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ANALYSIS OF AN IMMUNODOMINANT REGION OF INFECTIOUS BRONCHITIS VIRUS[1]

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We analyzed the antigenic fine-structure of an immunodominant region in the peplomer protein of infectious bronchitis virus. This region near the N-terminus of the S2 subunit is recognized by polyclonal antisera and by the majority of mAb that cross-react with denatured protein. Despite their involvement in neutralization, epitopes in this region were conserved in different serotypes. Epitopes of four mAb and two chicken antisera were localized by using prokaryotic expression of cDNA fragments, and overlapping peptides with lengths increasing from 3 to 12 residues (PEPSCAN). We found overlapping epitopes with lengths of 6, 9, 11, and more than 17 residues. The results indicate that the expression products are antigenically equivalent to denatured protein fragments. This suggests a general strategy for the localization of sequential epitopes in large proteins. We propose that the immunodominance of the N-terminal region of S2 is explained by features of the protein structure. Presumably, this region is a protruding protein segment of about 20 residues with a high local mobility, as indicated by the antigenicity of the peptides. The conservation of the sequence points to an involvement in a molecular recognition process during infection.

Avian IBV[4], a coronavirus, causes a highly contagious disease in chickens. The major obstacle to an effective vaccination is the continuous emergence of antigenic variants. Serologic differences between different IBV strains are most likely located on its peplomers, club-shaped structures projecting on the surface of the virion. Each peplomer consists of a dimer or trimer of the peplomer protein (E2), which is cleaved posttranslationally into two, noncovalently bound subunits, S1 and S2 (1). The S2 subunit consists of the carboxyl-terminal half of the E2 sequence and contains a transmembrane anchor and two long α-helices that probably form the stalk of the peplomer (2). The S1 subunit forms the bulbous upper part of the peplomer and is thought to be crucial for the protective immune response and the main target of antigenic variation of IBV. Serotype-specific neutralizing monoclonal antibodies bind to S1 (3) (Koch, G., unpublished data) which also contains hypervariable sequences (4–6). The neutralization epitopes on S1 are dependent on the native protein conformation (7) (Koch, G., unpublished data).

In contrast, conformation-independent epitopes are found almost exclusively in the S2 subunit (7). The N-terminal part of this subunit was shown to contain an immunodominant region recognized by several polyclonal antisera. Inasmuch as this region carries different neutralization epitopes and was not affected by antigenic variation, it was suggested to be a suitable starting point for the development of a synthetic IBV vaccine (7).

Regions carrying immunodominant epitopes have been described for several viruses, such as a number of picornaviruses (8, 9, 34) and HIV (10). In few cases, the epitopes have been delineated accurately (10–12). In addition, whether the location of these epitopes reflects intrinsic properties of the protein molecule is still a matter of dispute (13–15).

In this report, we describe an accurate mapping of the epitopes of four mAb that recognize the immunodominant region in the S2 subunit of IBV. Conservation of residues involved in the binding of neutralizing mAb suggests that this region is essential for peplomer structure or function. By two different methods, it is shown that the mAb bind to partially overlapping, but distinct epitopes. Our results suggest that the location of immunodominant regions may be predetermined by the protein structure, whereas within such regions the epitopes are selected by factors extrinsic to the protein structure.

MATERIALS AND METHODS

Virus strains. Virus strains were obtained from the Poultry Health Institute (Doorn, The Netherlands). Viruses were passaged once in the allantoic cavity of 10-day-old chicken embryos. Virus stocks were stored at −70°C.

Antisera and mAb. Virus-specific chicken sera were collected from animals infected with IBV as described (16). Ascites fluids were produced as described (17).

Cloning and sequencing of IBV peplomer genes. Virus growth, isolation of genomic RNA, cDNA synthesis, and nucleotide sequencing were carried out essentially as described previously (4) using either random pentamers, or specific primers of 15 nucleotides to generate cDNA. All DNA manipulations were performed essentially

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[4] Abbreviation used in this paper: IBV, infectious bronchitis virus.

as described by Maniatis et al. (18). Genomic cDNA clones containing the peplomer gene were sequenced using bacteriophage M13. A detailed description of the cloning procedures is published elsewhere (6).

Construction of IBV-pEX recombinants. Restriction-endonuclease sites in the cDNA sequences were used to obtain fragments of the peplomeric protein genes of IBV strains M41, D207, and D1466 for insertion into the expression plasmid pEX (22). An *AluI-PstI* fragment encoding the N-terminal S1 residues of D207 (Table I) was partially digested with Exonuclease III to remove 125 nucleotides containing in frame stop codons upstream of the ATG start codon. Exonuclease III digestion was at 16°C according the manufacturers (Stratagene, La Jolla, CA) conditions. *Escherichia coli* POP2136 was used (19) as host strain for the pEX plasmids. Recombinant plasmids were introduced into the cells via the $CaCl_2$ transformation procedure, performing the heat shock at 37°C. Recovery (1 h in LB), and growth on LB-ampicillin plates (100 μg/ml), were at 30°C. Recombinants were screened by PAGE of their expression products. Clones synthesizing fusion proteins of the expected m.w. were selected for further characterization. Plasmid DNA isolated from these clones was digested with various restriction enzymes and the insert sizes were determined as additional controls. The construction and selection of strain M41 expression clones have been described previously (7).

Insertion of epitope-encoding oligonucleotides. Synthetic genes for IBV-specific epitopes were synthesized as pairs of partially complementary oligonucleotides (Fig. 1). Two complementary oligonucleotides were mixed in an equimolar ratio (7 μM), incubated at 65°C for 5 min and then slowly cooled to room temperature. This yielded hybrids with free 5′ OH-groups and ends suitable for forced cloning into pEX (Fig. 1). Ligation conditions were essentially as described (20) using 50 ng ds-oligonucleotides with 100 ng linearized pEX. Ligation was performed in a 20 μl volume for 16 h at 18°C. Inserts of selected clones were checked by DNA sequencing.

Plasmid amplification and isolation of pEX fusion proteins. Clones of interest were grown overnight at 30°C in LB-ampicillin (100 μg/ml). To isolate pEX fusion proteins, cultures were diluted 50-fold in LB-ampicillin and grown at 30°C in 5 ml until an A_{600} of about 0.25. Transient expression was induced by incubation at 42°C for 120 min. Subsequently, cells were spun down and lysed in 250 μl 15% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0), and 0.12% (v/v) Triton X-100. After sonication for 15 min, the insoluble hybrid protein was spun down (30 s, 16,000 × *g*) and the viscous supernatant was removed. The pellets were resuspended in 250 μl 50 mM Tris-HCl, pH 8.0, 0.12% Triton X-100, 10 mM EDTA, sonicated and spun again, and finally stored at −20°C in 100 μl of the same buffer.

Western blotting. Proteins were fractionated by SDS-PAGE in a 7.5% gel and blotted to nitrocellulose membranes (BA85, Schleicher & Schuell, Dassel, FRG). All subsequent manipulations were in 0.5% (w/v) gelatine, 0.1% (v/v) Triton X-100 in PBS. The filters were incubated 1 h in a 1/500 dilution of the mAb, and after washing five times for 5 min, 1 h with a 1/5000 dilution of anti-mouse IgG (H + L) conjugated to alkaline-phosphatase (Promega BioTec, Madison, WI). For Western blot analysis with chicken sera a 1-h incubation with a 1/5000 dilution of antichicken IgG (H + L) (Miles Research, Elkhart, IN) was used. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates to visualize the binding of antisera (21).

PEPSCAN. A set of 47 tripeptides was synthesized and screened, essentially as described (11). Synthesis started with the N-terminus of S2, the first tripeptide containing amino acid 546–548 (Fig. 4), the second 547–549, etc. Nine more sets were synthesized, with peptide lengths increasing from 4 residues in the second set to 12 residues in the last one. Peptide recognition by mAb was tested in ELISA as described (11).

RESULTS

Recognition of expressed peplomer gene fragments by mAb. Table II lists mAb directed against the IBV peplomer protein. Serotype-specific neutralizing mAb all bind to conformation-dependent epitopes on the S1 subunit and can be divided in a number of different groups on the base of mutual competition experiments. One nonneutralizing mAb, 69.3, recognizes a conformation-independent epitope on S1 (Koch, G., unpublished data). mAb directed against the S2 subunit define conformation-independent epitopes and all belong to the same competition group. Most of these mAb have neutralizing activity.

To correlate the specificities of the mAb with a physical map of the IBV peplomer protein, cDNA fragments from the peplomer gene were expressed in pEX plasmids (22). In this system, heterologous expression leads to the synthesis of a carboxyl-terminal extension of the *cro-β*-galactosidase hybrid protein.

The relative positions of four cDNA clones spanning the peplomer gene from strain D207 are shown in Figure 2*a*. From these clones, initially five fragments were derived (Fig. 2*c*) and, depending on the reading frame, inserted in plasmid pEX1, pEX2, or pEX3 (Table II). The mobility of the expression products (Fig. 3) correlated with the predicted increase in m.w.

We tested the antigenicity of these proteins with the mAb listed in Table II by Western blotting. With the exception of mAb 69.3, none of the anti-S1 mAb recognized any of the pEX products. This is probably caused by the conformation-dependence of the epitopes and the absence of native conformation in the hybrid proteins (7). In contrast, 69.3 and most of the anti-S2 mAb (Table II) were able to bind to a pEX-IBV expression product. For most anti-S2 mAb tested (shown is only mAb 26.1, first six lanes of Fig. 3) binding was always to the product of clone pXD207-B, which contains residues 192–545 in S1 and 546–725 in S2.

TABLE I

pEX recombinants containing part of IBV peplomer gene

Subclone	Virus Strain	cDNA Clone	Enzymes Used to Prepare Fragment	Vector Preparation	Amino Acid Residues[a]
pXD207-A	D207	p304	*Pst* I -Exo III[b]	pEX2x*Eco* RIx*Pst* I	1–192
pXD207-B	D207	p303	*Pst* I -*Pst* I	pEX2x*Pst* I[c]	192–725
pXD207-C	D207	p19	*Pst* I -*Pst* I	pEX2x*Pst* I[c]	725–903
pXD207-D	D207	p12	*Hae* III-*Pst* I	pEX2x*Sma* I x*Pst* I	814–1005
pXD207-E	D207	p12	*Pst* I -*Pst* I	pEX2 x*Pst* I[c]	1005–1170
pXD207-XP	D207	p303	*Xba* I[d] -*Pst* I	pEX1 x*Sma* I[c]	543–726
pXD207-H	D207	p303	*Hinc* II-*Hinc* II	pEX3 x*Sma* I[c]	497–689
pXD207-Rsa	D207	p303	*Rsa* I -*Rsa* I	pEX2 x*Sma* I[c]	486–574
pXD1466-B	D1466	p385	*Bgl* II -*Bgl* II	pEX1 x*Bam* HI[c]	546–733
pXD1466-NB	D1466	p385	*Nde* I[d] -*Bgl* II	pEX3 x*Sma* Ix*Bam* HI	555–733
pXD1466-SN	D1466	p385	*Ssp* I -*Nde* I[d]	pEX1 x*Sal* I[cd]	443–555
pXM41-PS2	M41	p42	*Sau3* AI-*Pst* I	pEX3 x*Bam* HIx*Pst* I	398–620
pXM41-13.4	M41	p39	DNase I	pEX2 x*Sma* I[c]	537–569
pXM41-15.1	M41	p39	DNase I	pEX2 x*Sma* I[c]	548–566
pXM41-2.1	M41	p39	DNase I	pEX2 *Sma* I[c]	549–587

[a] The numbering corresponds to the alignment of Kusters et al. (6).
[b] Exonuclease III was used to remove in frame stopcodons preceding the AUG codon.
[c] After cleavage, the vector was treated with calf intestine phosphatase to reduce self-ligation.
[d] The cohesive end was filled in with Klenow enzyme.

```
          553                 560                 569
            S  C  P  Y  V  T  Y  G  K  F  C  I  K  P  D  G  L

#1  5'-gaTCTTGTCCATATGTAACTTATGGAAAATTTTGCATTAAACCTGATGGTCTT-3'
       3'-AACAGGTATACATTGAATACCTTTTAAAACGTAATTTGGACTACCAGAAagct-5'

#2  5'-gaTCTTGTCCATATGTAACTTATGGAAAATTTTGCa-3'
       3'-AACAGGTATACATTGAATACCTTTTAAA-5'

#3  5'-ccggaTGTCCATATGTAACTTATGGAAAATTTTGCt-3'
       3'-tACAGGTATACATTGAATACCTTTTAAAACGactag-5'

#4     5'-ccggaCCATATGTAACTTATGGAAAATTTTGCt-3'
          3'-tGGTATACATTGAATACCTTTTAAAACGactag-5'

#5        5'-ccggaTATGTAACTTATGGAAAATTTTGCt-3'
             3'-tATACATTGAATACCTTTTAAAACGactag-5'

#6  5'-ccggaTGTCCATATGTAACTTATGGAAAATTTt-3'
       3'-tACAGGTATACATTGAATACCTTTTAAAactag-5'

#7  5'-ccggaTGTCCATATGTAACTTATGGAAAAt-3'
       3'-tACAGGTATACATTGAATACCTTTTactag-5'

          S  C  P  Y  V  T  Y  G  K  F  C  I  K  P  D  G  L
          553                 560                 569
```

Figure 1. Sequences of double-stranded synthetic oligonucleotides aligned with the encoded amino acid residues of the peplomer protein. Capitalized nucleotides encode peplomer-specific amino acids. Ligation of oligonucleotide #1 in pEX1x*Bam*HIx*Sal*I, resulted in pXOligo-1; #2 in pEX1x*Bam*HIx*Pst*I resulted in pXOligo-2; #3 to #7 in pEX3x*Xma*Ix*Bam*HI resulted in pXOligo-3 to -7, respectively.

TABLE II

mAb against IBV peplomer protein

mAb	Neutralization Titer	Virus Specificity				pEX Fragment[a]		
		Peplomer subunit	IBV strain			pXD207-B	pXD1466-B	
			D207	M41	D1466	pXM41-PS2		
48.1	$>10^{3}$[b]	S1[b]	+	−	−	−	−	−
48.2	$>10^{3}$[b]	S1[b]	+	−	−	−	−	−
48.3	$>10^{3}$[b]	S1[b]	+	−	−	−	−	−
52.4	$>10^{3}$[b]	S1[b]	+	+	−	−	−	−
69.3	ND	S1[b]	+	+	+	+	+	−
26.1	640[c]	S2[c]	+	+	+	+	+	+
30.6	640[c]	S2[c]	+	+	+	+	+	+
31.4	0[c]	S2[c]	−	+	−	−	−	−
31.5	80[c]	S2[c]	−	+	−	−	+	−
31.7	320[c]	S2[c]	+	+	+	+	+	+
54.5	ND	S2[b]	+	+	+	+	+	+

[a] Only reactions with hybrid proteins that contain the N-terminal sequence of the S2 subunit are shown; other hybrid proteins were negative. The positions of the pEX fragments can be found in Figure 2.

[b] Data from Koch, G., manuscript in preparation.

[c] Data from Koch et al. (17). The neutralization titer indicates the reciprocal of the dilution that neutralizes the virus in a micro-neutralization assay. The subunit specificity was established by immunoprecipitation followed by SDS-PAGE, or by Western blotting. The virus specificity was derived from ELISA or dot-blot assays. The recognition of pEX expression products (Table I) was established by Western blotting.

For a more accurate localization of the epitopes, three smaller fragments encoding N-terminal sequences of the D207 S2 subunit were inserted in pEX plasmids (Fig. 2*d*, Table I). The positive reactions of the anti-S2 mAb with the expression products of all three plasmids (Fig. 3, Table III *columns c* to *f*) map the epitopes of S2 all to the same segment, the 29 N-terminal residues. This immunodominant region is also recognized by polyclonal chicken sera against D207 and D1466 (Table III, *columns g* and *h*) and polyclonal anti-M41 sera from different species (7).

mAb 69.3 recognized the expression products of pXD207-Rsa and pXD207-H but not of pXD207-XP (Table II, Fig. 2*d*). Thus, the epitope of mAb 69.3 is localized in the region 497–543.

All mAb against S2, except mAb 31.5, recognize strains with different serotypes. This cross-reactivity is also observed with expression products containing the S1–S2 junction from strain M41 or D1466 (Fig. 2*e*, Table I). This indicates that the residues involved in the recognition of the mAb are conserved among the serotypes.

Sequence conservation in immunodominant region. To verify the conservation of antigenic residues, we determined the N-terminal sequences of the S2 subunit from strains of different serotypes. Figure 4 shows an alignment with the published sequences of strains M41, M42, and 6/82 (4, 23, 24). In agreement with the data on S1 (6), three types of S2 sequences were found: from the serotype-A strains M41, M42, and H120; from the serotype B strains D207 and 6/82; and from the strains D1466 (serotype C) and V1397 (A/C).

Fine mapping with expression products. Four mAb, 26.1, 30.6, 31.7, and 54.5, were selected for fine mapping using either small expressed fragments from IBV strains M41 and D1466, or expression products encoded by oligonucleotides. Fragments used were: 1) D1466 restriction enzyme fragments (Table I); 2) DNase I generated fragments from M41, selected by immunoscreening with mAb 26.1 (7); and 3) a set of synthetic oligonucleotides spanning the conserved N-terminal region of S2.

The results of this fine mapping are shown in Table III. Apparently, the seven amino acid stretch (in one-letter code) PYVTYGK encoded by pXOligo-7 is sufficient for binding mAb 54.5. The proline at position 555 seems to be essential for antibody binding. One inconsistency was found: although clone pXD1466-NB expresses all these seven residues, it is negative in the binding assay. Presumably, the bulky side chains of the preceding leucine and isoleucine residues encoded by the vector shield the

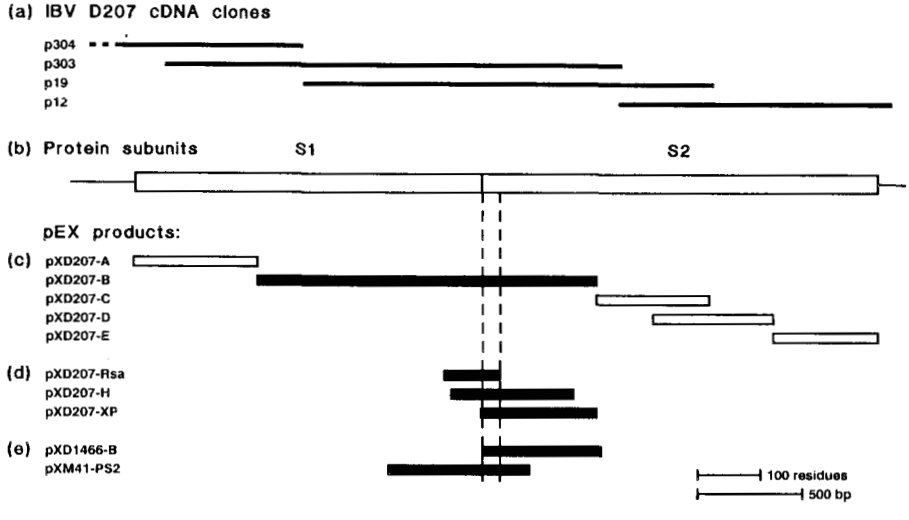

Figure 2. Map of pEX constructs containing major portions of the IBV peplomer gene. *a*, Positions of D207 cDNA inserts relative to (*b*) the S1 and S2 subunits of the peplomer protein; *c*, D207 pEX inserts spanning the whole peplomer gene; *d*, D207; and *e*, D1466 and M41 pEX inserts defining the immunodominant region. Clones indicated by *solid bars* are recognized by a number of anti-S2 mAb (Table II).

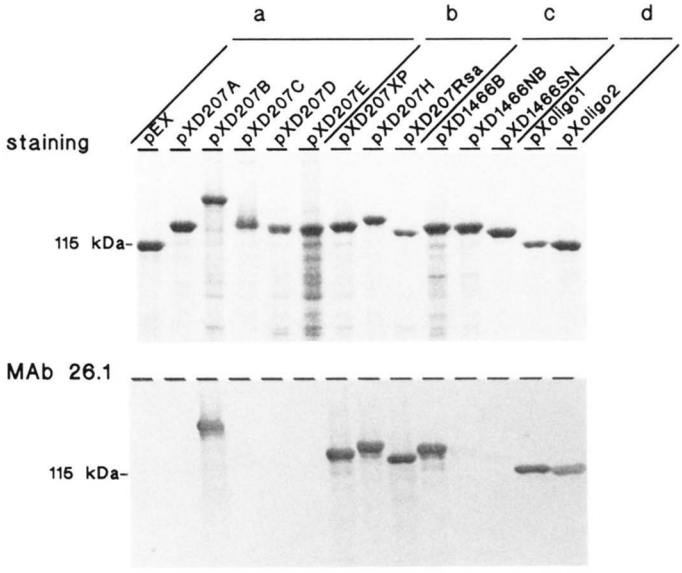

Figure 3. Gel electrophoresis and Western blot of hybrid proteins containing IBV peplomer sequences (for designation and location see Table I and Fig. 2). The m.w. of the expression product from a plasmid without insert (lane pEX) is 115,000. *Upper panel*, staining with Coomassie brilliant blue; *lower panel*, Western blot with mAb 26.1.

antigenic proline. Similar effects of residues preceding the actual antibody binding site were observed with synthetic peptides (see below).

The epitope of mAb 26.1 is longer than the 54.5 epitope: at least 10 residues are required for a detectable binding (pXOligo-3). Both cysteine residues at positions 554 and 563 are essential. Complete recognition, however, depends on the serine at position 553 (pXOligo-2), leading to an epitope length of 11 residues.

The epitope of mAb 30.6 is defined by the positive reactions of pXD1466-NB and pXM41.15.1, delineating a length of 12 residues.

The epitope of mAb 31.7 is exceptionally large. The 17 residues 553–569 from pXOligo-1 gave only a weak reaction. As follows from the binding of pXM41–2.1 (+++), pXDd1466-NB (−), pXD207-Rsa (+++), and pXM41–13.4 (+), respectively, both the sequences 549–554 and 570–574 are involved. Thus, a protein segment of at least 17 and at most 26 residues is required for maximal binding.

Finally, Table III (*columns g* and *h*) shows that the binding patterns of chicken D207 and D1466 antisera closely resemble the pattern of mAb 30.6.

PEPSCAN peptide synthesis. To validate the pEX epitope mapping data by an independent method and to refine the mapping of the epitopes, consecutive overlapping peptides were synthesized and tested by the PEPSCAN method. These peptides covered the N-terminal sequence of S2, with lengths increasing from 3 to 12 residues. The binding patterns of the four mAb with these peptides is shown in Figure 5.

For mAb 54.5 the minimum epitope length is reduced by one amino acid to six residues (555–560, PYVTYG). However, with increasing peptide length, there is again a clear effect of the preceding residues: preceding C-, NC-, or TNC-sequences inhibit the binding. Reactivity is regained when VTNC precedes the epitope in one of the undecapeptides.

mAb 30.6 binds to the nonapeptide containing residues 558–566 (TYGKFCIKP). This confirms the epitope mapping with the pEX products, and narrows it down to nine residues.

The PEPSCAN of mAb 26.1 shown in Figure 5 reveals a weak signal that, although in agreement with the pEX data, was not reproducible. Scans of mAb 31.7, when tested at very high concentrations of antibody, gave only weak and isolated signals (Fig. 5). Scans with the polyclonal chicken sera (not shown) were not successful.

TABLE III

Fine mapping of epitopes with pEX expression products[a]

(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
	«—S1—»«————————S2————————»						
	541 550 560 570 577	26.1	30.6	31.7	54.5	D207	D1466
pXD207-B	<RRSRRSITGNVTNCPYVTYGKFCIKPDGSISTIVPKE>	+++	+++	+++	+++	+++	+++
pXD207-RSA	<RRSRRSITGNVTNCPYVTYGKFCIKPDGSISTIVgir	+++	+++	+++	+++	+++	+++
pXD207-XP	aapRRSITGNVTNCPYVTYGKFCIKPDGSISTIVPKE>	+++	+++	+++	+++	+++	+++
pXD1466-B	saargSTSGNVTSCPYVTYGKFCIKPDGLVHIIVPEE>	+++	+++	+++	+++	+++	+++
pXD1466-NB	gddswspsvsaeliPYVTYGKFCIKPDGLVHIIVPEE>	-	+++	-	-	+	+
pXD1466-SN	<RRSRRSTSGNVTSCPYstcsqac*	-	-	-	-	-	-
pXM41- PS2	<RRFRRSITENVANCPYVSYGKFCIKPDGSIATIVPKQ>	+++	+++	+++	+++	nd.[b]	nd.
pXM41- 2.1	psvsaefpENVANCPYVSYGKFCIKPDGSIATIVPKQ>	+++	+++	+++	+++	nd.	nd.
pXM41-13.4	<RRFRRSITENVANCPYVSYGKFCIKPDGSgirrpaak	+++	+++	+	+++	nd.	nd.
pXM41-15.1	svsaefpTENVANCPYVSYGKFCIKPsss*	+++	+++	-	+++	nd.	nd.
pXOligo-1	dswspsvsaargSCPYVTYGKFCIKPDGLstcsqac*	+++	+++	+	+++	+++	+++
pXOligo-2	dswspsvsaargSCPYVTYGKFCsqac*	+++	-	-	+++	+	+
pXOligo-3	swspsvsaelipgCPYVTYGKFC*	+	-	-	+++	-	-
pXOligo-4	dswspsvsaelipgPYVTYGKFC*	-	-	-	+++	-	-
pXOligo-5	ddswspsvsaelipgYVTYGKFC*	-	-	-	-	-	-
pXOligo-6	swspsvsaelipgCPYVTYGKF*	-	-	-	+++	-	-
pXOligo-7	swspsvsaelipgCPYVTYGK*	-	-	-	+++	-	-

[a] +++, strong signal on Western blot, +, weak signal, −, no signal above background.

(a) pEX clone. (b) Carboxyl-terminal sequences of hybrid proteins. Residues encoded by the viral sequence are capitalized. The numbering of residues is as in Figure 4. *Asterisks* indicate carboxyl-terminal ends of hybrid proteins. *Arrows* indicate that the viral sequence extends in the given direction. Underscored residues in the oligonucleotide-derived products indicate differences between virus strains. (c), (d), (e), (f), Western blot binding patterns with mAb 26.1, 30.6, 31.7, and 54.5, respectively. (g) and (h), binding pattern of a polyclonal chicken D207 and D1466 antisera, respectively.

[b] nd.: not determined.

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        546 550       560       570       580       590       600
D207     SITGNVTNCPYVTYGKFCIKPDGSISTIVPKELEHFVAPLLNVTENVLIPDSFNLTVTDEY
6/82
M41          E  A      S          A     Q  Q        ___      N   ___
M42          E  A      S          A     Q  Q     F  ___      N   ___
H120         ES E      S          A     Q  Q        ___      N   ___
V1397     TS  ___S                LVHI  E VKDYTSL  _R_DY    N   ___
D1466     TS  ___S                LVHI  E VKDYTSL  _R_DY    N   ___
        546 550       560       570       580       590       600
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Figure 4. Amino acid sequences of the N-terminal region of S2. These sequences are numbered according to the S1 sequences published previously (6), S2 starting at residue 546. From strain D207, the full sequence is listed, from other strains only the differences with this sequence. The sequences of strains M41 and M42 are from Niesters et al. (4). The 6/82 sequence is from Binns et al. (23). Potential N-glycosylation sites are underlined.

DISCUSSION

Localization of conformation-independent epitopes. Often, epitopes are defined operationally by mutual competition of monoclonal antibodies. However, groups of mutually competing mAb may have different, although overlapping binding sites, as we found for epitopes within the immunodominant region of IBV. Obviously, mutual competition data should be interpreted in terms of antigenic "regions" or "sites," rather than in terms of epitopes.

Previously, we described an immunodominant region near the N terminus of the S2 subunit from the IBV peplomer protein, recognized by several polyclonal sera (7). Here, we show that the same region contains the majority of the conformation-independent epitopes recognized by mAb against the peplomer protein. Four epitopes were localized accurately by testing the binding of mAb to both prokaryotic expression products and synthetic peptides with different lengths. This method defines an epitope as the shortest sequence that is recognized specifically by the antibody. This does not exclude, however, contacts of other residues with the antibody. Further, no attempt was made to quantitate the strength of binding of the antibodies; instead, we essentially sought yes–no answers that likely reflect the biologic relevance of the interaction.

The results of both methods were found to be complementary. mAb 54.5 binds to expression products containing the sequence PYVTYGK, to peptides containing the residues PYVTYG and, much weaker, to the pentapeptide PYVTY. Preceding sequences may inhibit the recognition of this sequence: an AELI-sequence coded by the pEX vector in the expression product of pXD1466-NB (Table III), and the C-, NC-, and TNC- but not the VTNC-sequence present in the PEPSCAN-peptides (Fig. 5). These observations demonstrate that antibody binding may be influenced by side-chain interactions and/or secondary structure mediated by residues outside the actual recognition sequence.

The epitope of mAb 30.6 has been localized within 12 residues (PYVTYGKFCIKP) by the binding pattern of hybrid proteins and narrowed down to 9 residues (TYGKFCIKP) by the PEPSCAN data.

mAb 26.1 recognized an expression product carrying the decapeptide sequence CPYVTYGKFC, a preceding S (as present in the D1466 sequence) being necessary for maximum binding. This is based on the recognition of expression products only. Presumably the length of the epitope precluded a clear and reproducible PEPSCAN signal. Although a disulfide-bond between the two cysteine residues at both ends of the epitope seems an obvious possibility, this is not supported by experimental data: there is no effect on antibody binding upon treatment of the native viral Ag with 2-ME, SDS, heating, or the combination of these three (not shown).

The definition of the epitope of 31.7 relies only on the recognition of hybrid proteins. Interestingly, this epitope is at least 17 residues (553–569). This probably explains the negative results with peptides of 12 residues or less. Both ends of the epitope were required for complete binding. Nevertheless, mAb 31.7 is fully reactive with protein denatured by SDS and 2-ME indicating that the spatial structure of the long epitope, positioning both ends in the paratope of mAb 31.7, does not depend on the native conformation of the protein.

Two different chicken polyclonal antisera recognized the same hybrid proteins as mAb 30.6. However, the weak binding of the product of pXD1466-NB indicates an involvement of the C or NC residues preceding the PYVTYGKFCIKP sequence. Further, PEPSCAN tests with these sera did not allow a more precise mapping, leaving some uncertainty about the actual involvement of the carboxyl-terminal IKP sequence. The recognition pattern of the polyclonal chicken sera seems to correspond to one

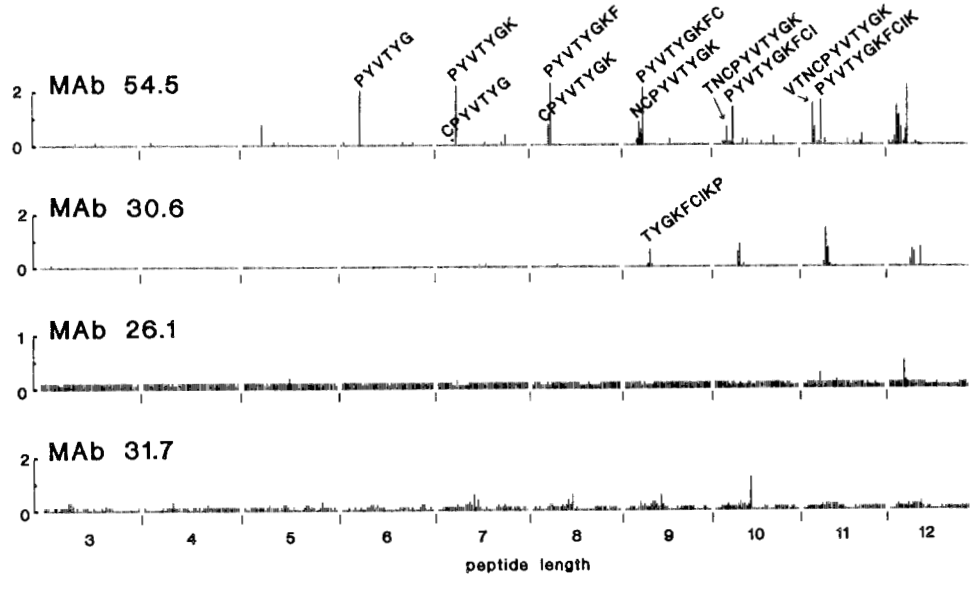

Figure 5. PEPSCAN patterns of peptides derived from the N-terminal sequence of S2. The 4 mAb indicated were tested on overlapping peptides of 3 to 12 residues, spanning the first 50 amino acid residues of the D207 S2 subunit. The numbers on the X-axis indicate the length of the peptide used. The numbers on the Y-axis represent the extinction at 450 nanometers. Notice the difference in ordinate scale with mAb 26.1. With a peptide length of three residues the first bar represents the residues 546–548, the second 547–549, etc. (numbering as in Fig. 4). With tetrapeptides the first bar represents residues 546–549, etc.

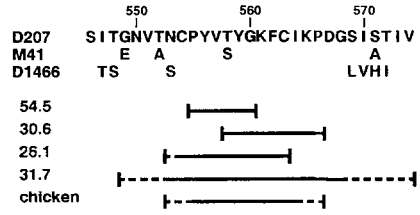

Figure 6. Relative positions of epitopes within the immunodominant region of IBV. The *upper portion* shows an alignment of IBV sequences from which the pEX products have been derived. Only the D207 sequence was tested by PEPSCAN. The *lower portion* summarizes the location of the epitopes of the 4 mAb and the polyclonal chicken D207 and D1466 antisera. The *dashed lines* indicate that the epitope boundaries have not been localized exactly, or that the omission of the corresponding residues resulted in a weaker signal on the Western blot.

or more epitopes overlapping with the epitopes of the mAb. Figure 6 summarizes the lengths and positions of the antigenic sequences within the immunodominant region.

No reaction was found between mAb recognizing the conformational epitopes in the S1 subunit and the pEX products. This observation indicates that prokaryotic expression products are antigenically equivalent to denatured protein fragments and, like peptides, cross-react only with epitopes that do not depend on the native protein conformation. This does not, however, exclude a local folding in the hybrid protein to combine different parts of a long epitope, as found for mAb 31.7.

With one exception, we found epitopes that are longer than the 4–8 residues considered as typical for linear epitopes (11, 25). This value may be biased by the method used to search for epitopes: PEPSCAN analysis with peptides of eight residues or less. As indicated by our data, a more complete database would include epitopes of up to 20 residues. This agrees with the typical size of a paratope (26, 27).

Our results suggest a general strategy for the localization of those epitopes that, as operationally indicated by the binding on Western blots, depend on the primary and not on the tertiary protein structure. First, hybrid proteins are used to make a complete inventory and to obtain approximate localizations. Then, systematic peptide synthesis may yield accurate localizations to a resolution of single residues. Although peptide synthesis by the PEPSCAN method seems suitable especially for shorter epitopes, the combination of pEX and PEPSCAN has been successful for epitopes of the coronaviruses MHV (28) and TGEV (W. P. A. Posthumus, unpublished results).

Immunodominant regions in viruses. Immunodominant regions have been found in several other viruses, such as foot and mouth disease virus (12), poliovirus (8) and in the gp41 (29) and gp120 (10) of human immunodeficiency virus. Typically, these regions are recognized by the majority of the polyclonal antisera. As we have found for IBV, such a region can also be recognized by mAb with different fine specificities. These data point to features intrinsic to the protein structure determining the location of the immunodominant regions. We propose that the role of extrinsic factors as host repertoire and self-tolerance (14) is limited to the selection of epitopes within these immunodominant regions.

Cross-recognition with denatured proteins or protein fragments has been described for most immunodominant regions (10–12). For the major antigenic site of foot and mouth disease virus, this has been explained by internal disorder on a protrusion on the virus surface (34). This is consistent with the hypothesis that local mobility is the most relevant intrinsic factor determining the localization of sequential epitopes (30). An alternative hypothesis proposes static surface accessibility as a condition sufficient for immunogenicity (31, 32). However, this would not explain the cross-recognition with denatured and nonnative structures.

Conservation of antigenic residues. Despite the significant neutralizing activity of mAb binding to the immunodominant region, most residues involved in antibody binding have been conserved between the different serotypes (Figs. 3 and 6), whereas there is considerable sequence variation in the flanking regions. In fact, there is only one continuous stretch of 10 conserved residues in the preceding S1 sequences of the same IBV strains (6). Sequence conservation to maintain the structure of the peplomer proteins is incompatible with the local flexibility and probable surface location, as indicated by the presence of conformation independent epitopes. Therefore, the sequence conservation in the region 550–570 is most likely explained by a molecular recognition process that is essential during the infection cycle.

The coronavirus peplomer proteins are essential for attachment of the virus to the cell and for membrane fusion (33, 35). For the murine cororonavirus MHV-A59, the epitopes of a mAb that inhibits cell fusion and protects against infection has been localized (28). With MHV, as with IBV, this epitope is (antigenically) conserved in different strains (35, 36). However, in an alignment of the IBV and MHV sequences (37), the MHV epitope was located about 150 amino acid residues downstream of the IBV epitope. For IBV, no neutralizing mAb recognizing the homologous region have been described sofar. Further, the region containing the IBV epitope has no sequence similarity with the MHV sequence, but is flanked by relatively conserved regions. This suggests that in the coronaviruses IBV and MHV, different regions are relevant for the function of the peplomer and for neutralization and protection.

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