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# A Clustering of RNA Recombination Sites Adjacent to a Hypervariable Region of the Peplomer Gene of Murine Coronavirus

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**Coronaviruses undergo RNA recombination at a very high frequency. To understand the mechanism of recombination in murine coronavirus, we have performed RNA sequencing of viral genomic RNA to determine the precise sites of recombination in a series of recombinants which have crossovers within the gene encoding the peplomer protein. We found that all of the recombination sites are clustered within a region of 278 nucleotides in the 5′-half of the gene. This region in which all of the crossovers occurred represents a small fraction of the distance between the two selection markers used for the isolation of these recombinant viruses. This result suggests that this region may be a preferred site for RNA recombination. The crossover sites are located within and immediately adjacent to a hypervariable area of the gene. This area has undergone deletions of varying sizes in several virus strains which have been passaged either *in vivo* or *in vitro*. These results suggest that a similar RNA structure may be involved in the occurrence of both recombination and deletion events.** 

## INTRODUCTION

Mouse hepatitis virus (MHV), the prototypic member of the Coronaviridae, is an enveloped virus which contains nonsegmented positive-sensed RNA of 32 kb in length (Lai and Stohlman, 1978; Pachuck *et al.*, 1989; Baker *et al.*, 1989). The virus particle is composed of three envelope proteins (S, M, and HE) and a nucleocapsid protein (N). The S protein (Spike protein, formerly named E2) forms the surface projections or peplomers, which are the outermost components of the virus surface. This protein is responsible for the attachment of virus to target cells and the induction of cell-to-cell fusion and is the target for neutralizing antibodies (Sturman and Holmes, 1983). Alterations in the S protein have been shown to result in changes in viral pathogenicity, as demonstrated by virus variants selected by escape from neutralization with monoclonal antibodies (Fleming *et al.*, 1986; Dalziel *et al.*, 1986). The peplomer is composed of two 90-kDa glycoprotein heterodimers, which are the cleavage products of a 180-kDa glycoprotein. The cleavage of the S protein is carried out by cellular proteases and is required for virus infectivity (Storz *et al.*, 1981; Yoshikura and Tajima, 1981; Sturman *et al.*, 1985). This protein most likely interacts with the receptors on the target cells. The functions of the other two envelope proteins are less well understood. The HE protein (formerly named E3 or gp65) contains an esterase activity but is present only in certain strains of MHV (Lai and Stohlman, 1981; Siddell, 1982; Makino *et al.*, 1983; Makino and Lai, 1989; Shieh *et al.*, 1989). The M protein (formerly named E1) is mostly embedded within the viral envelope (Armstrong *et al.*, 1984; Rottier *et al.*, 1984, 1986); however, monoclonal antibodies against M protein can also alter the pathogenicity of the virus (Fleming *et al.*, 1989).

All of the viral proteins are translated from separate mRNAs, which have a nested-set 3′-coterminal structure (Lai *et al.*, 1981; Leibowitz *et al.*, 1981). Only the 5′-terminus of each mRNA, representing the unique portion which does not overlap with the next smaller mRNA, is utilized for translation (Leibowitz *et al.*, 1982; Siddell, 1983). The S protein is translated from mRNA 3, representing a genetic region roughly 8.5 to 4.5 kb from the 3′-end of the genome. The complete sequence of this gene (gene 3, previously named gene C) has been reported for two different strains, A59 and JHM, of MHV (Schmidt *et al.*, 1987; Luytjes *et al.*, 1987). The two sequences are 93% homologous but differ in a major aspect in that A59 contains 156 nucleotides of additional sequence at the 5′-terminal one-third of the gene. The significance of these extra sequences in A59 is not known.

One unique feature of MHV replication is that it undergoes recombination at a very high frequency (Makino *et al.*, 1986). This phenomenon has been demonstrated in tissue culture (Lai *et al.*, 1985; Makino *et al.*, 1986, 1987; Keck *et al.*, 1987, 1988b) and in infected animals (Keck *et al.*, 1988a). Recombinants have been selected using temperature-sensitivity in replication,

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resistance to neutralization by monoclonal antibodies, or ability to induce cell-to-cell fusion as selection markers. Most interestingly, the recombinants obtained have crossovers not only in the genetic region between the two selection markers but very often also in other regions without selection pressure (Makino *et al.*, 1987; Keck *et al.*, 1988b), indicating an extremely high frequency of recombination. Although RNA recombination has also been observed in picornavirus (King *et al.*, 1982) and more rarely in brome mosaic virus (Bujarski and Kaesberg, 1986), the frequency of RNA recombination in coronavirus is exceptionally high. This finding suggests that coronaviruses have a unique mechanism of RNA replication, leading to a high frequency of RNA recombination.

To understand the mechanism of coronavirus RNA recombination, we have begun to use RNA sequencing to determine the precise crossover sites of recombinants. The characterization of RNA crossover sites may shed light on the RNA structure commonly associated with the occurrence of RNA recombination. We have chosen to examine a series of recombinants between the A59 and JHM-DL strains. These recombinants were obtained using temperature sensitivity in viral replication and viral sensitivity to neutralizing monoclonal antibodies as selection markers (Makino *et al.*, 1987). Previous oligonucleotide fingerprinting studies have indicated that all of these recombinants have a crossover site within the S gene of the virus. These recombinants have been used to map various biological determinants associated with the S protein (Makino *et al.*, 1987). RNA sequencing studies presented in this report reveal that all of the recombination sites are localized within a small region of the S gene. Furthermore, these sites are adjacent to an area which is subject to frequent deletions in several different MHV strains and some monoclonal antibody escape variants (Parker *et al.*, 1989). A model for RNA recombination in coronavirus is proposed.

## MATERIALS AND METHODS

### Viruses and cells

The DL isolate of the JHM strain is a large-plaque variant of the original JHM (Weiner, 1973) which had been passaged eight times in suckling mouse brain (Stohlman *et al.*, 1982). The JHM-X strain was a plaque-cloned isolate of MHV-JHM, originally obtained from Microbiological Associates, Bethesda, Maryland, and was maintained in DBT cells (Makino *et al.*, 1983), which are murine astrocytoma cells (Hirano *et al.*, 1974). The strains JHM-X-1a and -2c are different plaque isolates derived from a persistent infection of JHM-X in DBT cells (Makino *et al.*, 1983, 1984). All of the recombinants used in this study (Makino *et al.*, 1987) were obtained by co-infection with JHM-DL and one of two ts mutants, tsLA7 and tsLA12, of the A59 strain and selected with the JHM-specific neutralizing monoclonal antibodies, J2.2 and J7.2 (Fleming *et al.*, 1986). The viruses were grown on DBT cells as described previously (Makino *et al.*, 1986).

### Purification of viral RNA

Virus was prepared by a modification of procedures described by Makino *et al.* (1983). DBT cells were infected with virus at a multiplicity of infection (m.o.i.) of 1–5. Supernantant from infected cell cultures was harvested at 13 hr postinfection, and was clarified of cell debris by centrifugation at 8000 rpm for 20 min. Virus was precipitated by 50% saturation of ammonium sulfate for 1.5 hr at 4°, followed by centrifugation at 8000 rpm for 20 min. The pellet was resuspended in NTE buffer (0.1 *M* NaCl, 0.01 *M* Tris–HCl, pH 7.2, and 0.001 *M* EDTA) and the suspension was centrifuged at 2000 rpm for 10 min. The supernatant was placed on a discontinuous sucrose gradient consisting of 20, 30, 50, and 60% sucrose in NTE and centrifuged at 26,000 rpm for 3.5 hr at 4° in a Beckman SW 28 rotor. The virus band was collected from the interface between 30 and 50% sucrose, diluted sixfold in NTE, and applied to a second discontinuous gradient as above, and was spun for 14 hr. The virus at the 30–50% surcrose interface was diluted fivefold in NTE and pelleted by centrifugation at 40,000 rpm for 1.5 hr in an SW41 rotor. The virus pellet was resuspended in 400 $\mu$l of NTE, an equal volume of proteinase K buffer (2% SDS, 0.025 *M* EDTA, 0.2 *M* Tris pH 7.5, and 0.3 *M* NaCl) and 160 $\mu$g of proteinase K, and incubated for 30 min at 37°. The RNA was extracted twice with phenol/chloroform (1:1) and precipitated with ethanol. The pelleted RNA was resuspended in distilled $H_2O$ to a concentration of 1 $\mu$g/$\mu$l and was stored at −70°.

### RNA sequencing

Oligodeoxyribonucleotide primers of 18 nucleotides in length were synthesized on a 380-A DNA synthesizer (Applied Biosystems, Foster City, CA) and correspond to regions of homology between A59 and JHM-DL strains. The primers were used to sequence purified virion genomic RNA by a modification of the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). Briefly, 1 $\mu$g of template RNA was mixed with 20 ng of primer in a final volume of 6 $\mu$l and was heated at 100° for 2 min. KCL was added to the RNA/primer mixture to a final concentration of 0.17 *M* and the reaction mixture was incubated at 65° for 10 min.

A separate dideoxynucleotide mix was prepared for each of the four nucleotides as a 5× stock in a buffer containing 250 m*M* Tris–HCl, pH 8, 50 m*M* $MgCl_2$, 50 m*M* DTT, and 350 m*M* KCl. The dideoxy (dd) A mix contained 0.67 $\mu M$ dATP, 10 $\mu M$ dCTP, dGTP, TTP, and 2 $\mu M$ ddATP; the ddG mix contained 0.67 $\mu M$ dATP, 10 $\mu M$ dCTP and TTP, 5.3 $\mu M$ dGTP, and 5 $\mu M$ ddGTP; the ddT mix contained 0.67 $\mu M$ dATP, 10 $\mu M$ dCTP and dGTP, 2.5 $\mu M$ TTP, and 5 $\mu M$ ddTTP; the ddCTP mix contained 0.67 $\mu M$ dATP, 10 $\mu M$ dGTP and TTP, 5.3 $\mu M$ dCTP, and 5 $\mu M$ ddCTP. The RNA/primer mixture was divided into four tubes, each of which contained 1 $\mu$l of one of the 5× dideoxynucleotide mixture, 20 $\mu$Ci [$^{32}$P]dATP (3000 Ci/m*M*, ICN Radiochemicals), and 4 units of AMV reverse transcriptase in a buffer containing 50 m*M* Tris–HCl, pH 8, 10 m*M* $MgCl_2$, and 10 m*M* DTT, and incubated at 42° for 15 min. One microliter of a 5× "chase" dideoxy mix (2.5 m*M* of the appropriate ddNTP plus the three other dNTPs at a concentration of 0.5 m*M*) in a buffer containing 250 m*M* Tris–HCl, pH 8, 50 m*M* $MgCl_2$, 50 m*M* DTT, and 350 m*M* KCl was then added to the specific tubes for an additional 10 min at 42°. The reaction was stopped by the addition of a dye mix (10 m*M* EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue in deionized formamide). Samples were heated at 100° for 2 min and 4.5 $\mu$l of sample was applied to either 6 or 8% polyacrylamide gels according to the published procedures (Sanger *et al.*, 1977). Samples were electrophoresed for 2 or 3.5 hr at 2000 V. Gels were fixed in 10% methanol and 10% acetic acid for 20 min, dried, and exposed on X-ray film.

## RESULTS

### Sequence analysis of the parental strains

Before the precise crossover sites of the recombinants could be determined, the two parental strains used in our laboratory, JHM-DL and A59, had to be sequenced. In Fig. 1A, the sequences of the two parental strains along with the published sequence of MHV-JHM (Schmidt *et al.*, 1987) are compared for the region corresponding to nucleotides 1100 and 2100 from the 5′-end of the S gene, which encodes the peplomer protein. These 1000 nucleotides cover the genetic region which encompasses all of the recombination sites of the recombinants examined in this study (see below) and also represent the most diverged region in the S gene between the published A59 and JHM sequences (Luytjes *et al.*, 1987). It has previously been shown that the S genes of A59 and JHM are 93% homologous in overall sequence, with the amino terminal half (S1) showing less homology than the carboxyl terminal half (S2) (Luytjes *et al.*, 1987). The sequence shown in Fig. 1A is contained within the S1 region. Surprisingly, the sequence of JHM-DL used in our laboratory differs from the published JHM sequence, even though both were derived from the same source. These two strains share 98% homology (excluding the deleted sequence), while JHM-DL and A59 are 85% homologous. Most interestingly, both A59 and published JHM strains have large areas which are missing from the corresponding region of JHM-DL. The A59 strain is missing 156 nucleotides starting at position 1501, while the deletion in JHM encompasses 423 nucleotides beginning at position 1359. These deletions result in an S protein which is 52 amino acids smaller for A59 and 141 amino acids smaller for the published JHM strain. These results are presented diagrammatically in Fig. 1B. The deletions in both A59 and JHM occurred in the same region. No additional deletions in other areas of the S gene for either A59 or JHM were detected (data not shown).

### Identification of the recombination sites in A59/JHM-DL recombinants

Various synthetic oligodeoxyribonucleotides of 18 nucleotides in length corresponding to regions of homology between the two parental strains were used as primers for sequencing of virion genomic RNA. Because the two parental strains contain many nucleotide differences which are scattered throughout the S gene, the recombination sites could be determined by comparing specific nucleotide differences between the two strains. An example is shown in Fig. 2, in which a portion of the S1 gene of recombinant EL5 is compared with those of the two parental strains. EL5 contains A59-specific sequences 3′ to nucleotide 1308 of the S gene. The next base difference between A59 and JHM-DL, in the 5′ direction, occurs at nucleotide 1293; at this position, EL5 is identical to JHM-DL, as are the remaining nucleotides on the 5′ side. Thus, recombinant EL5 has a crossover site between nucleotides 1293 and 1308. Since JHM-DL and A59 are completely homologous within this region, the crossover site can only be localized to these 15 nucleotides. A comparison of RNA sequences of some of the recombinants with the parental strains is shown in Fig. 3A. Among all of the recombinants examined, EL3 has a crossover site closest to the 5′-end of the S gene, switching from JHM-DL sequences at nucleotide 1148 to A59-specific sequences beginning at nucleotide 1162. EL2 has a crossover site slightly closer to the 3′-end, switching from JHM-DL to A59 between nucleotides 1231 and 1236. The remaining seven recombinants have recombination sites between nucleotides 1291 and 1426, as shown in Fig. 3B. Three of the recombinants, EL1, EL5, and EL7, have the identical crossover site at nucleotides 1293 and 1307. The crossover site closest to the

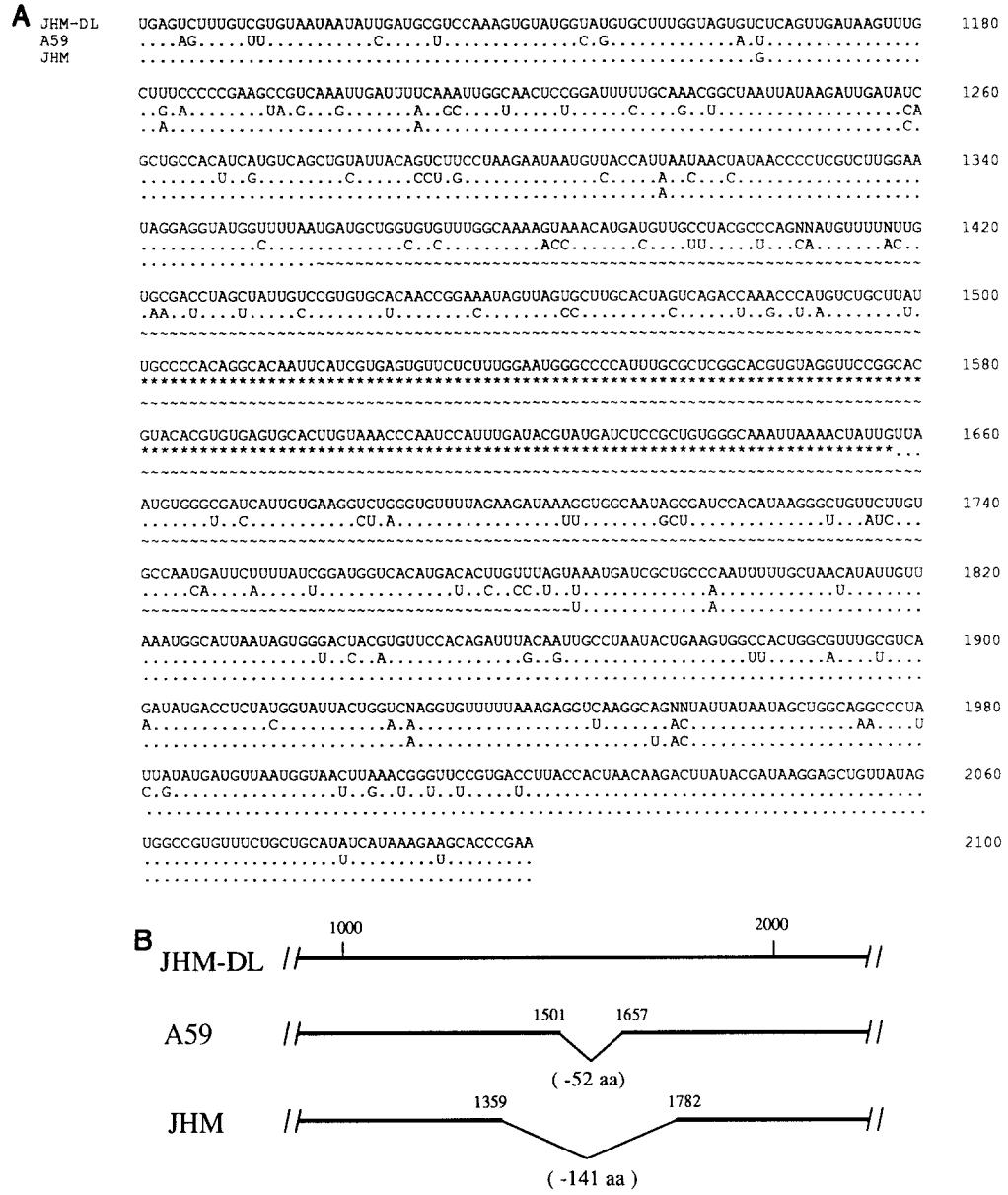


FIG. 1. Comparison of the nucleotide sequences of the S gene of JHM-DL, A59, and JHM. (A) Nucleotide sequences of the regions from nucleotides 1100 to 2100 from the 5′-end of the S gene. The area of deletion in A59 (nucleotides 1501–1657) is denoted by asterisks, while that of JHM (1359-1782) is denoted by the wavy lines. Only the diverged nucleotides are indicated. (B) Diagrammatic representation of (A). Nucleotides are numbered according to Luytjes *et al.* (1987). The sites of deletion are indicated by thin lines.

3′-end is in recombinant EL6, which is located between nucleotides 1423 and 1426. All of the recombination sites are clustered within 278 nucleotides of the S gene as shown in Fig. 3C. Because A59 and JHM-DL share many homologous nucleotides, the crossover in each recombinant could have occurred at any position between the two diverged nucleotides. Interestingly, the recombination sites in all of the recombinants examined are located in or adjacent to the deletion sites observed in A59 and the published JHM sequence (Fig. 1B).

## Occurrence of deletions in the S protein of other MHV strains

Since the recombination sites of all of the recombinants studied are located in or adjacent to the area which is deleted in A59 and JHM, it is possible that a relationship exists between recombination and the generation of deletions. To learn more about the mechanism of recombination, we examined other strains of MHV to determine if there are any structural features which are unique to this area. We chose to study JHM-

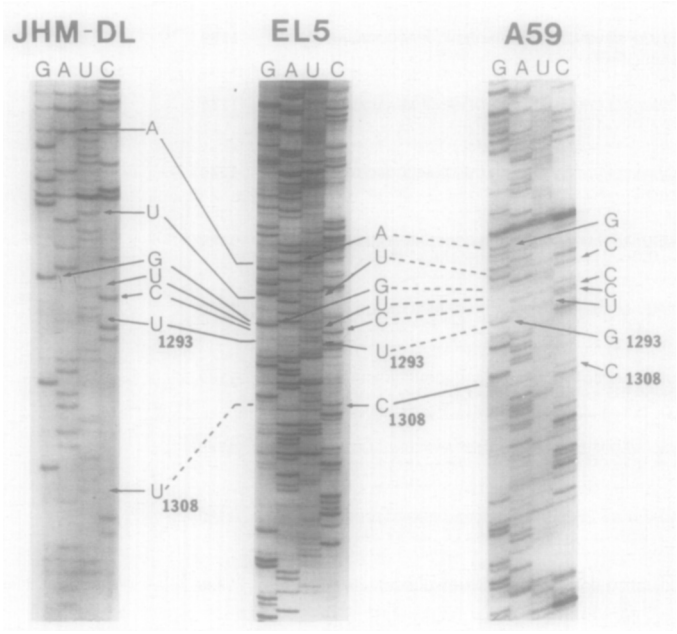


FIG. 2. RNA sequencing of the recombinant EL5 and its parental viruses JHM-DL and A59. RNA sequencing was performed using a primer corresponding to nucleotides 1381–1399 for A59 and EL5, and 1325–1343 for JHM-DL. Solid lines represent identical nucleotides while dashed lines represent nonhomologous nucleotides.

X because it has been shown to encode a smaller S protein when compared to JHM-DL (Taguchi and Fleming, 1989). Two variants of JHM-X, 1a and 2c, were also sequenced. These variants were isolated from a cell line which was persistently infected with JHM-X (Makino *et al.*, 1983, 1984). Previous oligonucleotide fingerprinting studies (Makino *et al.*, 1984) showed that these two viruses are missing an oligonucleotide which represents a genetic region where the recombination and deletions occurred in A59 and JHM-DL.

As shown in Fig. 4, JHM-X also has a 458-nucleotide deletion in the same general area of the S gene. This deletion starts at nucleotide 1336 and extends to 1794, encompassing 153 amino acids. The two variants, 1a and 2c, have the same deletion as the parental JHM-X, but both of them have an additional deletion of 15 nucleotides located slightly upstream, from nucleotides 1309 to 1324. The S protein encoded by these two isolates is 158 amino acids smaller than that of JHM-DL. These results are summarized in Fig. 4 which also shows the recombination sites.

The recombination sites and areas of deletion of all of the viruses studied are summarized in Fig. 5. The virus with the smallest deletion is A59, which is missing 52 amino acids, while JHM-X-1a and -2c have the largest deletion, encompassing 158 amino acids. All of the recombination sites either overlap with or are adjacent to the areas where deletions occurred in many strains of MHV.

## DISCUSSION

Coronavirus undergoes a very high frequency of RNA recombination (Makino *et al.*, 1986). The data pre-

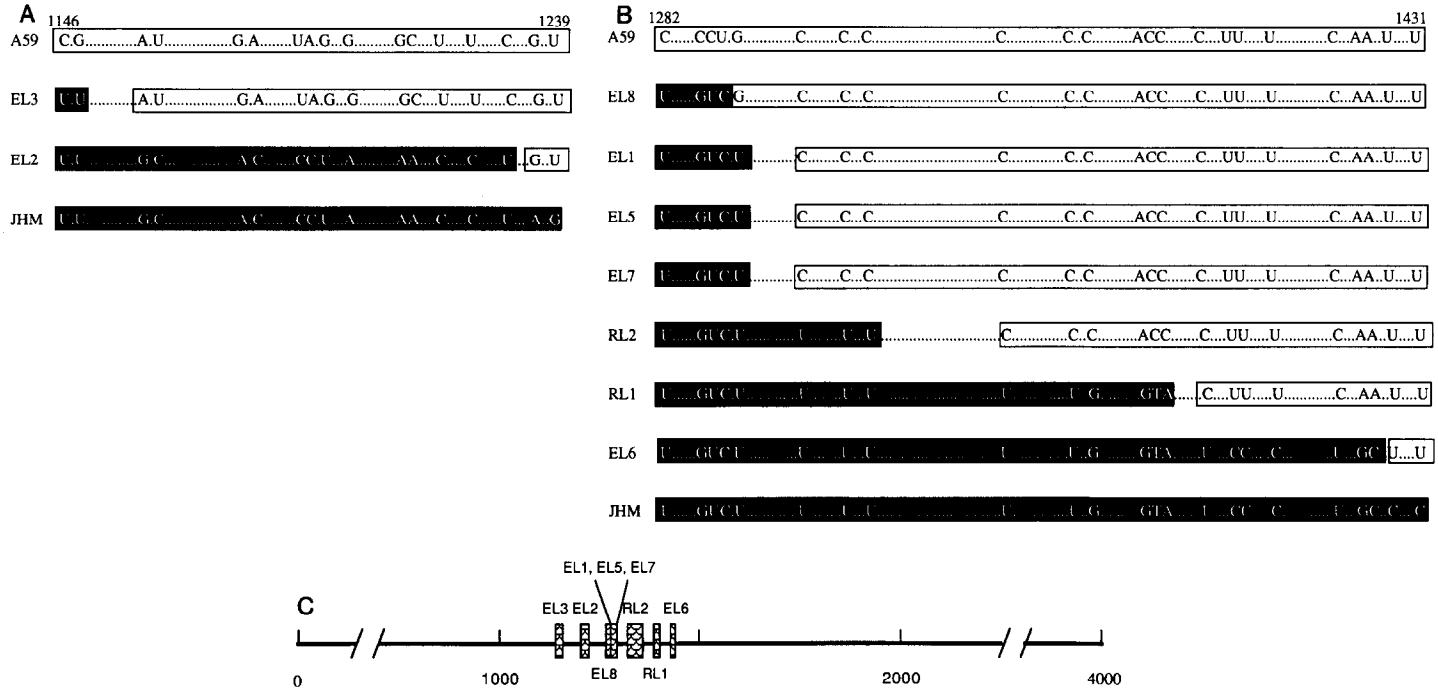


FIG. 3. Nucleotide sequences around the crossover sites of the recombinants. (A) 5′-most recombinants EL3 and EL2 are compared with JHM-DL (black) and A59 (white). Numbering of nucleotides is the same as in the legend to Fig. 1. (B) The crossover sites of the remaining seven recombinants. Nucleotides 1282–1432 are shown. (C) Diagrammatic summary of the crossover sites within the S gene. The filled rectangles represent the areas of crossover sites of each recombinant.

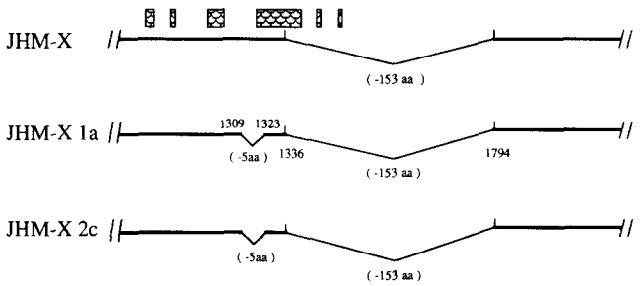


FIG. 4. Schematic representation of the structure of the S gene of JHM-X and the JHM-X variants 1a and 2c. The sites of deletion are indicated by thin lines. The sites of recombination (filled rectangles) in all of the recombinants studied are included for comparison.

sented in this report precisely determined the sites of recombination for a series of recombinant viruses with crossovers within the gene encoding the peplomer (S) protein. The crossover sites of the nine recombinants from two different crosses are clustered in the amino half of the protein. All of the recombination events occurred in a stretch of 278 nucleotides within the S gene, which is greater than 4 kb in total length. Seven of the recombination sites are within 135 nucleotides of each other; among these, three are located at the same site. This finding that the recombination sites are clustered within a short stretch of the S gene is of considerable interest. These recombinants were selected by using two selection markers (Makino *et al.*, 1987): resistance to monoclonal antibodies (MAb) specific for JHM-DL (Fleming *et al.*, 1986) and temperature sensitivity in virus replication. The MAbs used (J2.2 and J7.2) have been shown to react with the S protein (Fleming *et al.*, 1986). The ts mutants used were tsLA7 and tsLA12, both of which have an RNA (+) phenotype (data not shown). Preliminary sequence analysis of another set of recombinants between A59 and MHV-2, which were generated using the same ts mutants (Keck *et al.*, 1988b), suggests that the ts lesions of these mutants are located upstream of the S gene, probably within the gene encoding the HE protein (Shieh *et al.*, 1989) (data not shown). Thus, the potential sites of recombination between the two selection markers is greater than 1.5 kb. The fact that nine separate recombination events occurred in a small area within the potential recombination sites of more than 1.5 kb suggests that this area may be highly susceptible to recombination. It should be noted that several of these recombinants have been shown by oligonucleotide fingerprinting to have more than one crossover in the genome (Makino *et al.*, 1987). These additional sites were not selected by selection pressure and were scattered throughout the 32-kb genome. Thus, there may be other recombinational hot spots as well. It has previously been shown that poliovirus RNA recombination also follows a pattern of nonrandom distribution, suggesting the presence of preferred crossover sites (Tolskaya *et al.*, 1987).

The recombination sites of these recombinants are located closer to the 5′-end of the S gene than was previously estimated from oligonucleotide fingerprinting (Makino *et al.*, 1987). The fact that the oligonucleotide fingerprint mapping was not precise is not surprising

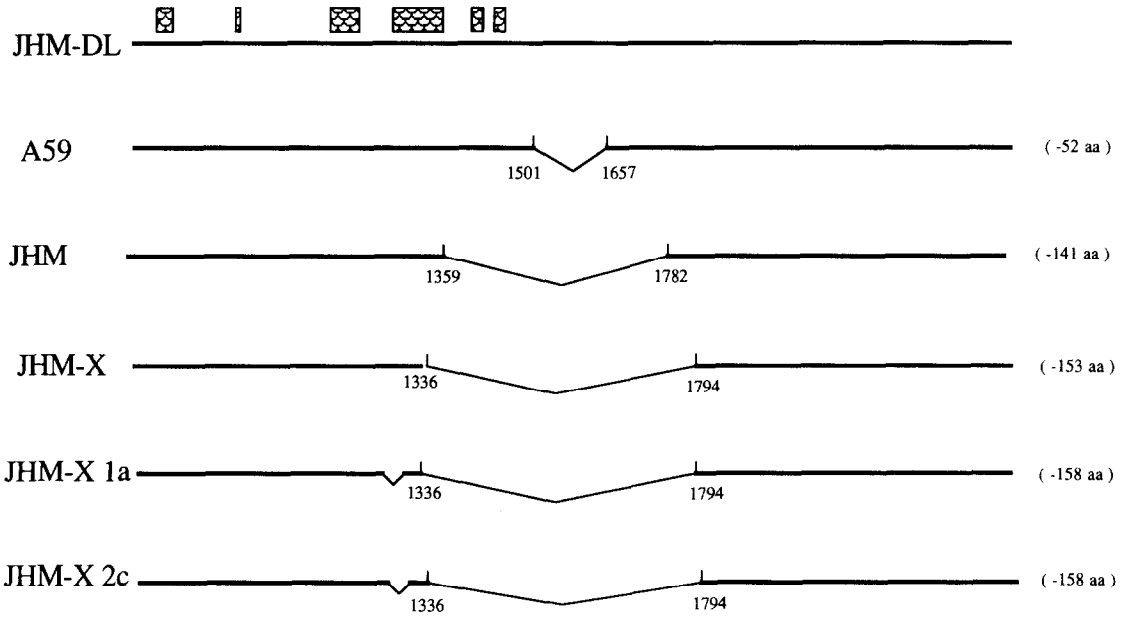


FIG. 5. Summary of recombination sites and deletions. Recombination sites are shown by filled rectangles above JHM-DL.

since oligonucleotide mapping relies on the estimates of RNA sizes based on agarose gel electrophoresis. We have previously used this series of recombinants to localize sites of neutralizing epitopes and neuropathogenic determinants of JHM strain to the carboxyl terminus of the peplomers (Makino *et al.*, 1987). Now that the crossover sites of these recombinants have been mapped within the N-terminal half (S1 region), the possible localization of both the neutralizing epitopes and neuropathogenic determinants has to be expanded to include not only the carboxyl terminal half (S2) but also the C-terminus of the S1 protein as well.

The recombination sites are located either within or immediately adjacent to an extremely hypervariable region of the S gene. This area, encompassing more than 400 nucleotides, is subject to frequent deletions of varying sizes. The deletions within this region occurred not only during *in vitro* passages in tissue culture (such as JHM-X, 1a, 2c, and possibly A59), but also during *in vivo* passages (probably the JHM strain sequenced by Schmidt *et al.* (1987)). We have recently found that a JHM strain isolated after passages in rat spinal cord, AtIIf (Morris *et al.*, 1989), also has a similar deletion of 441 nucleotides in the same region (La Monica *et al.*, in preparation). Furthermore, Parker *et al.* (1989) have recently found that many of their monoclonal antibody escape variants have a large deletion within the same area of the S gene. Thus, we conclude that this is a hypervariable region which is highly susceptible to deletions. It should be noted that the end points of these deletions are not at the same nucleotides. Also, JHM-X-1a and -2c have two deletions within the same general area. Thus, the deletions are probably not a sequence-specific event.

The fact that most of the recombination events occurred very close to the areas which are deleted in several MHV strains suggested that a similar RNA structure may be responsible for the generation of both recombination and deletion. It is possible that this hypervariable region has a very complex structure which may interrupt RNA synthesis. As a result, the "pausing" RNA intermediates may dissociate from the template. In the case of recombination, the dissociated RNA intermediates and RNA polymerase rebind to an RNA template derived from a different virus; the continuing RNA synthesis will result in a recombinant virus. For a deletion to occur, the RNA intermediates rebind to the original or homologous template but at a downstream location. Further RNA synthesis on these intermediates will lead to deletions. We have previously provided evidence for the generation of such RNA intermediates during MHV RNA synthesis (Baric *et al.*, 1985, 1987). Indeed, the computer analysis of the 1000 nucleotides surrounding the area of recombinations and deletions suggests the possible presence of extensive and stable secondary structures (data not shown). Additional experiments are needed to determine whether this is indeed the mechanism of coronavirus RNA recombination.

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