JOURNAL OF VIROLOGY, Mar. 1989, p. 1408–1412
0022-538X/89/031408-05$02.00/0

Vol. 63, No. 3

Amino Acid Sequence of a Conserved Neutralizing Epitope of Murine Coronaviruses

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Received 6 July 1988/Accepted 9 November 1988

We identified the binding site of monoclonal antibody 19.2, which cross-neutralizes several mouse hepatitis virus (MHV) strains, inhibits fusion of MHV-infected cells, and protects against lethal infection (P. J. Talbot and M. J. Buchmeier, Virus Res. 2:317–328, 1985). We used fusion proteins, generated by expression of fragments of the MHV A59 E2 gene in pEX plasmids, and synthetic peptides in a PEPSCAN.

The peplomer protein E2 of mouse hepatitis virus (MHV), a coronavirus (14), is associated with attachment to target cells and induction of cell fusion and elicits neutralizing antibodies (2, 17, 18). Competition assays with neutralizing monoclonal antibodies (MAbs) have suggested the presence of several major antigenic sites on the peplomer surface (9, 19, 21–24). Passive immunization with neutralizing MAbs could protect mice from acute encephalitis, but the animals developed a chronic demyelinating disease (1, 12, 24). Similar results were obtained with antigenic variants of MHV JHM which were selected for resistance to neutralization by neutralizing MAbs (3, 6, 24). It was not possible to generate escape mutants with MAb 19.2 (2), which inhibits cell fusion and cross-neutralizes several MHV strains, including A59 (19). This suggests that the binding site of MAb 19.2 (epitope A) is involved in vital functions of murine coronaviruses.

TABLE 1. MHV A59 E2 fragments and pEX expression plasmids used for mapping of the MAb 19.2-binding epitope

Fragment[a]		Restriction endonucleases[b]		pEX[c]	Treatment[d]	MAb[e]		
No.	Length	5′ position (nucleotide)	3′ position (nucleotide)			a	b	c
Library A								
2	1,261	*Sau*3A (163)	*Sau*3A (1424)	3 × *Bam*HI		−	−	−
3	1,364	*Taq*I (1124)	*Taq*I (2488)	2 × *Sma*I	K	−	−	−
4	469	*Sau*3A (2300)	*Sau*3A (2769)	1 × *Bam*HI		+	+	+
5	993	*Taq*I (2663)	*Taq*I (3656)	2 × *Sma*I	K	−	−	+
6	318	*Taq*I (3662)	*Taq*I (4269)	2 × *Sma*I	K	−	−	−
7	517	*Bam*HI (MCS)	*Hin*dIII (517)	2 × *Sma*I	K	−	−	−
9	1,034	*Rsa*I (1682)	*Rsa*I (2716)	3 × *Sma*I		+	+	+
11	606	*Hae*III (1906)	*Hae*III (2512)	2 × *Sma*I		−	−	−
12	714	*Hin*dIII (517)	*Pst*I (1231)	1 × *Sma*I	K	−	−	*
13	844	*Sau*3A (3130)	*Sau*3A (MCS)	3 × *Bam*HI		−	−	+
14	710	*Hae*III (2512)	*Hae*III (3222)	2 × *Sma*I		+	+	+
16	478	*Rsa*I (2716)	*Rsa*I (3194)	2 × *Sma*I		−	−	−
Sublibrary B								
18	334	*Bgl*I (2299)	*Tth*111I (2632)	1 × *Sma*I	K	+		
19	590	*Tth*111I (2632)	*Hae*III (3222)	1 × *Sma*I	K	−		
26	402	*Hin*cII (2172)	*Mbo*II (2585)	2 × *Sma*I	T	+		
27	637	*Mbo*II (2585)	*Hae*III (3222)	1 × *Sma*I	T	−		
Sublibrary C								
33	378	*Hin*cII (2172)	*Nsi*I (2550)	2 × *Sma-Pst*		−		
34	374	*Hin*cII (2172)	*Nsi*I (2546)	2 × *Sma*I	T	−		
36	596	*Nsi*I (2554)	*Hin*cII (3150)	1 × *Sma*I	T	−		
38	219	*Nsi*I (2550)	*Sau*3A (2769)	3 × *Pst*I		+		

[a] Fragment number and length refer to the numbers of the pEX subclones and the length of the E2-specific inserts.
[b] These are the positions in the E2 sequence of the restriction endonuclease recognition sites that were used for the isolation of fragments from plasmid pDGE2. In two cases, fragments were generated by using restriction endonuclease recognition sites from the multicloning region (MCS) of the reconstruct pDGE2.
[c] pEX lists the pEX expression plasmid and the restriction endonucleases that were used in subcloning.
[d] K and T indicate Klenow or T4 polymerase treatment, respectively.
[e] MAbs that were used: a, MAb 19.2; b, MAb 170.3; c, polyclonal serum. +, −, *, positive, negative, and ambiguously reacting clones respectively.

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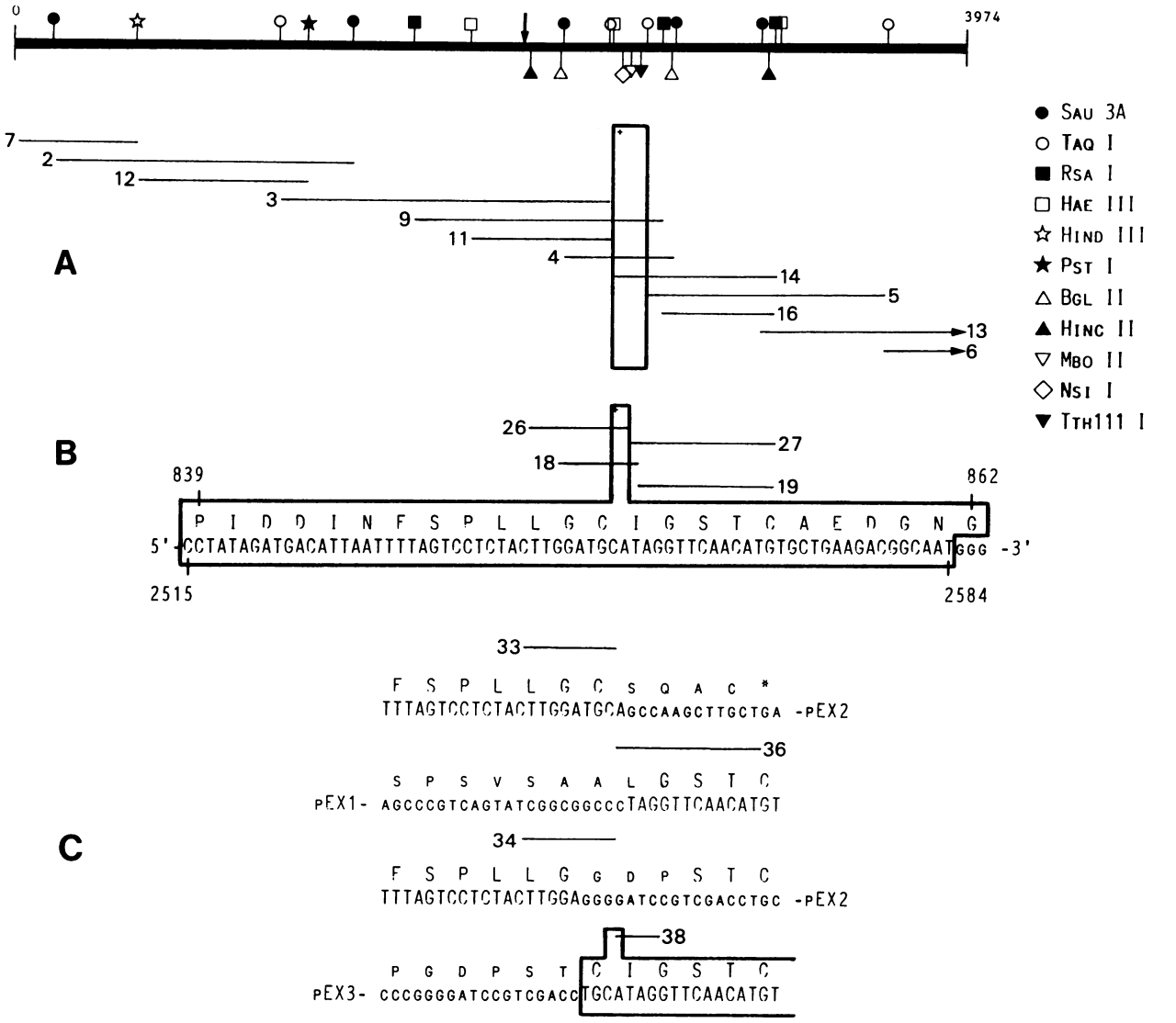

FIG. 1. Schematic representation of the MHV A59 E2 expression libraries. The upper bar symbolizes the MHV A59 peplomer protein E2 gene as presented by Luytjes et al. (10). Symbols (explained in the figure) indicate the restriction endonuclease recognition sites that were used in the creation of the expression libraries. The arrow points to the cleavage site between the E2 NH_2-subunit 90B and COOH-subunit 90A. Horizontal lines in panels A, B, and C show the positions of the E2 fragments, cloned in frame with the *cro-lacZ* hybrid gene of pEX expression plasmids (see text). A, B, and C refer to pEX library A and sublibraries B and C, respectively. pEX clones are numbered; details are listed in Table 1. Boxes border the E2 regions (defined by minimal fragment overlaps) that bind to MAb 19.2 (see Fig. 2). In panel B, the sequence of the binding region is boxed. Its start and end positions in the E2 sequence are indicated. In panel C, the sequences of the pEX-E2 junction regions of each selected fragment are represented. Large characters are MHV A59 sequences and small characters are pEX sequences. The single-letter amino acid code is used.

We wanted to investigate the possible role of epitope A in fusion or receptor binding. The important first step was to determine its position on the E2 gene of MHV A59 (10) and to elucidate its amino acid sequence. MAb 19.2 binds to the E2 protein under denaturing conditions (20). This suggested that we would be able to define the binding site by using the pEX plasmid bacterial expression system, which generates linear unglycosylated β-galactosidase fusion proteins (15, 16).

Construction of expression libraries. pEX plasmids and appropriate restriction fragments of plasmid pDGE2, a full-length reconstruct of the peplomer gene based on cDNA clones (H. Vennema, manuscript in preparation), were selected on the basis of the E2 sequence (10). A detailed list of restriction endonucleases, generated fragments, and pEX plasmids that were used is presented in Table 1. Use of restriction enzymes and DNA-modifying enzymes and plasmid preparation procedures were as specified by the manufacturer or as described by Maniatis et al. (11). When necessary, fragments were treated with T4 DNA polymerase or Klenow fragment and cloned into *Sma*I-digested pEX plasmids. Clones 19 and 27 were generated by deleting a fragment from clone 14, and clone 38 was similarly derived from clone 4. Constructs were verified on the DNA level for correct orientation of inserted fragments and checked for subcloning artifacts (data not shown). All clones contained insert DNA oriented with the *lacZ* gene (see reference 15). We created three expression libraries: library A, consisting

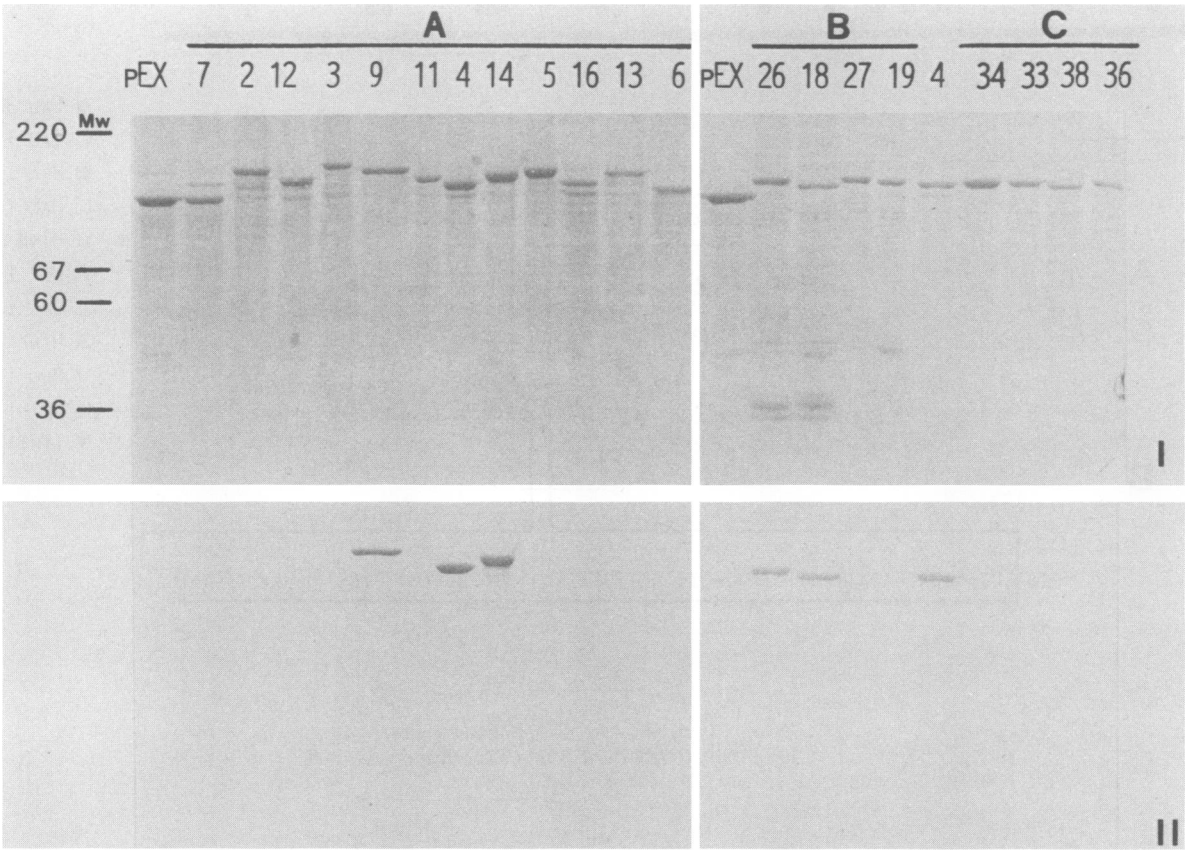

FIG. 2. Polyacrylamide gel electrophoresis of the MHV A59 E2 expression (sub)library fusion proteins and Western blot immunoscreening with MAb 19.2. pEX fusion proteins separated by polyacrylamide gel electrophoresis were blotted onto nitrocellulose filters. Remaining proteins in gels were stained with Coomassie blue (panel I), and filters were used in an immunoscreening with MAb 19.2 (panel II). A, B, and C refer to the different expression libraries (Fig. 1 and text). Numbers represent the individual pEX and E2 clones (Fig. 1; Table 1). pEX is the original *cro-lacZ* gene product (117 kilodaltons). The positions of molecular size markers are indicated in kilodalton scale.

of a set of overlapping fragments of the entire E2 gene, and sublibraries B and C, containing subfragments of library A (Fig. 1; Table 1).

Screening of expression libraries. Initial immunoscreening of recombinant clones (colony blot) and small-scale isolation of fusion proteins (5 ml) were performed as described by Lenstra et al. (9a). Large-scale (500 ml) isolation of β-galactosidase fusion proteins and subsequent blotting and immunoscreening procedures were as described previously (W. Luytjes, Ph.D. thesis, University of Utrecht, Utrecht, The Netherlands, 1988). Western blots (immunoblots) of the expression libraries were screened with either polyclonal rabbit anti-MHV antiserum or anti-MHV E2 MAbs 19.2 or 170.3. Secondary antisera were either goat anti-rabbit- or rabbit anti-mouse-peroxidase conjugates (Nordic). All antisera were diluted 1:1,000.

The expression products of clones 4, 9, and 14 (library A) reacted with the MAb 19.2 (Fig. 1A and Fig. 2, section A). The other clones from this library were negative. The E2-specific inserts of the positive clones had an overlap of 50 amino acids, positions 839 to 888 in the E2 sequence. Table 1 shows that the same region was recognized by MAb 170.3, which is consistent with the fact that it competes with MAb 19.2 (2, 19). The polyclonal serum also recognized this region but in addition reacted with clones 5 and 13 and showed an ambiguous reaction with clone 12. Further definition of the overlap was performed with sublibrary B. Representative fragments 18, 19 and 26, 27, indicated in Fig. 1B and specified in Table 1, reduced the overlap region that is recognized by MAb 19.2 to amino acid positions 839 to 862. (Fig. 1B and Fig. 2, section B). Sublibrary C contained fragments which were generated by *Nsi*I cleavage of this region. From the four different types of fragments, representative clones 33 and 38 (sticky ended) and 34 and 36 (blunt ended) were selected (Fig. 1C; Table 1). Only the fusion protein derived from recombinant 38 could bind MAb 19.2 (Fig. 2, section C).

The shortest E2-specific fusion protein that reacted was thus defined by the overlap between clones 38 and 26. Clone 38 contains only part of the epitope, which probably explains the weak response. Cys-851 and Ile-852 are apparently important since deletion of these amino acids (clone 36) abolishes the binding. Furthermore, clone 34, which can be regarded as a substitution mutant which has amino acids Cys-851, Ile-852, and Gly-853 altered, did not bind to MAb 19.2. We therefore suggest that Cys-851 and Ile-852 are essential for binding.

Mapping of epitope with synthetic peptides (PEPSCAN). To establish in detail which amino acids compose epitope A, we constructed 42 overlapping nonapeptides as described previously (7, 8), covering amino acids 839 to 888 of the E2 protein. Nonapeptide 1 consisted of the residues 839 to 847, nonapeptide 2 consisted of residues 840 to 848, and so on. All peptides were tested against MAb 19.2 (diluted 1:100) in

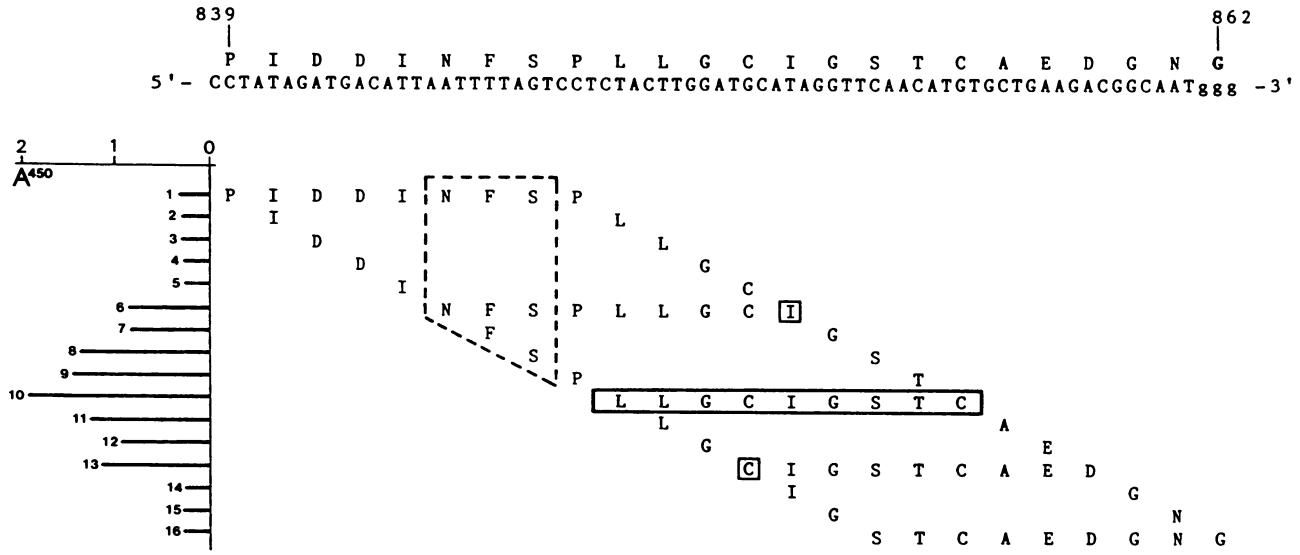

FIG. 3. PEPSCAN of MHV A59 E2 fragments against MAb 19.2. Sixteen synthetic nonapeptides (from a set of 42) covering amino acid positions 834 to 862 in the MHV A59 E2 sequence (22) were tested in an enzyme-linked immunosorbent assay at 450 nm against MAb 19.2. The extinctions are plotted for each peptide. The upper lines represent the nucleotide and (single-letter) amino acid sequence of the region covered by these peptides. Selected peptides are represented with their complete amino acid sequence; from others, only the extreme N-terminal and C-terminal residues are shown. The solid horizontal box marks the peptide with the strongest binding to MAb 19.2. Boxed isoleucine and cysteine residues represent essential amino acids for antibody binding. The interrupted line boxes a potential site for N-linked glycosylation.

an enzyme-linked immunosorbent assay for extinction at 450 nm (PEPSCAN) (7, 8). The results obtained for the first 16 peptides are shown in Fig. 3. Peptide 10 displayed the strongest antibody-binding activity. Peptides 1 to 5 and 14 to 16 did not react with the antibody nor did peptides 17 to 42 (data not shown). Deletion of Cys-851 or Ile-852 decreased the binding activity to background levels (compare peptides 13 and 14 and 5 and 6, respectively). Again only those peptides containing Cys-851 and Ile-852 in tandem could bind to MAb 19.2.

MAb 19.2 binds to the glycosylated native peplomer protein but apparently does not recognize the sugar chain since it also binds to the unglycosylated fusion proteins. Asn-844, which can potentially be glycosylated (13), will thus probably not be part of the epitope. The same could be true for the surrounding amino acids because of probable steric hindrance between the antibody and the sugar side chain. We therefore suggest that the antibody-binding site stretches from amino acid positions 848 to 856 (nucleotide positions 2544 to 2568) in the E2 sequence of MHV A59: NH_2-L-L-G-C-I-G-S-T-C-COOH. The sequence of the antibody-binding region is identical in MHV JHM and A59 (10), which explains the cross-neutralizing property of MAb 19.2. The reaction with the polyclonal serum shows that the C-terminal half of the E2 protein contains additional immunogenic regions with linear epitopes. We are currently investigating these epitopes using our pEX expression libraries.

The specific role of the sequence of amino acids that bind MAb 19.2 is not clear, since the mechanism of neutralization by this MAb is unknown. Preliminary results indicate that fusion proteins and synthetic peptides containing the region that is recognized by MAb 19.2 can protect mice against a lethal challenge of MHV (unpublished data).

Conserved peplomer domains are located upstream and downstream of the binding site of MAb 19.2 (4). These conservations, as we have proposed for the coiled-coil sequences further downstream (5), probably reflect conserved vital peplomer structures. This could mean that the domain which contains the recognition site of MAb 19.2 is similarly exposed in different coronaviruses. It will be important to establish for these viruses whether their corresponding peplomer domains also elicit neutralizing or fusion-inhibiting antibodies.

We thank J. A. Lenstra for setting up the pEX expression system, P. J. Bredenbeek for the original MHV clones and for technical advice, and M. C. Horzinek for critical reading of the manuscript. We are indebted to M. J. Buchmeier for a gift of the monoclonal antibodies, M. J. M. Koolen for the polyclonal serum, K. K. Stanley for providing his pEX plasmids, and O. Raibaud for *Escherichia coli* pop2136. We thank Karin Mink for preparation of the pEX plasmid DNA and W. Puyk and D. Kuperus for technical assistance.

W.L. was supported by a grant from Duphar B.V., Weesp, The Netherlands.

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