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RNA recombination of coronaviruses: Localization of neutralizing epitopes and neuropathogenic determinants on the carboxyl terminus of peplomers

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ABSTRACT Murine coronaviruses undergo RNA recombination at a very high frequency. We have obtained a series of recombinant viruses using neutralizing monoclonal antibodies in conjunction with temperature-sensitive markers. All of the recombinants obtained have a crossover within gene C, which encodes the peplomer protein of the virus. The genetic structure of these recombinants suggests that the antigenic regions recognized by these neutralizing monoclonal antibodies are localized on the carboxyl-terminal one-third of the peplomer protein. Since the two monoclonal antibodies used are also associated with the critical determinants of virus neuropathogenicity, we conclude that both the neutralizing antibody binding sites and determinants of pathogenicity are localized at the carboxyl-terminal one-third of the peplomer. The variation of crossover sites in different recombinant viruses also allowed precise mapping of additional antigenic sites. RNA recombination thus presents a powerful genetic tool, and the carboxyl-terminal localization of the biological functions of peplomers suggests a distinct conformation of these viral membrane proteins.

Mouse hepatitis virus (MHV), a coronavirus, is an enveloped virus containing two envelope glycoproteins, E1 and E2, and a nucleocapsid protein, N (1). The N protein has a molecular weight of 60,000 and is closely associated with viral RNA. The E1 glycoprotein has a molecular weight of $\approx 25,000$ and probably serves as a matrix protein. The E2 glycoprotein, a 180-kDa heterodimer, forms the projecting spikes or peplomers on the surface of the virus particle and is involved in the attachment of virus to target cells, in the induction of cell-to-cell fusion, and in the elicitation of neutralizing antibodies (1-3). Competitive binding studies have identified at least six major antigenic sites on the E2, three of which are involved in virus neutralization (refs. 4 and 5; J.O.F., unpublished observation). Studies on the antigenic variants of the JHM strain of MHV suggest that E2 is important for determining the pathogenic potential of the virus (6, 7). For instance, neutralization-resistant variants isolated following sequential treatment of wild-type JHM with two anti-E2 monoclonal antibodies (J.7.2 and J.2.2) have greatly reduced ability to cause either encephalitis or demyelination (8). Thus, the two antigenic sites recognized by these antibodies are important components of the neuropathogenic determinants of the virus. It is, therefore, of interest to identify the functional domains of these antigenic sites.

MHV contains a single piece of single-stranded RNA genome of positive polarity (9). The RNA contains at least seven genes, termed genes A through G in the 5'-3' order, based on the finding that, in infected cells, six subgenomic and one genomic mRNAs are expressed (10). These mRNAs have a 3'-coterminal, nested-set structure, i.e., the sequence

of each mRNA is contained entirely within the next larger mRNA (10). Only the 5'-specific portions, which do not overlap with the next smaller mRNAs, are translated. Furthermore, each mRNA contains a leader sequence of ≈ 70 nucleotides, which is derived from the 5' end of the genome and joined to the mRNAs by a mechanism of "leader-primed transcription" (11, 12). Despite the fact that MHV genome is a single, nonsegmented RNA, our laboratory has demonstrated that MHV can undergo RNA-RNA recombination at an extremely high frequency (13), reminiscent of the genetic reassortment of viruses with segmented RNA genomes. This observation suggests that fragmented RNA intermediates might be generated during replication of MHV RNA, probably by a mechanism of discontinuous and nonprocessive RNA synthesis (13, 14). The availability of RNA recombinants provides a powerful genetic tool to study the gene functions of MHV.

In this study, we devised a selection scheme to isolate RNA recombinants with possible crossovers within gene C, which encodes the E2 protein. These recombinants reveal that the two antigenic sites important for virus neutralization and neuropathogenicity are surprisingly mapped within the carboxyl-terminal one-third of the peplomer protein. The various classes of crossover sites also allowed us to precisely determine other antigenic sites on the protein.

MATERIALS AND METHODS

Viruses and Cells. The DL isolate of JHM strain of MHV (15) was used throughout this study. Two temperature-sensitive (ts) mutants of the A59 strain of MHV, designated LA7 and LA12, were also used (J. Egbert and S.A.S., unpublished data). These two mutants have RNA (+) phenotype. The viruses were grown in either L cells or DBT cells, a mouse brain tumor cell line (16), as described (13).

Isolation of Recombinants. The DBT cells were infected with the JHM strain and with ts mutant LA7 or LA12 at a multiplicity of infection of 5 each at 39°C (nonpermissive temperature for ts mutants). The released virus was harvested 16 hr after infection. The virus was then mixed with JHM-specific anti-E2 monoclonal antibodies J.2.2 and J.7.2 (3) and incubated at 37°C for 45 min. The amount of each monoclonal antibody used had been determined to be sufficient to reduce the titer of the virus by $\approx 4 \log_{10}$ units. Thus, the combined use of the two antibodies reduced the parental JHM virus titer to nearly zero. Viruses surviving neutralization were isolated by plating at limited dilution on DBT cells. Isolated plaques were picked and propagated either in the presence or in the absence of the same monoclonal antibodies at 39°C. Individual isolates were studied by two-dimensional RNase T₁-resistant oligonucleotide fingerprinting of genomic

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Abbreviations: MHV, mouse hepatitis virus; ts, temperature sensitive.

RNA to determine whether they were recombinants or revertants derived from the ts parent.

Oligonucleotide Fingerprinting. The ^{32}P -labeled genomic and intracellular RNAs were prepared as described (10, 13). The intracellular RNA was separated by electrophoresis on urea agarose gels (17). The individual RNA species was extracted by the method of Langridge *et al.* (18). The RNA was digested with RNase T₁ and separated by two-dimensional polyacrylamide gels as described (10).

Antibody Binding Assays. The JHM-specific anti-E2 monoclonal antibodies used have been described (3). None of them except J.7.5 cross-react with A59. Likewise, the A59-derived anti-E2 monoclonal antibodies (29) used do not react with JHM. The ELISA tests were performed as described (6).

RESULTS

Genetic Structure of Recombinants Between A59 and JHM Isolated Using ts Markers and Strain-Specific Monoclonal Antibodies. To obtain MHV recombinants with possible crossovers within the gene encoding the E2 protein, we devised an experimental protocol that utilized two strains of MHV, each of which contained a specific selectable marker. One was the RNA(+) ts mutant, either LA7 or LA12, of the A59 strain. The other selection marker was the antigenic sites recognized by the neutralizing monoclonal antibodies J.7.2 and J.2.2, which are specific for the E2 protein of JHM and do not react with A59 (3). Viruses, which grew at 39°C and were resistant to neutralization by monoclonal antibodies, were selected and examined by oligonucleotide fingerprinting (Fig. 1). At least four types of RNA recombinants were detected, all of which contain crossovers within gene C.

Among the eight viruses isolated from the cross between LA12 and JHM, four are A59 revertants. The other four are recombinants with one of the two following genetic structures. The first one, RL-1, has a single crossover within gene C. The 3' end of gene C and the remaining 3'-downstream

genes are derived from A59, whereas the 5' sequence upstream from the crossover site is derived from JHM (Fig. 2). This genetic map was established by comparing the oligonucleotide fingerprints of the recombinant and the parental viruses (Fig. 1). The map position of every oligonucleotide has been determined for both parental viruses (10, 19). The second recombinant, RL-2, has two crossover points. One crossover occurs in the 3' end of gene A, and the other is in gene C, at approximately the same position as in RL-1. Thus, the RL-2 recombinant has A59 sequence at both the 5' and 3' ends, and has the JHM sequence in gene B and part of gene C. Both RL-1 and RL-2 appear to have undergone a similar crossover event within gene C.

Since the fingerprint of the genomic RNA is too complex to allow for precise determination of the crossover points, we examined the oligonucleotide fingerprints of the intracellular virus-specific mRNA 3, which is the functional mRNA for gene C (10) and, in the case of RL-1, a distinct RNA, 3a, which represents the 3'-end one-third of gene C (20) (Fig. 3A). These mRNAs have a 3'-coterminal nested-set structure, i.e., they contain sequences of the gene in question and all the 3'-downstream genes. The mRNA 3 of RL-1 contains both A59- and JHM-derived oligonucleotides. All of the A59-derived oligonucleotides are localized in the 3' half of gene C and 3'-downstream genes, whereas the JHM-derived ones are in the 5' half of gene C. The crossover point was more precisely determined by oligonucleotide fingerprinting of mRNA 3a, which represents a new RNA initiation site within gene C of the recombinants. This RNA species is synthesized by all of the recombinants containing a JHM-specific leader RNA (Fig. 3A), including B-1 recombinant virus identified (20). It is notable that mRNA 3a of RL-1 contains mostly A59 sequences but also four JHM-specific oligonucleotides—oligonucleotides 8, 9, 27 and 4. Oligonucleotide 8 represents the leader sequence derived from the 5' end of the genome, and the remaining JHM oligonucleotides are mapped in the middle of gene C (19). In view of the nested-set structure of

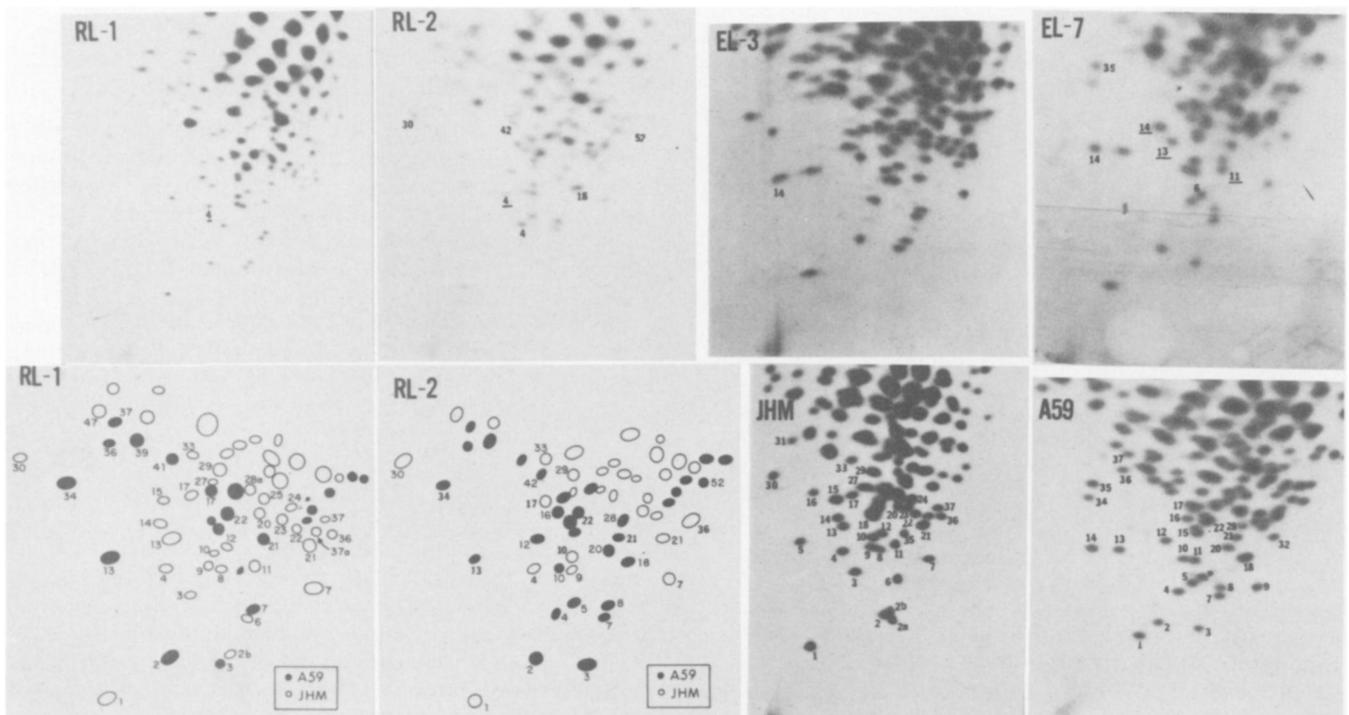


FIG. 1. Oligonucleotide fingerprints of the genomic RNA of recombinant and parental viruses. The schematic drawings of the fingerprints of RL-1 and RL-2 are also presented. The numbering of oligonucleotides is according to Lai *et al.* (10) for A59 and to Makino *et al.* (19) for JHM. EL-3 and RL-1 are identical, and EL-7 and RL-2 are identical except that the spots numbered in the fingerprints are missing in the corresponding recombinants. The JHM-specific spots are underlined.

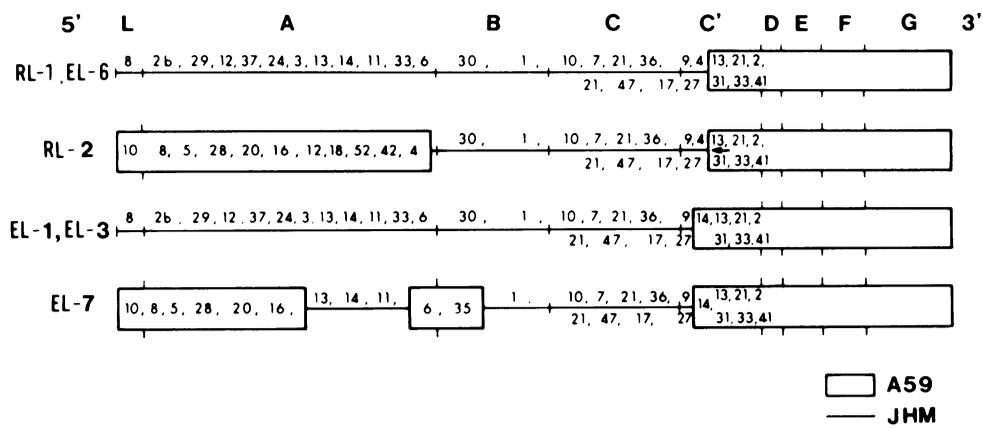


FIG. 2. Schematic drawing of the genetic structure of the recombinants. The data were derived from Fig. 1. The positions of each oligonucleotide have been determined (10, 19). L represents the leader region. A through G are the seven genes described (10). C' corresponds to mRNA 3a. The A59-specific spots are presented within the box. The crossover points in the C genes of RL-1 and RL-2 are nearly identical except that RL-2 contains an additional small A59-specific oligonucleotide marked by the arrow (see Fig. 3B).

MHV mRNAs, this result clearly establishes that the cross-over point of RL-1 virus is mapped within the RNA 3a-coding region. Since mRNA 3a has a molecular weight of 1.7×10^6 , in contrast to 2.6×10^6 for mRNA 3, the mRNA 3a represents a transcript from the region corresponding to the 1.5-kilobase (kb) area at the 3' end of gene C, which spans 4.2 kb (22). Therefore, the E2 of the RL-1 virus must consist of mostly JHM sequences with approximately one-third of the sequences at carboxyl terminus being derived from A59.

Comparison of mRNA 3 of RL-2 with that of RL-1 shows that all of the large T_1 oligonucleotides in the mRNA 3 of these two viruses are identical except that RL-2 contains the A59-specific leader oligonucleotide, oligonucleotide 10, instead of the JHM-specific one (oligonucleotide 8 in RL-1), reflecting the fact that the 5' end of RL-2 genome is derived from A59. In addition, RL-2 contains a small T_1 -oligonucleotide (pointed by an arrow in Fig. 3B) not found in RL-1. This oligonucleotide has been mapped in gene C of A59 (10). Thus,

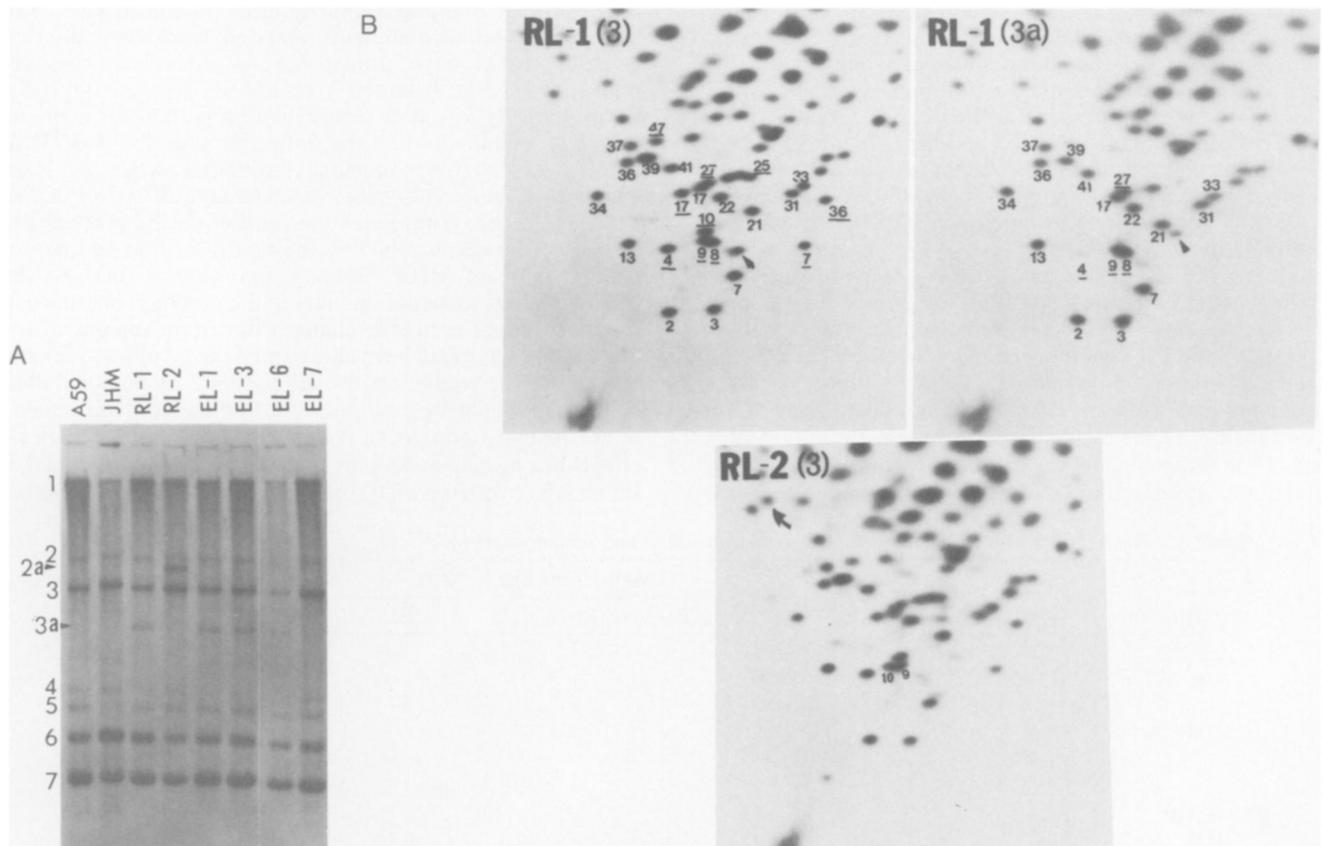


FIG. 3. Structure of the intracellular RNAs of recombinant viruses. (A) The patterns of intracellular RNAs of each recombinant. Note the presence of RNA 3a in recombinants RL-1, EL-1, EL-3, and EL-6. RL-2 has an additional RNA 2a, the nature of which is not clear. (B) The oligonucleotide fingerprints of RNAs 3 and 3a of RL-1 and RL-2. The JHM-derived oligonucleotides are underlined. The spots indicated by an arrow in RL-1 RNAs are not present in the fingerprint of the viral genome and are likely the leader-body junction oligonucleotides (21). The spot denoted with an arrow in RL-2 is the spot found only in RL-2.

RL-2 has a crossover site slightly upstream in gene C of the crossover site in RL-1. In other words, RL-2 has a slightly longer A59-derived sequence in gene C.

All four isolates derived from the cross between LA7 and JHM are recombinants. One of the recombinants, EL-6, has a genetic structure identical to that of RL-1 (data not shown). The other three recombinants, EL-1, EL-3, and EL-7, have crossover sites slightly on the 5' side of the crossover site in RL-2. This conclusion is supported by the presence of A59-specific oligonucleotide 14 and the loss of JHM oligonucleotide 4 in these three recombinants (Figs. 1 and 2). Recombinant EL-7 has three additional crossover sites in genes A and B and thus contains three discontinuous stretches of A59 sequences.

Antigenicity and Pathogenicity of Recombinant Viruses. Genetic analysis of the recombinants shows that gene C of these viruses contains mostly JHM sequences except for the 3'-terminal one-third, which is derived from A59. The substitution of A59 sequences at the carboxyl-terminal one-third of E2 encoded by gene C is most likely responsible for the escape of the recombinants from neutralization by the JHM-specific anti-E2 monoclonal antibodies. Thus, essential antigenic sites recognized by the two monoclonal antibodies are most likely localized within the carboxyl-terminal one-third of E2, corresponding to the 1.5-kb region at the 3' end of gene C. The antigenic properties of these viruses were examined by ELISA using JHM-specific monoclonal antibodies (Table 1). Monoclonal antibodies J.7.2 and J.2.2, which recognize two topographically distinct E2 regions (6), designated sites A and B, respectively, did not recognize any of the recombinant viruses. Other JHM-specific monoclonal antibodies, e.g., J.2.6 and J.1.2, which recognize site A, also did not bind to any recombinants. These results suggest that the 1.5-kb segment at the 3' end of JHM gene C codes for the antigenic determinants essential for these antibody binding sites.

We have also tested monoclonal antibodies specific for E2 of A59. Two of the monoclonal antibodies, A.2.3 and A.3.10, have previously been shown to recognize an antigenic site, A59 site A, which has antigenic determinants in common with the site A of JHM (J.O.F., unpublished data). A.2.3 bound to all of the recombinants, whereas A.3.10 bound to only recombinants RL-2, EL-1, EL-3, and EL-7 and not recombinants RL-1 or EL-6. Since the latter two recombinants have the smallest A59-derived sequence in gene C, these data suggest that the antigenic site recognized by A.3.10 is localized within the region encoded by the sequence close to oligonucleotide 14. In contrast, A.1.4, which recognizes another antigenic site on A59 (29), did not bind to any of the recombinants. These results suggest that the crossover sites of all of the recombinants are localized within site A of A59, and that the A59 site B is localized within the amino-terminal

two-thirds of the E2 protein. To ensure that recombination had not induced dramatic changes in overall conformation of peplomers in recombinant viruses, we also tested the JHM monoclonal antibody J.7.5, which recognizes site A of both JHM and A59. As shown in Table 1, J.7.5 binds to all of the recombinants.

In prior studies with neutralization-resistant variants of JHM, we have shown that the antigenic sites recognized by J.7.2 and J.2.2 are critical for the neuropathogenicity of the virus. Specifically, J.7.2 binds to a region essential for paralytic disease, and J.2.2 is associated with determinants necessary for fatal encephalitis (8). Since the recombinants have lost JHM determinants at both these sites, we sought to determine the pathogenicity of representative recombinant viruses RL-1 and RL-2 by intracerebral inoculation in 6-week-old C57BL/6 mice. Whereas the parental JHM has a 50% lethal dose of 4 plaque-forming units, neither RL-1 nor RL-2 were lethal at 1000 plaque-forming units. Further, no paralytic disease was observed in these mice. Although mice appeared clinically normal after infection with RL-1 or RL-2, histopathological examination showed subclinical encephalitis acutely and mild demyelination subacutely. These findings are similar to those observed with neutralization-resistant variants with mutations affecting both the J.7.2 and J.2.2 recognition sites (8). This result suggests that critical neuropathogenic sites have been lost in RL-1 and in RL-2 and confirms the map location of these determinants to the carboxyl terminus of E2 protein.

DISCUSSION

In this study RNA-RNA recombination was used to map antigenic sites and pathogenic determinants of murine coronaviruses. The recombinants isolated from two different genetic crosses have similar but not identical crossover points, at ≈ 1.5 kb from the 3' end of the gene encoding the E2 protein. By virtue of recombination within this gene, all of the recombinants lost the antigenic sites for two JHM-specific neutralizing monoclonal antibodies, which are associated with neuropathogenic determinants of the virus (8). Thus, the carboxyl-terminal one-third of the E2 protein is at least partially responsible for the neutralization and neuropathogenicity of MHV, although we cannot rule out the possibility that sequence changes at the carboxyl one-third of E2 have caused antigenic changes in distant regions of the protein. We have also been able to map the location of several other epitopes recognized by different E2 monoclonal antibodies. It should be cautioned that antigenic determinants often depend upon local conformational or distant steric effects in a protein molecule (23, 24), and any linear model of E2 may be oversimplified. Nevertheless, there is substantial

Table 1. Binding of anti-E2 monoclonal antibodies to parental and recombinant MHV

Antigenic site	mAb	Anti-E2 binding to virus									
		JHM	RL-1	RL-2	EL-1	EL-3	EL-6	EL-7	LA7	LA12	A59
A59 "B"	A.1.4	-	-	-	-	-	-	-	+	+	+
A59 "A"	A.3.10	-	-	+	+	+	-	+	+	+	+
	A.2.3	-	+	+	+	+	+	+	+	+	+
JHM "A"	J.7.5	+	+	+	+	+	+	+	+	+	+
	J.1.2	+	-	-	-	-	-	-	-	-	-
	J.2.6	+	-	-	-	-	-	-	-	-	-
	J.7.2	+	-	-	-	-	-	-	-	-	-
JHM "B"	J.2.2	+	-	-	-	-	-	-	-	-	-

Binding was determined by ELISA (6). Viruses were assayed at equivalent antigenic densities. An OD reading 50% or greater of that achieved with homologous virus was considered positive (+). An OD of less than 50% was considered negative (-). A59 "A" and "B," and JHM "A" and "B" denote the antigenic sites on the E2 protein of A59 and JHM, respectively, recognized by the monoclonal antibodies as determined by competitive binding studies (6, 8, 29). A59 "A" and JHM "A" partially overlap while the "B" sites of A59 and JHM do not overlap with each other or with other sites. The monoclonal antibodies (mAb) designated by A were derived from A59, whereas those designated by J were from JHM.

agreement between a linear relationship with the monoclonal antibody binding sites and the genetic structure of recombinants, suggesting that such a linear map may reflect real spatial arrangement of antigenic sites on E2.

The localization of the neutralization epitopes on the carboxyl-terminal one-third of E2 was unexpected. The sequence of gene C, which encodes the E2 protein, has suggested that the carboxyl terminus of the protein is inserted in the viral envelope (22). Thus, the carboxyl half of the E2 would be expected to be closer to the envelope, whereas the amino half would be more exposed. However, the 180-kDa E2 peplomer is cleaved by cellular proteases into two subunits, 90A and 90B (25). This cleavage is required for the fusion-inducing activity of MHV (25). It is likely that the cleavage of peplomer might expose the carboxyl half of the peplomer. Since the 90A subunit contains palmitic acid (25) and likely represents the carboxyl-terminal half of E2, this subunit probably carries the major neutralization epitopes and neuropathogenic determinants. Furthermore, since only antibodies recognizing site A of E2 inhibit virus-induced cell-to-cell fusion (ref. 2; W. Gilmore and J.O.F., unpublished observation), the fusion-inducing activity of MHV may also be localized at the carboxyl-terminal one-third of E2. These structural arrangements suggest that the MHV peplomers are likely to have unusual conformation, with the carboxyl half of the protein being exposed.

The recombinants obtained in this study have crossovers within a small region of gene C. The presence of additional JHM sequences in gene A of RL-2 and EL-7 recombinants suggests that the ts lesions of LA7 and LA12 are in gene C or part of B. The relative proximity of the crossover sites in these recombinants strongly suggests that these ts lesions are probably localized very close to the antigenic sites recognized by the monoclonal antibodies. Thus, these lesions may be localized in the amino-terminal two-thirds of gene C. The finding that some of the recombinants have additional crossover sites in a region where no selectable marker was used further confirms the high frequency of coronavirus RNA recombination (13). The high frequency of recombination also made possible the isolation of different recombinant viruses with minor variations in their crossover sites. The availability of these recombinants allowed for a fine genetic mapping of coronaviral functions, such as monoclonal antibody binding sites, which had not been achieved with recombinants (26, 27) or reassortants (28) of other RNA viruses. Thus, RNA recombination of coronavirus provides a particularly powerful genetic tool.

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