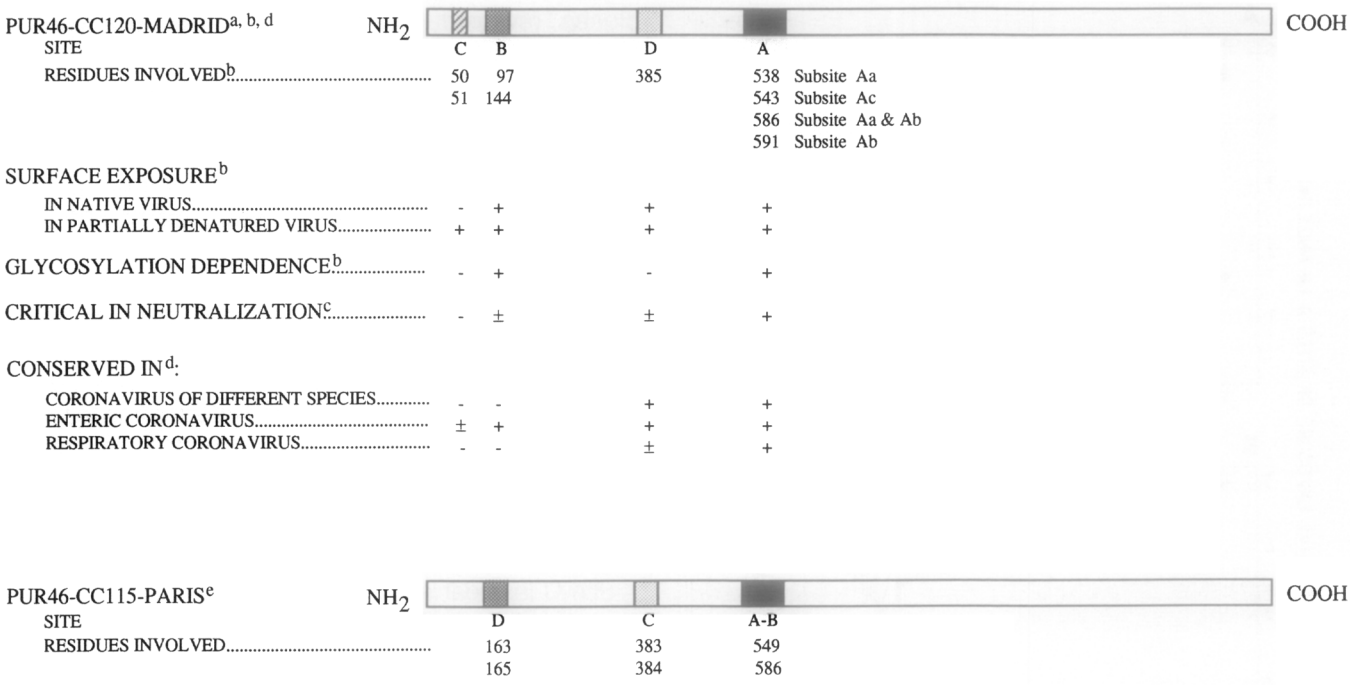

FIG. 8. Summary of the amino acids contributing to the antigenic sites of the S glycoprotein of two clones of the PUR46 strain of TGEV, and of the properties of the antigenic sites. The diagram shows the position of the residues involved in the formation of the antigenic sites of two clones of the PUR46 virus with a common origen (Bohl, 1972). The antigenic sites were defined by competitive binding assays with MAbs and named according to Correa *et al.* (1988) (PUR46-CC120-MADRID) (a) and Delmas *et al.* (1987) (PUR46-CC115-PARIS) (e). In the diagram, the residues implicated in the antigenic sites, as derived from sequence differences between parental virus and escape mutants selected with MAbs, or by PEPSCAN analysis (Correa *et al.*, 1990; Delmas *et al.*, 1990; this study) are shown. The properties of the antigenic sites, are deduced from this study (b) or from other publications: (c) Jiménez *et al.* (1986); Correa *et al.* (1988); Suñé *et al.* (1990); Delmas *et al.* (1986); Delmas *et al.* (1990); and (d) Sánchez *et al.* (1990). A site was defined as conserved (+) if at least part of it was present in most of the isolates. Sites with a minor presence or which were completely absent in certain isolates were referred as ± or −.

of the virus with 2.5% SDS and 5% 2-mercaptoethanol (Fig. 7; Correa *et al.*, 1990); (ii) it is represented by synthetic nonapeptides derived from TGEV S-glycoprotein (Fig. 5); (iii) it is present in recombinant products expressed in bacteria, which do not reconstitute the native S protein (Correa *et al.*, 1990); and (iv) it is formed in the absence of glycosylation (Fig. 7). Since nonapeptides with the sequence (-P-P-N-S-D-V-) are strongly bound by MAbs 5B.H11 and 6A.A6 under denaturing conditions, this peptide can be used as a reporter to control the expression of the spike protein gene or other heterologous genes. In addition, because binding and sequencing studies indicate that site C is not present in the respiratory variants of TGEV (Sánchez *et al.*, 1990); C. M. Sánchez and L. Enjuanes, unpublished results), this peptide could be useful to discriminate serum from TGEV or PRCV infected animals.

The sequence of the linear site C deduced by PEPSCAN (P-P/S-N-S-D/E-V/A) strongly resembles the sequence of the mimotopes P-P/S-N/H-S-D/E-A selected by site C-specific MAbs from the random-sequence expression library (Lenstra *et al.*, 1991; see above). Serine (S) at position 51 is probably the most essential aminoacid of site C, since it is the only residue conserved in the 12 peptides recognized by epitope scanning (Fig. 5) and in six mimotopes (Lenstra *et al.*, 1991).

Site C is not exposed on native virus, as determined by cRIA (Fig. 6). This agrees with: (i) the absence of this site on the surface of TGEV-infected cells, as determined by immunofluorescence or antibody-dependent complement-mediated cell lysis (Laviada *et al.*, 1990), and (ii) the lack of detection of this site by immune-electron microscopy with gold-labeled MAbs (C. Suñé, M. V. Nermut, J. L. Carrascosa, and L. Enjuanes, unpublished results). In contrast, the antigenic sites A, B, and D were detected on the surface of infected cells and on whole virions.

Site D

The glycine (G) residue at position 385 was found to be essential for the epitope of site D-specific MAb 1D.G3 (Table 4). The other site D-specific MAb 8D.H8 recognized both mutants selected by MAb 1D.G3 (Fig. 4). Epitope 8D.H8 has previously been localized within the residues 387 to 392 by PEPSCAN analysis (Post-

humus *et al.*, 1990). Our data indicate that site D has at least two different epitopes. In the study reported by Posthumus *et al.* (1990) it has been shown that other site D-specific MAbs recognized peptides consisting of the residues 381 to 387 and 378 to 386. The location of the site D within the 378 to 392 region agrees with work reported by Delmas *et al.* (1990). They found substitutions of the residues 383 (Ser) and 384 (Tyr) with escape mutants selected by their MAbs specific for an antigenic site homologous to site D. Glycosylation does not appear to be essential for keeping antigenicity at this site when the S protein purified from ensambled virus was analyzed (Fig. 7).

Antigenic structure of TGEV

Comparison of the residues involved in the antigenic sites of the S glycoprotein of two clones of the PUR46 strain of TGEV (Fig. 8), one clone (PUR46-CC120-MADRID) studied by our group (Correa *et al.*, 1988, 1990; this study) and another clone (PUR46-CC115-PARIS) studied by Delmas *et al.* (1990) have shown that sites A, B, and D are located within the same regions of the S protein. Site C was only identified by our group, probably because this site is not exposed in native virions.

In vitro tests, using swine testis cells, indicate that antigenic sites A and D are the major inducers of neutralizing antibodies. The residues that contribute to these sites have been conserved in enteric and respiratory strains of TGEV (PUR46-CC120-MADRID, PUR46-CC115-PARIS, PUR46-115-UTRECHT, MIL65, the British isolate FS772/70), and in feline infectious peritonitis virus (based on sequence data: Rasschaert *et al.*, 1987; Jacobs *et al.*, 1987; Wesley, 1990, Britton *et al.*, 1990; F. Gebauer, C. M. Sánchez, and L. Enjuanes, unpublished data). This suggests that these residues must be important for the replication of viruses of the TGEV group (Sánchez *et al.*, 1990). However, they may not be relevant for the protection *in vivo* against coronaviruses, since antigenic variation would generate the same or similar substitutions to those observed in our mutants. We are currently testing the potential use of the antigenic peptides for diagnosis and protection.

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