Localization of antigenic sites of the E2 glycoprotein of transmissible gastroenteritis coronavirus

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Four antigenic sites of the E2 glycoprotein of transmissible gastroenteritis virus were defined by competitive radioimmunoassays of monoclonal antibodies (MAbs). Here, we describe the localization of these sites by testing the antigenicity of protein fragments and prokaryotic expression products of E2 gene fragments, and by sequencing of MAb-resistant (*mar*) mutants. Partial proteolysis of purified E2 protein allowed the isolation of a 28K fragment recognized by both site A- and site C-specific MAbs. An antiserum against this fragment bound to a synthetic peptide containing residues 1 to 18 and to an expression product containing residues 1 to 325. The same expression product was recognized by site C-specific MAbs. These data indicate that residues within the sequence 1 to 325 contribute to site C and possibly also to site A. Sequencing of *mar* mutants that escaped neutralization by site A-specific MAbs indicated that residues 538 and 543 also belong to site A. The binding of site-specific MAbs to expression products led directly to the localization of sites B and D, between residues 1 to 325 and 379 to 529, respectively. The first 37% of the polypeptide chain of E2 appears to be more immunogenic than the rest of the sequence.

Introduction

Transmissible gastroenteritis (TGE) virus is a coronavirus that causes enteric disease in swine of all ages. The disease is especially severe in newborn animals less than 2 weeks old, in which mortality approaches 100% (Siddell *et al.*, 1983; Sturman & Holmes, 1983; Saif & Bohl, 1986). The virus has a single-stranded, positive-sense RNA genome of more than 20 kb (Brian *et al.*, 1980; Rasschaert *et al.*, 1987) and three structural proteins: E2, N and E1, of 1447, 382 and 262 amino acids, respectively (Kapke & Brian, 1986; Laude *et al.*, 1987; Rasschaert & Laude, 1987; Jacobs *et al.*, 1987). The E2 glycoprotein is responsible for the induction of neutralizing antibodies (Garwes *et al.*, 1978).

On the E2 glycoprotein a minimum of four antigenic sites (A, B, C and D) have been defined; site A is the most relevant for virus neutralization and contains three subsites (Aa, Ab and Ac) (Jiménez *et al.*, 1986; Correa *et al.*, 1988).

In this report we used monoclonal antibodies (MAbs) as specific probes for these sites. We tested the binding of these MAbs to E2 protein fragments generated by partial proteolysis or by expression of E2 gene fragments in pEX vectors (Stanley & Luzio, 1984; Lenstra *et al.*, 1989). An N-terminal 28K peptide could be isolated which contained the antigenic site C and possibly also part of site A. The use of recombinant antigens led to localization of sites B and C within residues 1 to 325 and of site D within residues 379 to 529. The sequencing of *mar* mutants that escaped the neutralization by site A-specific MAbs indicated that residues 538 and 543 are also essential for site A. This led to approximate localization of the antigenic sites, all four within the first 543 of the 1447 residues of the peplomer protein.

Methods

Virus purification. The Purdue strain of TGE virus (PUR46.C1) (Bullido *et al.*, 1988) was grown on ST cells (McClurkin & Norman, 1966) and purified as previously described (Correa *et al.*, 1988).

Monoclonal antibodies. The hybridomas producing the MAbs were obtained and characterized, as previously described (Jiménez *et al.*, 1986; Correa *et al.*, 1988; Sanz *et al.*, 1985). The MAbs used and their properties are summarized in Table 1.

Purification of E2 glycoprotein. E2 protein was purified by phase separation with Triton X-114, essentially as described by Bordier (1981) and Ricard & Sturman (1985). Briefly, purified TGE virus (0·5 mg/ml in 200 mM-ammonium acetate pH 7·4) was disrupted by incubation with 1% (w/v) Triton X-114 at 0 °C for 20 min. The two phases were then separated by heating at 30 °C for 3 min and

Table 1. *TGE virus-specific MAbs*

MAb	Specificity*	Neutralizing activity†
1B.B1	Aa	8·1
1B.C1	Aa	6·1
1G.A7	Aa	5·4
1D.E7	Ab	6·0
1B.B5	Ac	2·5
1A.F10	Ac	1·8
6A.C3	Ac	>9·0
1D.B12	B	<0·3
1B.H11	B	<0·3
5B.H1	C	<0·3
6A.A6	C	<0·3
1D.G3	D	<0·3
8D.H8	D	0·7

* Capitals and lower case letters refer to antigenic sites and subsites, respectively (Correa *et al.*, 1988).

† Expressed as the neutralization index, or the $-\log_{10}$ of the ratio of the virus concentration (p.f.u./ml) with or without MAb.

centrifugation at 13000 *g* for 2 min. The aqueous phase was collected and the detergent phase was extracted twice with 200 mM-ammonium acetate. The aqueous phases were pooled and all fractions were analysed by SDS–PAGE.

Fragmentation of E2 glycoprotein. Triton X-114-purified E2 protein (1 μg/μl) was digested at 37 °C, with *Staphylococcus aureus* V8 protease in 125 mM-Tris–HCl pH 6·8 in the presence of 0·5% SDS, using a protein to enzyme ratio of 50 : 1 (w/w). After a 6 h incubation a second dose of the enzyme was added and after 18 h the reaction was stopped by freezing.

Analysis by SDS–PAGE and immunoblotting. The reactivity of polyclonal or antigenic site- and subsite-specific MAbs (Table 1) with E2 fragments was studied by immunoblotting (Towbin *et al.*, 1979). Briefly, the fragments were separated by SDS–PAGE (Laemmli, 1970) in the presence or absence of 2-mercaptoethanol (2-ME) and with 0·1% SDS in the sample buffer if indicated. After equilibration of the gel with 20% methanol in 25 mM-Tris, 192 mM-glycine pH 8·3, the fragments were electrophoretically transferred to a nitrocellulose membrane filter, which was subsequently washed in 500 mM-NaCl, 20 mM-Tris–HCl pH 7·5 (Tris-buffered saline; TBS). The incubation was continued at room temperature for 2 h in washing buffer (TBS supplemented with 0·1% Tween 20) containing 5% bovine serum albumin. The membrane was incubated overnight at 4 °C with undiluted hybridoma supernatants specific for TGE virus (Table 1 and legends of the corresponding figures). The nitrocellulose paper was washed, incubated at room temperature for 1 h with a 2×10^3-fold dilution of rabbit anti-mouse immunoglobulin, washed and incubated at room temperature for 2 h with ^{125}I-labelled Protein A (1×10^6 c.p.m./ml; 3×10^7 c.p.m./μg protein). Alternatively, the MAbs used were ^{125}I-labelled (1×10^6 c.p.m./ml; 1×10^7 c.p.m./μg protein) (Greenwood *et al.*, 1963). The nitrocellulose paper was washed and subjected to autoradiography.

Fractionation of E2 protein peptides. Triton X-114-purified glycoprotein was dissolved in 125 mM-Tris–HCl pH 6·8 and 0·5% SDS at 1 μg/μl and digested with V8 protease as described above. The E2 fragments were fractionated by HPLC using two columns in series, a Protein Pak 300 sw (Waters, 7·5 mm × 30 cm) and a Protein Pak 125 (7·8 mm × 30 cm). Elution with 0·1 M-ammonium acetate and 0·05% SDS was carried out at room temperature and at 0·8 ml/min. The proteins were collected in five fractions (Fig. 6). The peak fraction was lyophilized, dissolved in electrophoresis sample buffer with 0·1% SDS but without 2-ME, incubated at 35 °C for 5 min and separated by SDS–PAGE (12% polyacrylamide). A gel band containing the 28K fragment was cut out, homogenized and extracted at 4 °C overnight in 0·1 M-ammonium bicarbonate pH 7·8. The purity of the 28K fragment was tested by SDS–PAGE and by HPLC on the reverse phase Ultrapore RPSC C3 column of 4·6 mm × 7·6 cm and 30 nm pore diameter (Beckman). Peptides were eluted using a gradient of 0·1% trifluoroacetic acid (TFA) (solvent A) and acetonitrile : 2-propanol (1 : 1, v/v) with 0·1% TFA (solvent B). Constant flow gradient elution (0·5 ml/min) was controlled with a Model 721 programmer (Waters).

Production of an antiserum against the 28K fragment. After elution from an SDS–PAGE gel and removal of the SDS by electroelution three 1 μg doses of 28K fragment were subcutaneously injected into BALB/c mice at 2 week intervals, the first dose in complete Freund's adjuvant, the second in incomplete Freund's adjuvant and the third in phosphate-buffered saline. Ten days after the last dose the mice were bled. Another set of mice was immunized following a similar protocol with native virus.

Expression of E2 gene fragments in pEX vectors. E2 gene fragments were inserted as described (Lenstra *et al.*, 1989) in the pEX expression plasmid (Stanley & Luzio, 1984) using the restriction sites in the E2 peplomer gene as indicated. Recombinant plasmids were introduced into the *Escherichia coli* strain pop 2136 by the $CaCl_2$ transformation procedure. Unless otherwise stated all DNA manipulations used were done essentially as described by Maniatis *et al.* (1982) and Davis *et al.* (1986). Recombinant plasmids were amplified at 30 °C and then transient expression was induced by shifting to 42 °C for 90 min. The *cro*–β-galactosidase hybrid proteins were extracted as Triton X-100-insoluble pellets (Stanley & Luzio, 1984; Lenstra *et al.*, 1989). Briefly, cells were sedimented (10 min at 5000 *g*), resuspended in 100 μl 15% (w/v) sucrose, 50 mM-Tris–HCl pH 8·0 and 50 mM-EDTA and treated with lysozyme (1 mg/ml) for 10 min. After addition of 140 μl 0·2% (v/v) Triton X-100 in 10 mM-Tris–HCl pH 8·0, 1 mM-EDTA, the suspension was sonicated in a bath sonicator for 15 min. After pelleting of the insoluble fusion protein (10 min at 10000 *g*) and removal of as much of the viscous supernatant as possible, the pellet was resuspended in Triton X-100 buffer. Sonication, centrifugation and removal of the supernatant were repeated (once or twice) until the viscosity disappeared. Finally, the pellet was resuspended in 2·5% SDS and 5% 2-ME for electrophoresis. The expression products were analysed by immunoblotting.

Peptide synthesis. A peptide (Cys-Asp-Asn-Phe-Pro-Cys-Ser-Lys-Leu-Thr-Asn-Arg-Thr-Ile-Gly-Asn-Gln-Trp-Asn), containing the N-terminal 18 residues of the mature E2 protein (Rasschaert & Laude, 1987; Jacobs *et al.*, 1987) coupled to a cysteine, was synthesized in an Applied Biosystems synthesizer 430A using t-Boc amino acids and customer protocols. The peptide was dissolved in 0·1% TFA, purified by reverse-phase HPLC on an Ultrapore RPSC C3 column (Beckman) to remove non-peptide impurities and conjugated to keyhole limpet haemocyanin (KLH) via the cysteine residues, as described (Correas *et al.*, 1986). Briefly, 3 mg of KLH was activated by incubating at room temperature for 3 h in the presence of a 20-fold molar excess of the bifunctional reagent *m*-maleimido-benzoyl-*N*-hydroxy-sulphosuccinimide ester (sulpho-MBS) in 50 mM-phosphate buffer, 1 mM-EDTA pH 7·0. Unreacted sulpho-MBS was removed by dialysis against the same buffer. The peptide (1·6 mg) was coupled to the activated KLH by incubation overnight at 4 °C with a 20-fold excess of the peptide. Remaining binding sites were blocked by the addition of 50 μl of 0·4 M-cysteine and incubation for 3 h at room temperature.

Dot blot assay. The synthetic peptide conjugated to KLH, or KLH alone, was resuspended in PBS in the presence of protease inhibitors (1 mM-PMSF, 0·1 mM-TLCK and 0·1 mM-TPCK). About 100 ng of each protein sample in 4 μl of buffer was adsorbed per well for 1·5 h at room temperature onto a nitrocellulose membrane filter, prewashed for 15 min with TBS (see above), using a Bio-Dot (Bio-Rad) microfiltration apparatus. After washing with TBS under vacuum the nitrocellulose was removed from the filter holder, washed in TBS and non-specific binding sites were blocked with TBS containing 5% BSA and 0·1% Tween 20. Incubation with HPLC-purified ^{125}I-labelled MAbs (10^6 c.p.m./ml; 1×10^7 c.p.m./μg protein) was performed at room temperature for 1·5 h. The filters were washed, dried and processed for autoradiography.

Selection of MAb escape (mar) *mutants*. TGE virus mutants resistant to neutralization by site A-specific MAbs were selected as described previously (Jiménez *et al.*, 1986; Correa *et al.*, 1988). Single *mar* mutants resistant to MAbs 1B.B5, 1G.A7 and 1B.C1, and the double mutant *dmar* 1B.B5–1B.B1 were antigenically characterized (Jiménez *et al.*, 1986; F. Gebauer & L. Enjuanes, unpublished). Their infectivity was reduced less than $10^{0.45}$-fold by the MAb used for their isolation, which neutralized the original virus (PUR46) between $10^{2.4}$- and $10^{8.1}$-fold.

Cloning of E2 gene cDNA in the Bluescript phagemid. E2 gene cDNA was synthesized as described (Villanueva *et al.*, 1983; Efstratiadis *et al.*, 1976) and cloned in the Bluescript SK M13$^-$ plasmid (Stratagene) (F. Gebauer & L. Enjuanes, unpublished). Three DNA fragments, which included the nucleotides −8 to 1587, 1135 to 3329 and 3330 to 4628 of the genomic RNA of the TGE virus strain PUR46.C1, were cloned. Recombinant DNA techniques were performed essentially by standard methods (Maniatis *et al.*, 1982; Davis *et al.*, 1986).

DNA and RNA sequencing. The DNA purified from the Bluescript–TGE virus plasmids, and the RNA purified from virions, was sequenced by oligodeoxynucleotide primer extension and dideoxynucleotide chain termination procedures (Sanger *et al.*, 1977; Zimmern & Kaesberg, 1978). For RNA sequencing we used the primer 3′ TCTGTTGTATCACCCACATG 5′, complementary to nucleotides 1980 to 2000 of the E2 gene. Sequence data were assembled and analysed using the computer programs of the Genetics Computer Group (University of Wisconsin).

Results

Purification of E2 glycoprotein

Purified TGE virus was solubilized with Triton X-114 and its protein components were separated by phase fractionation. An SDS–PAGE analysis of the aqueous phases, collected in three extractions, and of the detergent phase is shown in Fig. 1. Approximately 85% of the E2 protein was recovered in a pool of the first and second aqueous phases; a third extraction did not substantially increase the yield. The detergent phase contained practically only the N and E1 protein.

Controlled fragmentation of E2 glycoprotein

E2 protein was digested with *S. aureus* V8 protease for different periods of time. Prolonging the digestion to over 26 h did not further change the observed pattern of fragments. As deduced from the positions of the glutamate residues in the E2 sequence (Rasschaert & Laude, 1987; Jacobs *et al.*, 1987) this was still a partial digestion. The binding of MAbs specific for antigenic sites A, B, C and D (as determined by immunoblotting) to the V8 protease fragments is shown in Fig. 2. All the MAbs reacted with undigested E2 protein (not shown) and, although many different E2 fragments were detected by silver staining (Fig. 2, lane 1), site A-specific MAbs recognized peptides of 28K and of around 66K (Fig. 2, lane 2). The recognition by one MAb of more than one peptide may be explained by the partial digestion. Site B-specific MAbs did not bind any of the V8 protease fragments on the immunoblot (Fig. 2, lane 3). Site C-specific MAbs selected two fragments of 28K and 30K (Fig. 2, lane 4) and site D-specific MAbs recognized a fragment of 50K (Fig. 2, lane 5). Site A-specific MAbs bound the whole E2 protein more strongly than any of the fragments.

To determine whether the three antigenic subsites from site A (a, b and c; Correa *et al.*, 1988) were in the same V8 fragments, immunoblots were performed with subsite-specific MAbs (Fig. 3). The identical patterns suggest that all three subsites are contained in the same fragment.

Expression of E2 glycoprotein antigenic sites in pEX–TGEV vectors

The E2 gene fragments expressed by pEX vectors (pEX–TGEV) are summarized in Fig. 4 and Table 2. Nine overlapping inserts, numbered 1 to 9, respectively, accounted for 98% of the E2 gene. Bacteria transformed with pEX–TGEV constructs were selected by identifying hybrid proteins larger than the *cro*–β-galactosidase

Table 2. *E2 gene* fragments expressed by pEX–TGEV recombinants*

Fragment number	Nucleotide fragment†	E2 protein fragment
1	−8–1136	1–378
2	976–1674	326–558
3	1588–2021	530–673
4	1675–2021	559–673
5	1819–2238	607–746
6	2022–2760‡	675–(919)‡
7	2622–3477	875–1159
8	3447–3717	1150–1239
9	3478–4255	1160–1418

* The E2 gene from TGE virus has 4341 nucleotides, encoding a protein of 1447 residues (Rasschaert *et al.*, 1987; Jacobs *et al.*, 1987).

† Numbers are relative to the start of the coding sequence.

‡ Insert 6 was derived from clone B1 (Jacobs *et al.*, 1987) using the *Pst*I site from the polylinker. It contains the *Hpa*I site at nucleotide 2619 and the E2 fragment encoded by this insert includes at least residues 675 to 919.

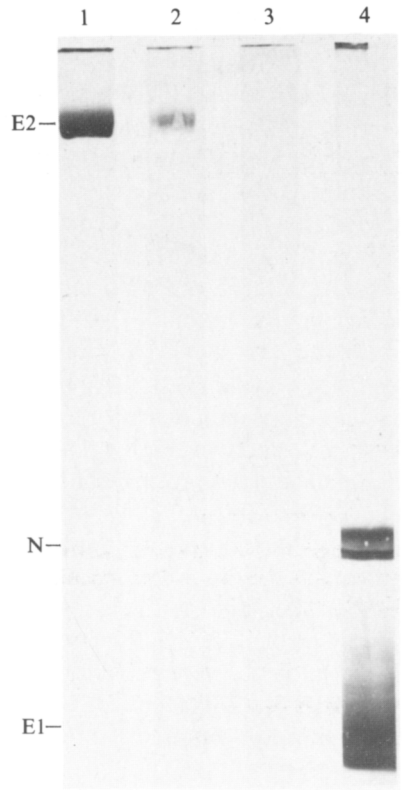


Fig. 1

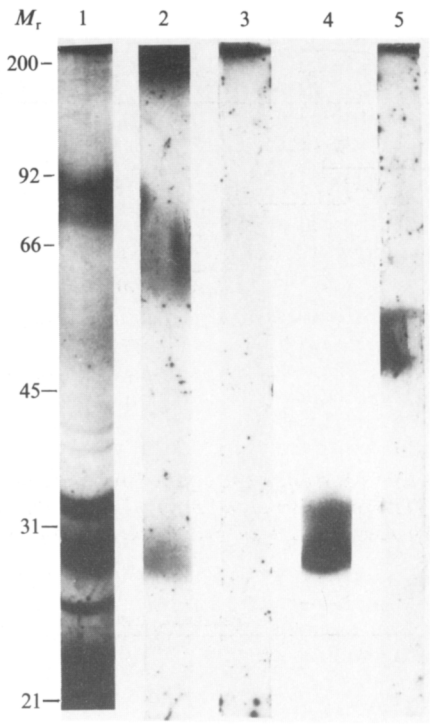


Fig. 2

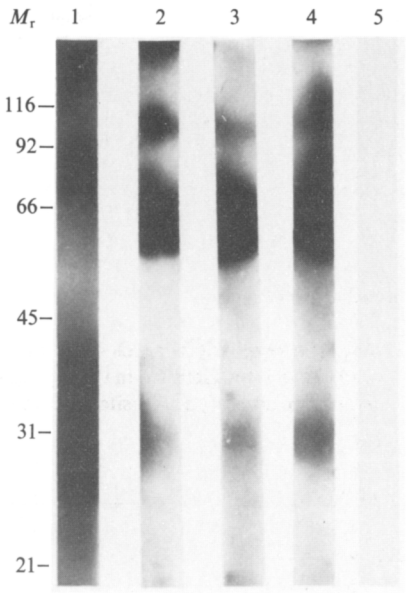


Fig. 3

Fig. 1. SDS–PAGE analysis of the Triton X-114 fractionation of TGE virus proteins. Purified TGE virus was disrupted with Triton X-114 and the solubilized proteins were separated in two phases. The detergent phase was twice re-extracted with the aqueous buffer. The left-hand side shows the position of the structural proteins of TGE virus. Lanes 1, 2 and 3 show the contents of the three aqueous phases in the order in which they were collected, lane 4 shows the contents of the detergent phase.

Fig. 2. Recognition of E2 fragments by site-specific MAbs on immunoblots. Purified E2 protein was digested with V8 protease from *S. aureus*. The fragments obtained by protease digestion observed after silver staining (lane 1), were incubated with MAbs specific for the different antigenic sites: MAbs 1G.A7, 1A.F10 and 6A.C3 (site A, lane 2), MAb 1D.B12 (site B, lane 3), MAb 5B.H1 (site C, lane 4) and MAb 1D.G3 (site D, lane 5). M_r, relative molecular mass $\times 10^{-3}$.

Fig. 3. Immunoblotting of E2 fragments with subsite-specific MAbs. V8 protease fragments from E2 protein were incubated with TGE virus-specific polyvalent antiserum (lane 1), or MAbs specific for the different antigenic subsites: MAb 1G.A7 (subsite a, lane 2), MAb 1D.E7 (subsite b, lane 3), MAb 1A.F10 (subsite c, lane 4), and a control MAb (lane 5).

protein. The correlation of the size of these proteins with the size of the insert (not shown) indicated complete expression.

On immunoblots, the site-specific MAbs recognized only the expression products of inserts 1 and 2. Fig. 5 shows representative results of the binding of different MAbs to TGE virus proteins and to expression products. Four site A-specific MAbs (1G.A7, 1D.E7, 1A.F10 and 6A.C3) tested did not bind to the expression products. One site B-specific MAb (1B.H11) and two site C-specific MAbs (5B.H1 and 6A.A6) bound to the product of insert 1. In contrast, two MAbs (1D.G3 and 8D.H8), which are site D-specific, recognized products of the plasmid containing insert 2, but not any of the other products.

E2 protein fragments involved in the formation of the antigenic sites

Fragments from V8 protease-digested E2 protein were subjected to HPLC gel filtration. Five fractions (Fig. 6*a*) were analysed by SDS–PAGE and silver staining (Fig. 6*b*) or by immunoblotting with the MAbs specific for site A (Fig. 6*c*), site C (not shown) or site D (Fig. 6*d*). Both site A- and site C-specific MAbs recognized a 28K fragment in fraction d, well separated from a 50K fragment containing site D in fraction c. To remove an 18K fragment in fraction d, the 28K fragment was eluted from SDS–PAGE gels. SDS–PAGE (Fig. 6*e*) or reverse-phase HPLC on an Ultrapore RPSC C3 column (not shown) revealed only one component (although a second

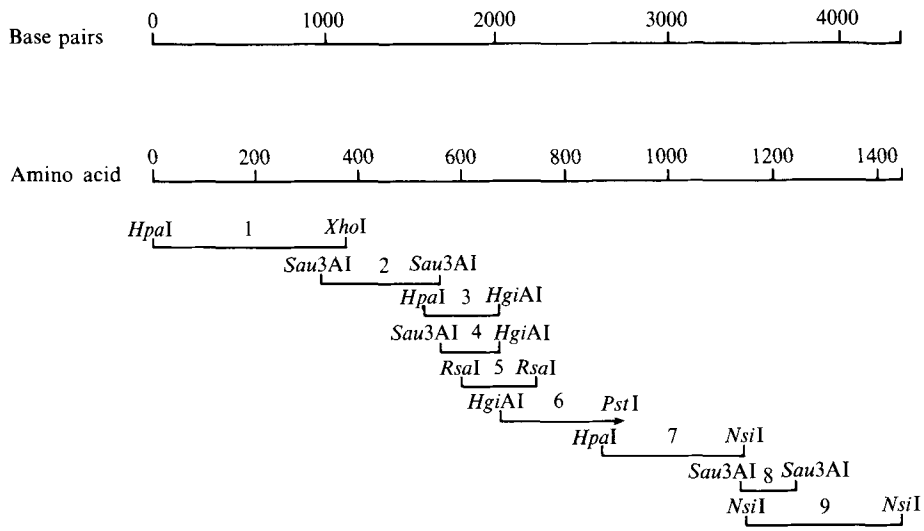


Fig. 4. E2 gene fragments cloned in pEX vector. Location of the nine partially overlapping E2 gene fragments, named 1 to 9, from the 5′ end to the 3′ end, inserted in the expression vector pEX (Stanley & Luzio, 1984), using the indicated restriction endonuclease insertion sites. For insert 6, a *Pst*I site from the polylinker region of the cDNA clone B1 was used (Jacobs *et al.*, 1987).

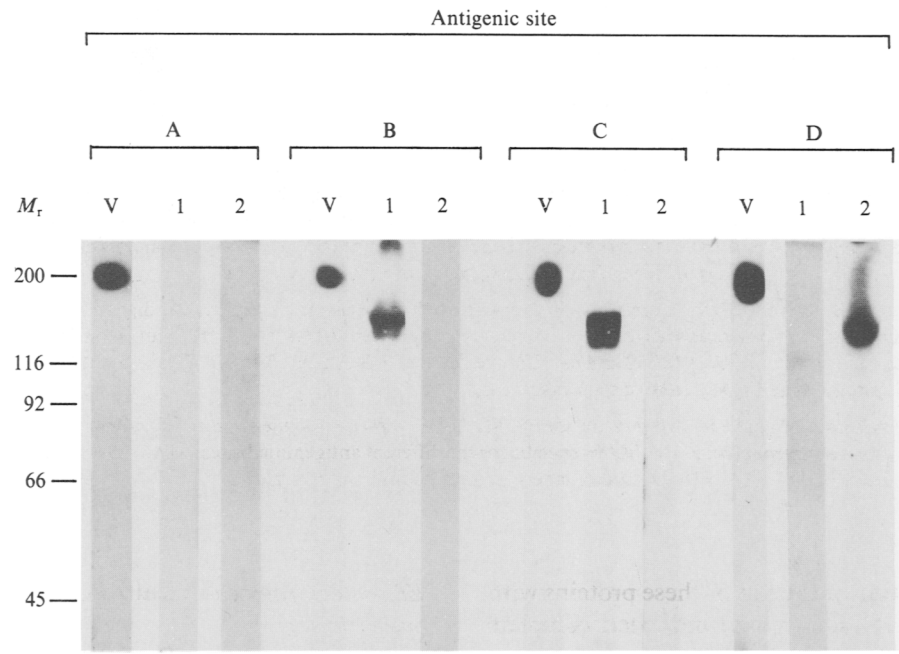
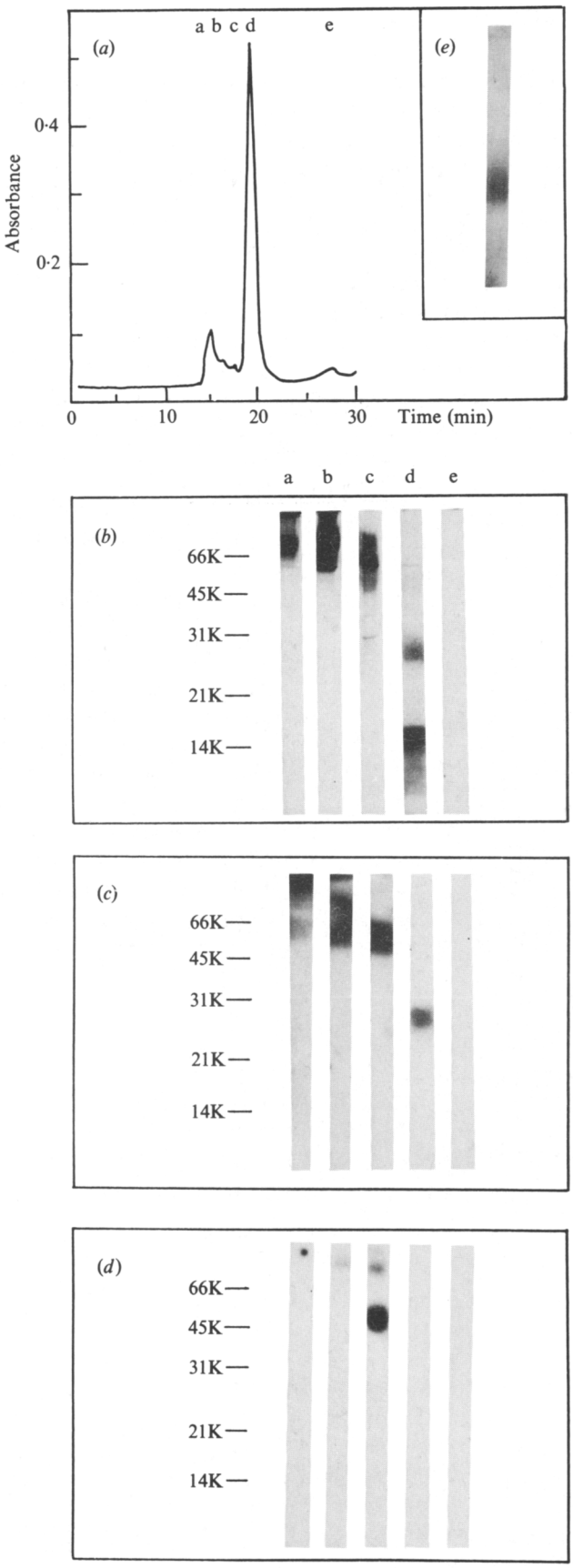


Fig. 5. Immunoblotting of pEX–TGEV expression products with MAbs. Lane V contains TGE virus proteins, lanes 1 and 2 the expression products of inserts 1 and 2, respectively. Antigens had been incubated in the presence of 2·5% SDS and 5% 2-ME. A, site A-specific MAbs (1G.A7, 1D.E7, 1A.F10 and 6A.C3); B, site B-specific MAb (1B.H11); C, site C-specific MAbs (5B.H1 and 6A.A6) and D, site D-specific MAb (1D.G3).

28K component present in a proportion lower than 10% could not be ruled out).

Mapping the 28K E2 protein fragment involved in the formation of antigenic sites A and C to the E2 gene

BALB/c mice were immunized with the 28K fragment and the resulting serum was used to screen expression products of pEX–TGEV plasmids on immunoblots (Fig. 4). The positive reaction with products of insert 1 (Fig. 7*a*), but not with products of the other inserts, indicated that the 28K fragment involved in site A and C formation was encoded by insert 1 sequences which did not overlap with insert 2, that is, by the 5′ end of the E2 gene (Fig. 4).



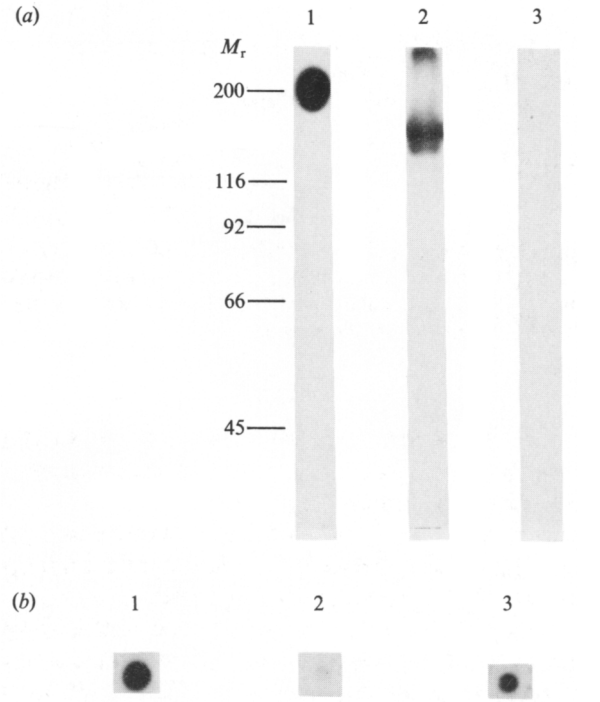


Fig. 7. Binding of antiserum raised against the 28K E2 protein fragment to pEX–TGEV expression products or to a synthetic peptide from the N-terminal end of E2 protein. (*a*) Immunoblot of TGE virus proteins (lane 1) or pEX–TGEV expression products of insert 1 (lane 2) and 2 to 9 (lane 3), incubated with a murine antiserum against the 28K fragment. (*b*) Immunodot analysis of the 28K-specific antiserum with TGE virus (1), KLH (2) and with a synthetic peptide containing the 18 N-terminal residues of the E2 protein, conjugated to KLH (3).

To determine whether the 28K fragment started at the N-terminal end of E2 protein, a peptide including the first 18 N-terminal amino acids was synthesized with an additional cysteine residue at its amino-terminal end. After conjugation of this peptide to KLH dot blot assays showed that the antiserum against the 28K fragment(s) bound to TGE virus E2 protein and to the peptide–KLH conjugate (Fig. 7*b*, lanes 1 and 3, respectively), but not to KLH alone (Fig. 7*b*, lane 2).

Nucleotide differences between the sequences of the mar *mutants selected from TGE virus and the original* (*wt*) *virus*

The E2 gene of the clone PUR46.C1 of TGE virus and of the double *mar* mutant *dmar* 1B.B5–1B.B1 were cloned

Fig. 6. Purification of the 28K fragment of the E2 protein. V8 protease fragments of E2 glycoprotein were separated by gel filtration HPLC on two Protein Pak columns (*a*). Five major fractions, a, b, c, d and e, were analysed by SDS–PAGE and silver staining (*b*), or by immunoblotting with site A- (*c*) or site D- (*d*) specific MAbs (1G.A7 and 1D.G3, respectively). Site C-specific MAbs gave the same pattern as the site A-specific MAbs (not shown). (*e*) 28K fragment purified by preparative SDS–PAGE.

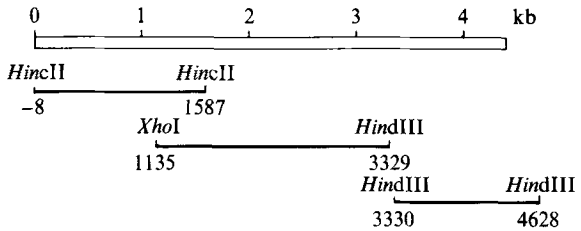


Fig. 8. E2 gene cloning strategy. cDNA from both clone PUR46.C1 of TGE virus and from the double *mar* mutant 1B.B5–1B.B1 selected from PUR46.C1 were cloned in the Bluescript plasmid using the indicated restriction enzyme fragments.

in the Bluescript vector. Three cDNA fragments covering the E2 gene of each virus were inserted using the strategy summarized in Fig. 8. The first 1950 nucleotides of the 5′ end of each gene were sequenced on these plasmids. Three nucleotide differences were detected (Table 3), which corresponded to residues 538, 543 and 631 of the E2 protein. To determine whether these nucleotide differences were present in the consensus population of the genomic RNA, direct RNA sequencing was performed, using the wt virus, *dmar* 1B.B5–1B.B1, and the mutants selected with one MAb, i.e. *mar* 1B.B5, *mar* 1G.A7 and *mar* 1B.C1. The sequence differences detected (Table 3) in the *dmar* 1B.B5–1B.B1 RNA were located in the same nucleotide positions as detected by DNA sequencing. In addition, analysis of *mar* mutants selected with MAbs specific for subsite a (1G.A7, 1B.C1) or c (1B.B5) indicates that the substitution at position 538 is associated with subsite a and the substitutions at positions 543 and 561 with subsite c.

Discussion

In this paper, we describe a correlation between the antigenic structure of the TGE virus E2 protein and its physical map. MAbs specific for four antigenic sites were used to screen protein fragments as well as the expression products of E2 gene fragments. In addition, the sequencing of *mar* mutants, selected from TGE virus stocks with site A-specific MAbs, was used to locate site A antigenic residues.

The preparation of protein fragments was facilitated by a convenient Triton X-114 phase separation procedure, which gave a high yield of purified E2. Digestion with staphylococcal V8 protease resulted in the isolation of a 28K fragment, which was identified as being N-terminal, since it induced an antiserum that bound to only the recombinant product of insert 1 (Fig. 7 and Table 2), and to a synthetic peptide containing the 18 N-terminal residues of E2 protein, although the presence of a different 28K fragment, also copurified by HPLC and SDS–PAGE with the N-terminal fragment, cannot be completely ruled out. However, this is not likely, since the polyvalent antiserum induced by the 28K fragment recognized recombinant products only of insert 1.

Antigenic site A is complex and discontinuous. MAbs specific for this site possibly bound to the 28K fragment identified as the N terminus. In addition, site A must contain the residues around positions 538 and 543, which are substituted in the *mar* mutants selected with site A-specific MAbs. These MAbs did not recognize any of the prokaryotic expression products, which are most suitable for the localization of linear epitopes (Lenstra *et al.*, 1989). Residue 538 is most likely involved in the

Table 3. *Nucleotide sequence differences between TGEV wt and TGEV* mar *mutants*

Clone	Nucleic acid sequenced	Antigenic subsite specificity of MAb‡	Nucleotide sequence		Base changed	Amino acid change		
			In wt virus	In *mar* mutant		Residue	From	To
Bluescript PUR46	DNA	c/a	AAG	CAG	1612	538	Lys	Gln
dmar 1B.B5–1B.B1*			GGT	GAT	1628	543	Gly	Asp
			GTT	GCT	1892	631	Val	Ala
PUR46 *dmar* 1B.B5–1B.B1	RNA†	c/a	AAG	CAG	1612	538	Lys	Glu
			GGU	GAU	1628	543	Gly	Asp
			GUU	GCU	1892	631	Val	Ala
PUR46 *mar* 1B.B5	RNA	c	GGU	GAU	1628	543	Gly	Asp
			GUU	GCU	1892	631	Val	Ala
PUR46 *mar* 1G.A7	RNA	a	AAG	AUG	1613	538	Lys	Met
PUR46 *mar* 1B.C1	RNA	a	AAG	ACG	1613	538	Lys	Thr

* The sequences of the 5′ end 1950 nucleotides of the PUR46.C1 strain of TGE virus and of the double *mar* mutant *dmar* 1B.B5–1B.B1 were obtained using cDNAs cloned in the Bluescript plasmid.

† RNA sequencing was performed on RNA from purified virions.

‡ The antigenic subsites were defined as described by Correa *et al.* (1988).

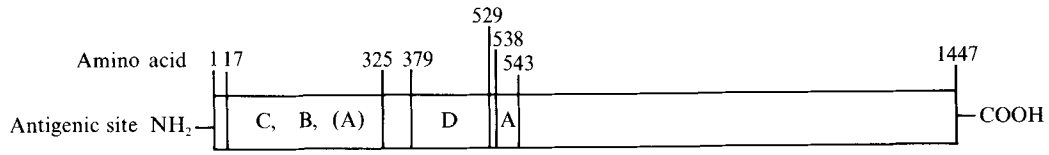


Fig. 9. Location of the antigenic sites of E2 glycoprotein. Site A is discontinuous and formed by residues around positions 538 to 543 and possibly also by residues between 17 and 297. Site B is located between 1 and 325 and site D between 379 and 529. The relative order of sites C and B and part of site A is not defined.

formation of subsite Aa, since this residue was substituted in three mutants selected with different subsite Aa-specific MAbs.

Amino acids 543 and 631 may be involved in the formation of subsite Ac, since these two residues were changed in both *dmar* 1B.B5–1B.B1 and the *mar* 1B.B5 mutants. Recognition of synthetic nonapeptides that contain residue 543 by subsite Ac-specific MAb 1A.F10 (L. Enjuanes, W. P. A. Posthumus and R. H. Meloen, unpublished results) suggests that the relevant residue difference, which facilitated the escape of the *mar* 1B.B5 mutant from neutralization by the corresponding MAb, is in position 543. The change in residue 631 may have been incidental during the cloning of the *mar* 1B.B5 mutant, which was used to select *dmar* 1B.B5–1B.B1. The precise location of the three antigenic subsites of site A will require the sequencing of more *mar* mutants, which is in progress.

The antigenic site A of TGE virus may resemble one of the neutralization epitopes of foot-and-mouth-disease virus, shown recently to be formed by two separated antigenic regions (Thomas *et al.*, 1988; Parry *et al.*, 1989). Hu *et al.* (1987) reported that a TGE virus-neutralizing MAb recognized E2 protein fragments expressed in *E. coli*, representing residues 378 to 601; this region contains the residues 538 and 543 involved in site A formation.

Fig. 9 summarizes the location of the antigenic sites. Site C is encoded only by insert 1 (Fig. 4 and 5 and Table 2), which is in agreement with its detection in the N-terminal 28K fragment. Site D is expressed by only pEX insert 2, leading to its localization between residues 379 and 529. More accurate localizations are being derived by sequencing MAb non-binding mutants and by epitope scanning (Geysen *et al.*, 1984). Data on the location of sites C and D by PEPSCAN technology confirmed the results presented here (L. Enjuanes, W. P. A. Posthumus, I. Correa, R. Meloen and J. A. Lenstra, unpublished results).

Interestingly, the four antigenic sites A, B, C and D, which were the target of the 1015 independently derived TGE virus hybridomas selected in our laboratory (Correa *et al.*, 1988), are encoded by an RNA segment that represents less than 37% of the complete E2 gene. This indicates that the E2 peplomer has a relatively small immunogenic area. In the model of de Groot *et al.* (1987) this area would be located in the globular part of the peplomer, which is more exposed than the fibrillar, C-terminal portion of the E2 dimer.

In an alignment of the peplomer sequences of TGE virus and feline infectious peritonitis virus, only 30% of the first 274 residues were found to be identical, contrasting with the 94% identity in the other 1173 residues (Jacobs *et al.*, 1987). This may reflect the variability of some antigenic domains in the globular part of the E2 molecule, the segment where we have located the four antigenic sites. In fact, while site A has been determined to be highly conserved (Jiménez *et al.*, 1986) the antigenic sites B, C and D are altered in related coronaviruses (Callebaut *et al.*, 1988; L. Enjuanes & C. M. Sánchez, unpublished results).

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