

## ANTIGENICITY OF THE PEPLIMER PROTEIN OF INFECTIOUS BRONCHITIS VIRUS

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**Abstract**—To study the antigenic structure of the peplimer protein of the avian coronavirus infectious bronchitis virus, fragments from the peplimer gene were generated by restriction-enzyme cleavage or by limited DNase digestion and inserted in the *Escherichia coli* expression plasmid pEX (Stanley and Luzio, 1984). The antigenicity of the expression products was tested using a number of polyclonal antisera and monoclonal antibodies.

The polyclonal antisera recognized different sets of epitopes in the 1162-residue sequence. The N-terminal region of one of the two subunits, S2, was recognized by all polyclonal sera and by two monoclonal antibodies. This clearly immunodominant region contains at least two adjacent or overlapping epitopes, one of which has been localized within 18 residues.

The epitopes found as antigenic pEX expression products do not coincide with the regions in the S1 subunit that have been found to contain hypervariable sequences. We suggest that these regions constitute conformation dependent neutralization epitopes that cannot be detected in the pEX system. The relevance of our findings for vaccine development is discussed.

### INTRODUCTION

Coronaviruses act as causative agents of a number of infectious diseases in domesticated animals (for a review about these positive stranded RNA viruses, see Siddell *et al.*, 1983; Sturman and Holmes, 1983). Conventional vaccines do not exist or are rendered ineffective by antigenic variation. This latter mechanism is thought to be relevant for the epidemiology of infectious bronchitis virus (IBV), which causes a highly contagious respiratory disease in chickens. After recent outbreaks of the disease, new serotypes have been isolated that may have originated from the live attenuated strains used for vaccination (Kusters *et al.*, 1987). Obviously, recombinant-DNA expression products or peptide vaccines would offer a valuable alternative for conventional vaccines.

The antigenic properties of IBV or other coronaviruses most likely reside in the peplimer (Cavanagh *et al.*, 1984), a club-shaped spike projecting from the viral membrane and consisting of a dimer or trimer of the E2 peplimer protein (Cavanagh, 1983). Analysis of the amino acid sequences of the peplimer proteins from representatives of the three main coronavirus serotypes (De Groot *et al.*, 1987) has led to a model, in which two

long  $\alpha$ -helices are located in the C-terminal half of the monomer subunit. In the oligomer, those helices intercalate and form a coiled-coil structure, which constitutes the stalk of the peplimer. The trans-membrane segment at the C-terminal end serves as membrane anchor. The N-terminal half is thought to form the spherical outer part of the peplimer.

In IBV, proteolytic cleavage results in an N- and a C-terminal subunit, designated S1 and S2, respectively. S1 is thought to be the most relevant part for the antigenic properties of IBV (Cavanagh *et al.*, 1986). It is recognized by strongly neutralizing monoclonal antibodies (Mockett *et al.*, 1984; Koch *et al.*, unpublished). Furthermore, isolated S1 is capable of eliciting neutralizing antisera (Cavanagh *et al.*, 1986). By comparing the amino acid sequences of different strains, it was found that most amino acid substitutions, likely to reflect antigenic variation, have occurred in the S1 subunit (Niesters *et al.*, 1986; Binns *et al.*, 1986; Kusters *et al.*, unpublished).

In this study, we have localized epitopes by expressing parts of the peplimer sequence in a prokaryotic expression system, and measuring the antigenicity of the expression products (Stanley and Luzio, 1984). Most probably, this method detects epitopes that are essentially conformation independent. Antigenic regions were almost exclusively found in the S2 subunit, suggesting that the antigenic variation in S1 reflects conformation dependent epitopes. One immunodominant region has been localized near the N-terminus of S2. This region was recognized by

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; IBV, infectious bronchitis virus; MAb, monoclonal antibody; PBS, phosphate-buffered saline.

all polyclonal antisera tested as well as by two neutralizing monoclonal antibodies, and contains at least two different epitopes. The sequence of this region may be the starting point for the development of peptide vaccines.

#### MATERIALS AND METHODS

##### *cDNA clones, antisera and bacterial strains*

The cDNA clones p39 and p42 of the peplomer gene of IBV strain M41 in the vector pUC9 have been described previously (Niesters *et al.*, 1986).

Rabbit antisera against M41 were raised by intramuscular injection of purified virus (1 mg protein), lysed by Triton X-100, in Freund's complete adjuvant, followed by the same dose in Freund's incomplete adjuvant after two and three weeks, respectively. Mouse antiserum was raised by intraperitoneal injection of 100  $\mu$ g virus protein, followed by a second dose after 1 month. Chicken antisera were collected from convalescent (chickens 1 and 3) or hyperimmune (chickens 2 and 4) animals, infected with IBV strain M41 (chickens 1 and 2) or strain D207 (chickens 3 and 4).

The preparation of the monoclonal antibodies 26.1 and 31.5 against IBV has been described previously (Koch *et al.*, 1986).

Alkaline phosphatase-conjugated goat anti-(mouse IgG, H+L) and goat anti-(rabbit IgG, Fc) were from Promega (Madison); anti-(chicken IgG, H+L) conjugates were from Nordic, Tilburg, The Netherlands (horseradish peroxidase) or from Miles (alkaline phosphatase).

As pEX host strain, *E. coli* pop 2136 was used, which was constructed by Dr Raibaud (Institut Pasteur, Paris).

##### *Construction of pEX clones*

Unless mentioned otherwise, all DNA manipulations used were done essentially as described by Maniatis *et al.* (1982) or Davis *et al.* (1986).

Genomic cDNA clones p39 and p42 were cut with restriction enzymes as indicated in Fig. 1. Fragments were isolated by agarose gel electrophoresis onto NA-45 DEAE cellulose membranes (Schleicher and

Schuell). The *Hinf*I ends of h1 were made blunt by treatment with the Klenow fragment of DNA polymerase. Depending on the desired reading frame, the fragments were ligated in compatible sites of pEX1, pEX2 or pEX3 (Stanley and Luzio, 1984), which had been treated with calf intestine phosphatase if linearized by a single digestion.

Recombinant plasmids were introduced into *E. coli* strain pop 2136 via the  $\text{CaCl}_2$  transformation procedure. Recombinants expressing the IBV gene fragments were selected by immunoscreening with a rabbit antiserum or, when the expression product was not recognized antigenically, by hybridization followed by gel electrophoresis of the protein expression products. As hybridization probe, a mixture of *Hind*III/*Hinf*I fragments of the insertion from plasmid p39 was used, which could be isolated free from the 2.7-kb pUC fragment by gel electrophoresis.

##### *Construction of a DNase-fragment library*

After isolating the 4.0-kb insertion of plasmid p39 by agarose gel electrophoresis, limited DNase digestion was carried out with 1  $\mu$ g DNA and 1 ng DNase (Worthington) in 10  $\mu$ l 20 mM Tris-HCl (pH 7.5), 1.5 mM  $\text{MnCl}_2$  and 0.1 mg/ml BSA at 22 C for 6 min (first 5  $\mu$ l of the reaction mixture) or 10 min (remaining 5  $\mu$ l). As judged by gel electrophoresis and autoradiography after end-labelling with the Klenow fragment of DNA polymerase, fragments of 20–500 bp were generated. These fragments were made blunt-ended by treatment with T4 DNA polymerase, followed by treatment with the Klenow fragment. After sequential extractions with phenol/chloroform and ether, the fragments were ligated directly in pEX2 DNA, which had been cut with *Sma*I and dephosphorylated with calf intestine phosphatase. The reaction was carried out overnight at 16 C in aliquots of 20  $\mu$ l containing 200 ng vector, 10 ng DNase fragments and 10 units T4 ligase. Transformation of *E. coli* pop 2136 with 1  $\mu$ g DNA via the Hanahan procedure yielded 9000 colonies, 80% of which hybridized with the p39 insertion probe (see above). The size of the insertions ranged from 50 to 500 bp.

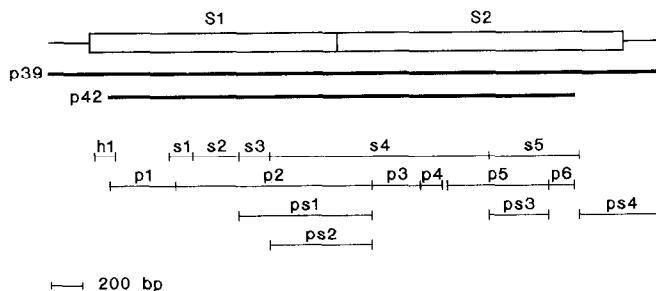


Fig. 1. Restriction-enzyme fragments from the peplomer gene that have been expressed. Thick lines indicate the insertions of the peplomer cDNA plasmids p39 and p42. h, *Hinf*I fragment; p, *Pst*I fragment; ps, *Sau*3AI-*Pst*I fragment; s, *Sau*3AI fragment. By a single nucleotide difference between the two plasmids, one of the *Sau*3AI sites in the IBV sequence in p42 is not present in p39.

This rapid cloning procedure obviates the use of linkers (Haymerle *et al.*, 1986), but generates nevertheless a sufficient number of clones from a low amount of insert DNA.

#### *Immunoscreening of bacterial colonies*

Immunoscreening of bacterial colonies transferred to nitrocellulose membranes (BA85 from Schleicher and Schuell) was performed as described (Stanley, 1983), using a gelatin/Triton X-100/PBS buffer and an *E. coli* lysate to quench aspecific reactions. The dilutions of the primary antisera were 1:1000 for polyclonal sera and 1:100 or 1:500 for ascites fluids containing monoclonal antibodies. Binding of antisera to colonies was visualized using alkaline phosphatase-conjugated secondary antisera (diluted 1:5000) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates (Blake *et al.*, 1984).

#### *Nucleotide sequence analysis of pEX plasmids*

To localize DNase fragments from pEX clones that were positive after immunoscreening or to check the correct reading frames for the expression of restriction fragments, DNA from pEX/IBV recombinants was isolated by the alkaline-lysis method, cut with two restriction enzymes flanking the insertion and with *Hind*III to cleave the pEX vector sequence. The resulting mixture of fragments was ligated in M13mp9 DNA cut with the appropriate enzymes. Subsequently, the nucleotide sequence of the insertion was determined by the dideoxynucleotide termination method and compared to the sequence of the IBV M41 peplomer gene.

#### *Isolation of expression products*

Expression of the pEX fusion gene, leading to the synthesis of a hybrid  $\beta$ -galactosidase protein, was induced in a 5 ml exponential culture ( $OD^{600}$  ca. 0.25) by incubation at 42°C for 90 min. Hybrid proteins were purified as Triton X-100 pellet (Stanley and Luzio, 1984). Cells were spun down (10 min at 5000 g), resuspended in 100  $\mu$ l 15% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, and treated with lysozyme (1 mg/ml) for 10 min. After addition of 140  $\mu$ l 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. The lysozyme digestion as well as use of the sucrose buffer appeared essential for the purity of the final preparation. After spinning down the insoluble hybrid protein (10 min at 10,000 g) and removing the viscous supernatant as completely as possible, the pellet was resuspended in Triton X-100 buffer. Sonification, centrifugation and removal of the supernatant were repeated (1–2 times) until no viscosity remained. Finally, the pellet was resuspended in 250  $\mu$ l PBS.

#### *Dot blots; Western blotting*

After adding concentrated SDS-containing lysis

buffer (Laemmli and Favre, 1973), hybrid proteins were spotted via a manifold on nitrocellulose membranes or fractionated by SDS-polyacrylamide gel electrophoresis (7.5%), followed by Western blotting. Hybrid proteins thus immobilized were analysed as described for the filters of the immunoscreening (see above). Binding of chicken antisera was visualized using peroxidase-conjugated secondary antisera (diluted 1:1000) and 3'-3' diaminobenzidine and  $H_2O_2$  as substrates.

## RESULTS

#### *Subcloning strategy and characterization of clones*

In the pEX expression system, foreign gene fragments are inserted at the 3' end of a *cro-lacZ* expression vector (Stanley and Luzio, 1984). Expression of this gene is under control of the lambda  $P_R$  promoter and induced by inactivation of the temperature sensitive *cI* repressor at 42°C. The resulting expression products precipitate inside the cell and are thus protected against proteolysis. After solubilization, the product of the foreign gene fragment, linked to the C-terminal residue of the  $\beta$ -galactosidase part, may be recognized by appropriate antisera.

Figure 1 shows the *Hin*fl, *Pst*I, *Sau*3AI and *Sau*3AI/*Pst*I fragments that have been excised from the genomic cDNA plasmids p39 or p42 (Niesters *et al.*, 1984) and expressed in pEX plasmids. The *Hin*fl site in the region encoding the N-terminal signal sequence of the peplomer protein allowed the cloning of the 5' part of the coding sequence without the stop codons just upstream of (and in frame with) the ATG start codon.

As judged from an SDS-polyacrylamide gel [Fig. 2(A)], the hybrid proteins have sizes predicted by the lengths of the insertions. Exceptions were ps4, which contains the stop codon of the peplomer gene, and the 144 bp fragment p4, which was inserted twice, both fragments expressed in tandem and in the same reading frame. Analysis of the sequence showed that only in the case of h1 and p4, hybrid proteins of (almost) the same size could be obtained after translation in a wrong reading frame; the proper reading frames of these clones have been checked by sequencing. Fragment p1 starts with a stretch of 12 dG residues (originating from p42); apparently, this did not disturb the reading frame by ribosome slippage along the homopolymer track (Stanley, 1983).

The IBV peplomer sequences contained in the hybrid proteins are listed in Table 1. Only residue 1064 has not been overlapped by an expression product. Further, a reactive region exactly on the junction of two fragments or in the gap between p4 and p5 can only be detected in an overlapping fragment if this fragment does not contain other reactive regions. In the theoretically worst case, the reactivity of 8% of the total peplomer sequence would not be detected, assuming an epitope size of 10 residues.

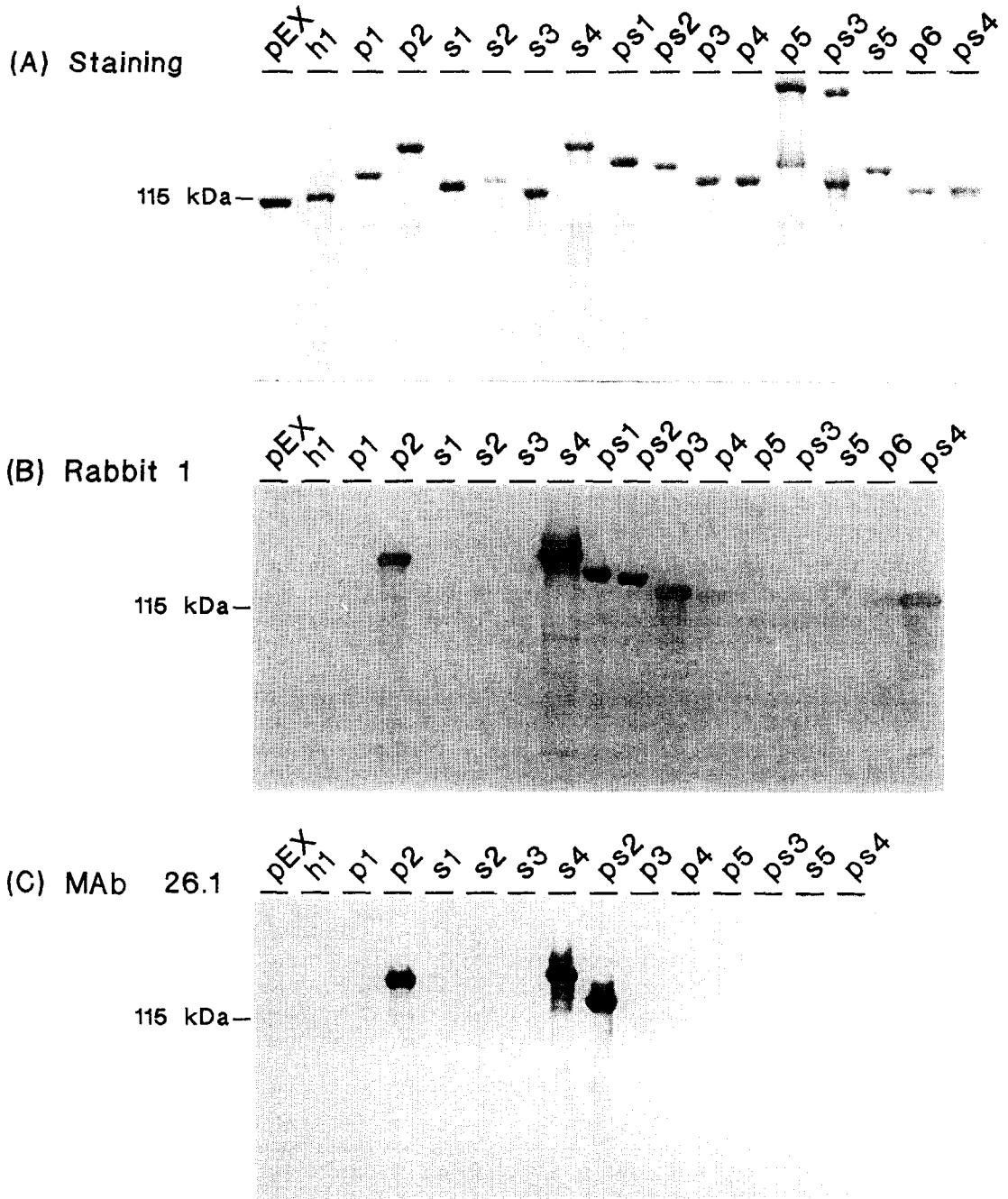


Fig. 2. Gel electrophoresis and Western blots of hybrid  $\beta$ -galactosidase proteins. The molecular weight of hybrid proteins from pEX-plasmids without insertions (lanes pEX) is 115,000. (A) Staining with Coomassie brilliant blue; (B) Western blot with a rabbit anti-IBV serum; (C) Western blot with MAb 26.1.

#### *Regions recognized by polyclonal antibodies*

In Fig. 2(B), a Western blot of the IBV/pEX hybrid proteins, incubated with a rabbit antiserum, is shown. Only three regions were recognized strongly, one contained in p2, s4, ps1 and ps2, the second in p3 and s4, and the third in ps4. Weaker signals were observed for the regions contained in p4, in p5 while not in ps3, and in the overlap of s5 and p6, respectively.

Similar experiments have been carried out with another rabbit antiserum, with a mouse antiserum and with four antisera from chickens, infected with IBV strain M41 (chickens 1 and 2) or with strain D207 (chickens 3 and 4). The binding patterns are summarized in Fig. 3. Because of high background signals of the chicken antisera, only strong signals could be scored. Apparently, each antiserum recognized a different set of epitopes. The number of

Table 1. IBV/pEX recombinants

| Fragment | Linearized vector                   | Peplomer sequence residues |
|----------|-------------------------------------|----------------------------|
| h1       | pEX3 × <i>Hin</i> fl                | 10-56                      |
| p1       | pEX1 × <i>Pst</i> I                 | 48-186                     |
| p2       | pEX2 × <i>Pst</i> I                 | 186-612                    |
| s1       | pEX3 × <i>Bam</i> HI                | 172-224                    |
| s2       | pEX1 × <i>Bam</i> HI                | 223-324                    |
| s3       | pEX1 × <i>Bam</i> HI                | 323-390                    |
| ps1      | pEX1 × <i>Bam</i> HI × <i>Pst</i> I | 323-612                    |
| ps2      | pEX3 × <i>Bam</i> HI × <i>Pst</i> I | 390-612                    |
| s4       | pEX3 × <i>Bam</i> HI                | 390-867                    |
| p3       | pEX1 × <i>Pst</i> I                 | 611-717                    |
| p4       | pEX2 × <i>Pst</i> I                 | 717-766                    |
| p5       | pEX1 × <i>Pst</i> I                 | 775-997                    |
| ps3      | pEX3 × <i>Bam</i> HI × <i>Pst</i> I | 867-997                    |
| s5       | pEX3 × <i>Bam</i> HI                | 867-1064                   |
| p6       | pEX2 × <i>Pst</i> I                 | 997-1045                   |
| ps4      | pEX3 × <i>Bam</i> HI × <i>Pst</i> I | 1065-1162                  |

The positions of the restriction-enzyme fragments of the peplomer gene are shown in Fig. 1. The fragments were ligated to linearized pEX1, -2 or -3 as indicated. The last column indicates which parts of the amino acid sequence of the peplomer protein are synthesized by the recombinants after induction of the expression.

antigenic regions as defined by the restriction fragments varies from 1 (mouse) to 6 (rabbit 1). One region, corresponding to fragment ps2, was recognized by all homologous and heterologous antisera.

#### Epitopes recognized by monoclonal antibodies

Most neutralizing monoclonal antibodies described so far are reported to recognize the S1 subunit of the peplomer protein (Mockett *et al.*, 1984; Koch *et al.*, unpublished results). We tested MAb 13, reacting with S1 or S2 (Niesters *et al.*, 1987), MAb A38 (Mockett *et al.*, 1984) as well as MAbs 48.2, 48.3, 51.2 and 52.4 (Koch *et al.*, unpublished results). None

of these sera bound any of the pEX products, most likely because the epitopes of these antisera are conformation dependent. However, the lack of binding of other, non-neutralizing monoclonal antibodies recognizing conformation independent epitopes on the peplomer protein (Niesters *et al.* 1987), suggests that other factors than the conformation dependence may affect the reactivity of monoclonal antibodies with pEX hybrid proteins.

Koch *et al.* (1986) described a number of monoclonal antisera with weak neutralizing activity that bound to the S2 subunit on Western blots. One of these, MAb 26.1, recognized all IBV strains tested and has a neutralization titre of about  $10^3$ . Figure 2(C) shows a Western blot of hybrid proteins screened with this MAb. The strong binding to fragments p2, s4, ps1 and ps2 localizes the epitope of MAb 26.1 between residues 390 and 612, the same region that is recognized by all polyclonal sera. Since subunit S2 starts at residue 538, the epitope must lie in the N-terminal region (residues 538 to 612) of S2. The same localization has been found for the epitopes of another S2-specific MAb 31.5.

To further localize the epitope of MAb 26.1, a library of small random DNase fragments in pEX has been constructed. After immunoscreening, 0.2% of the colonies were found to react positively with MAb 26.1. Six clones were selected for sequence analysis after subcloning in M13 replicative-form DNA. The results are summarized in Fig. 4. All clones contain nucleotides 1620-1673, restricting the localization of the epitope of MAb 26.1 to residues 541-558.

Figure 5 shows the antigenicity of four expression products of the DNase fragments to MAb 26.1 and to rabbit 1 antiserum; the results with the other

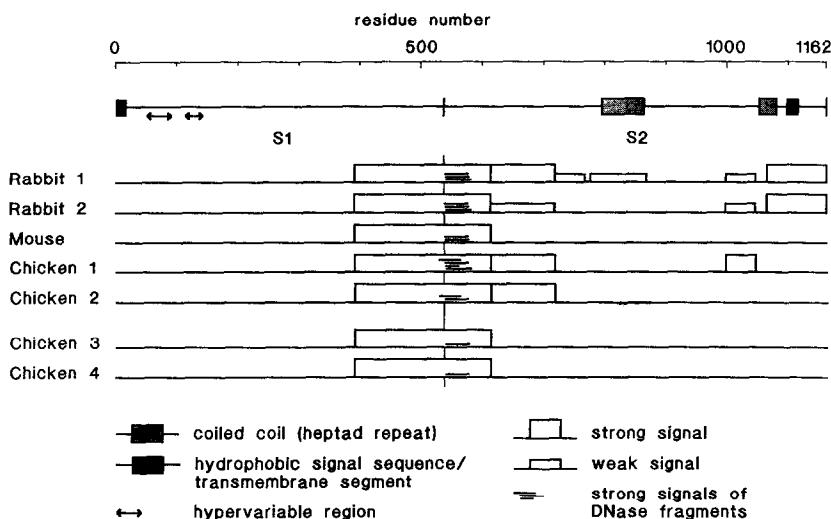


Fig. 3. Reaction patterns of polyclonal antisera with hybrid proteins containing IBV peplomer sequences. The top part of the figure shows the regions coding for the peplomer subunits S1 and S2 and the locations of characteristic sequence segments. Chicken 3 and 4 denote heterologous antisera from chickens infected with IBV strain D207. With the chicken sera, the background was too high for the detection of weak signals.

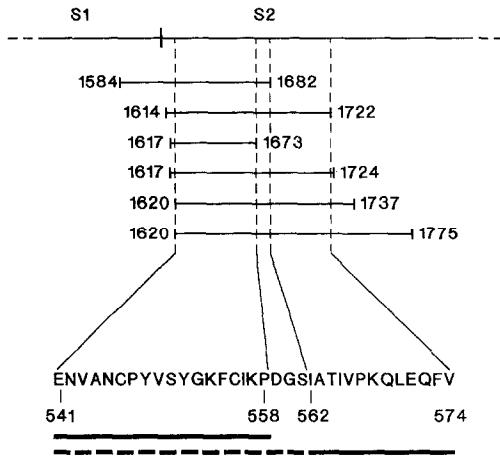


Fig. 4. Positions of overlapping DNase fragments selected from a random library by MAb 26.1. The beginning and end positions of the DNase fragments within the coding region are indicated by nucleotide numbering. Sequence analysis showed that fragment 1617–1673 had been inserted in pEX upstream of another DNase fragment (3316–3366), which is expressed in an artificial reading frame and adds three serines to residues 540–558. The upper heavy line indicates the region sufficient for recognition by MAb 26.1 and by the chicken 1 antiserum (residues 541–558). The lower heavy line indicates the region recognized by MAb 31.5 and by the rabbit and mouse polyclonal antisera: the essential residues 562–574 (uninterrupted part) and the possible contribution of residues 541–561 (dashed part).

antisera and fragments are listed in Table 2. The antiserum from chicken 1 binds to the expression products of the same fragments as MAb 26.1, indicating the recognition of the same or an adjoining epitope. The results with antiserum of chicken 2 were clouded by a particularly high background reaction, but the clear positive reactions with two of the fragments indicate a binding to the same region. Interestingly, the negative reactions of fragments (1584–1682) and (1617–1673) demonstrate that the epitope(s) of MAb 31.5 and of the sera of rabbit 1, rabbit 2 and mouse are located partially or completely in the region 562–574.

The positions of the DNase defined regions reactive with the polyclonal sera are graphically represented in Fig. 3 by short horizontal lines. The delineations of the individual epitopes are indicated in

Fig. 4 by heavy lines, the dashed line indicating the possibility that the region 541–561 contributes to the epitopes recognized by the MAb 31.5 and by the rabbit and mouse antisera.

In indirect ELISAs on Western blots with intact viral proteins, MABs 26.1 and 31.5 have different specificities (Koch *et al.*, 1986, unpublished results). MAB 26.1 recognizes the S2 subunit of all strains tested, while MAB 31.5 only binds to S2 of the H120/B222/M41 serotype. The same specificity was found with pEX expression products: MAB 26.1 binds to the hybrid proteins of strains M41, D207 and D1466; MAB 31.5 only to M41 products. An accurate definition of the epitope of MAB 26.1 and other epitopes in the same region, based on the sequences of several IBV strains, on PEPSCAN peptide synthesis (Geysen *et al.*, 1984) and on competition experiments will be published elsewhere (Kusters *et al.*; Koch *et al.*, in preparation).

## DISCUSSION

### *The localization of continuous epitopes*

In the pEX system, the expression product of an inserted DNA fragment constitutes the C-terminus of a hybrid  $\beta$ -galactosidase protein, which precipitates in the cell. Prior to the assay of its antigenicity, the hybrid protein is solubilized by SDS and transferred to a nitrocellulose filter. Presumably, epitopes found by cross-reaction with antisera are essentially conformation independent. The localization of epitopes within stretches of 10–20 residues in several coronaviruses (Fig. 4, unpublished results) demonstrates that the binding of antibody does not depend on particular flanking sequences, and that any native-like folding of the epitope in the hybrid protein is confined to the same small region.

Classically, continuous epitopes are localized by testing the antigenicity of peptides (Atassi, 1984). Prokaryotic expression of subgenomic fragments in pEX or in  $\lambda$ gt11 (Nunberg *et al.*, 1984; Mehra *et al.*, 1986) offers a convenient alternative and is not limited to small proteins. We have combined the expression of large (130–1400 bp) restriction fragments to localize antigenic regions, and of small DNase fragments to delineate epitopes more accurately. Another strategy is the subcloning of small

Table 2. Binding to IBV M41 antisera of expression products of DNase fragments from the peplomer gene

| Nucleotides | Amino acid residues | MAB  |      | Polyclonal antisera |    |   |     |     |     |     |   |
|-------------|---------------------|------|------|---------------------|----|---|-----|-----|-----|-----|---|
|             |                     | 26.1 | 31.5 | R1                  | R2 | M | Ch1 | Ch2 | Ch3 | Ch4 |   |
| 1584–1682   | 529–561             | +    | –    | –                   | –  | – | +   | +   | /   | /   | / |
| 1614–1722   | 539–574             | +    | +    | +                   | +  | + | +   | +   | /   | /   | / |
| 1617–1673   | 540–558             | +    | –    | –                   | –  | – | +   | –   | /   | /   | / |
| 1617–1724   | 540–575             | +    | +    | +                   | +  | + | +   | –   | +   | +   | / |
| 1620–1737   | 541–579             | +    | +    | +                   | +  | + | +   | +   | +   | /   | / |
| 1620–1775   | 541–592             | +    | +    | +                   | +  | + | +   | +   | +   | /   | / |

Antigenicities have been assayed on dot or Western blots. Nucleotides are numbered from the AUG start codon. R1, rabbit 1; R2, rabbit 2; M, mouse; Ch1, chicken 1; Ch2, chicken 2; Ch3, chicken 3 (anti-D207); Ch4, chicken 4 (anti-D207); +, reaction as intense as positive control (ps2); –, reaction not higher than background control (pEX without insertion); /, not determined.

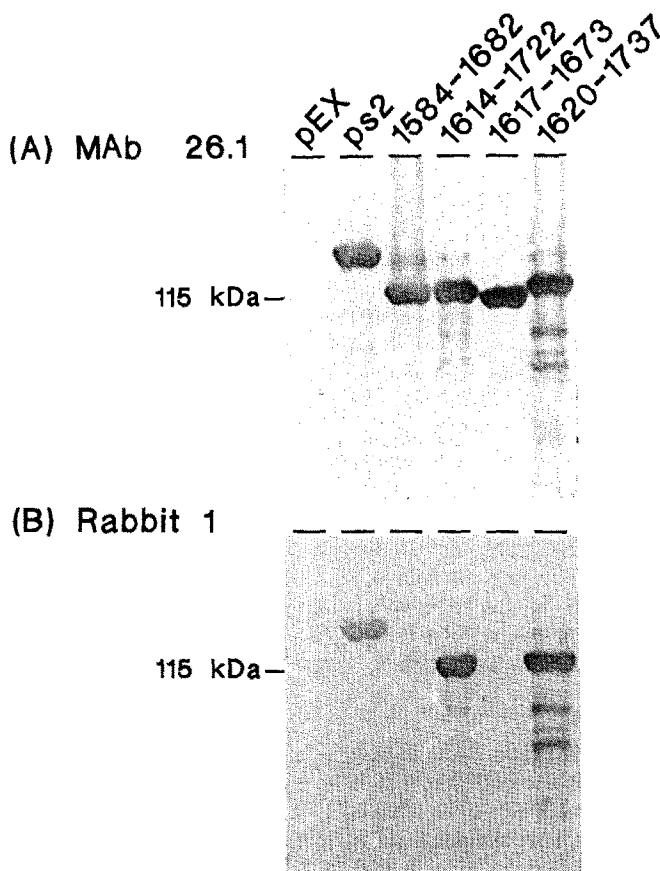


Fig. 5. Western blots of hybrid proteins from pEX recombinants containing DNase fragments with (A) MAb 26.1 and (B) rabbit antiserum. Numbers denote the positions of the DNase fragments. pEX and ps2 served as negative and positive controls, respectively.

restriction fragments, identifying positives with immunoscreening (De Groot *et al.*, Kusters *et al.*, Luytjes *et al.*, unpublished results). This obviates the sequence analysis of DNase fragments, but depends on the presence of suitable restriction sites or on the availability of homologous sequences.

Another new and complementary approach to the localization of continuous epitopes is the synthesis of a large number of peptides coupled to a solid phase, the so-called PEPSCAN (Geysen *et al.*, 1984). This method has been used in conjunction with the pEX expression system for the localization of a neutralization epitope of the peplomer protein of mouse hepatitis virus (Luytjes *et al.*, unpublished results). However, not all monoclonal antibodies that cross-react with pEX expression products bind to PEPSCAN peptides spanning the antigenic region (Posthumus and Melen, unpublished results). For instance, MAb 26.1 did not recognize any of the overlapping nonapeptides spanning the N-terminal region of the S2 subunit. Whether this should be explained by the lengths of the peptides or by local conformations in the hybrid proteins will be the subject of further study.

#### *The location of epitopes*

The reactivity patterns of the different polyclonal antisera (Fig. 3) outline the variability of the humoral immune response against the IBV peplomer protein at the molecular level. There are also a number of other noteworthy features.

Firstly, all antisera recognize in the 1162-residue sequence a relatively small (1–6) number of regions, predominantly in the S2 subunit; the actual number of epitopes per antiserum depends on the degree of clustering of epitopes within the antigenic regions. So, if the whole surface of the proteins is potentially antigenic (Berzofsky, 1985; Geysen *et al.*, 1987), this is clearly not the case for the determinants detected as antigenic pEX hybrid proteins.

Secondly, the N-terminal part of the S2 subunit is recognized by all polyclonal antisera tested and by a number of monoclonal antibodies. Within this region, the monoclonal and polyclonal antibodies recognized different epitopes (Table 2). The MAbs have neutralization titres ranging from 10 to 1000 (Koch *et al.*, 1986), suggesting a role of this region in the neutralization of the infection.

Thirdly, the regions found to be antigenic do not coincide with the hypervariable regions in the S1 subunit (Niesters *et al.*, 1986; Binns *et al.*, 1986). These regions are supposed to be part of neutralization epitopes that are the targets of antigenic variation. In fact, although the S1 subunit is thought to induce the protective immunity against IBV (Cavanagh *et al.*, 1986), we found it to be relatively, if not completely, devoid of antigenicity (Fig. 3).

As the most obvious explanation, we propose that the neutralization epitopes in S1 are conformation dependent and cannot be mimicked by pEX hybrid proteins. This would also explain the lack of reaction of the neutralizing MAbs that were directed against epitopes in S1. Apparently, these epitopes are of the same category as the conformation dependent neutralization epitopes of the haemagglutinin of influenza virus (Wiley *et al.*, 1981). In contrast, the epitopes localized in the N-terminal region of S2 may be comparable to the epitopes of foot-and-mouth disease virus, detectable by cross-reaction with peptides (Geysen *et al.*, 1984; Rossman *et al.*, 1985).

#### *Prospects for vaccine development*

Continuous epitopes often correspond to relatively mobile regions in the protein structure (Westhof *et al.*, 1984; Van Regenmortel, 1987). By virtue of their inherent cross-reactivity with short peptides, these regions are the prime candidates for the development of peptide vaccines (Van Regenmortel, 1987). Thus, for the N-terminal region of the IBV S2 subunit, structural considerations may outweigh the relatively weak neutralization, if compared with the epitopes in S1 (Mockett *et al.*, 1984; Koch *et al.*, unpublished results).

The N-terminal sequences of S2 from strains of different serotypes are relatively constant (Kusters *et al.*, submitted). MAb 26.1, recognizing this region, has a broad serotype specificity (Koch *et al.*, 1986). Further, M41 sequences cross-react with chicken antisera against D207, belonging to a different serotype, (Fig. 3, Table 2) and vice versa (not shown). Apparently, this region is not subject to antigenic variation. This would be another advantage of the epitopes found in S2.

Thirdly, the recognition of the N-terminal S2 sequence by several polyclonal antisera indicates that this region is strongly immunodominant. This would favour a response in all vaccinated animals.

These considerations may become relevant as soon as the technology for designing effective peptide vaccines becomes available.

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