RNA RECOMBINATION OF CORONAVIRUS

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Summary

We have previously shown that Mouse hepatitis virus (MHV) can undergo RNA-RNA recombination at a very high frequency (S. Makino, et al., J. Virol. 57, 729-737, 1986). To better define the mechanism of RNA recombination, we have performed additional crosses involving different MHV strains. We have obtained recombinant viruses with multiple cross-overs. The isolation of such recombinants further indicates the high frequency of coronavirus RNA recombination. By using cell fusion as a selection marker, we have also obtained recombinants between MHV-2 and A59 strains. Some of these recombinants have cross-overs in the 3'-end genes of the genome, thus demonstrating that recombination could occur along the entire genome. Finally, we have obtained recombinants by selecting with neutralizing monoclonal antibodies. These recombinants have cross-overs within gene C which encodes the peplomer protein. The genetic structure of these recombinants allowed us to determine the important domains of the peplomer proteins.

Introduction

Mouse hepatitis virus (MHV) contains a single piece of single-stranded, nonsegmented and positive-sensed RNA genome (Lai and Stohlman, 1978, 1981; Wege et al., 1978). The RNA is replicated through a full-genome-length negative-strand RNA (Lai and Stohlman, 1978, 1981; Lai et al., 1982), which is then used to transcribe a genome-size RNA and six subgenomic mRNAs. Evidence has been presented that the synthesis of subgenomic RNAs utilize a unique mechanism of "leader-primed transcription" (Soe et al., this volume). However,the mechanism of replication of full-size genomic RNA is not yet clear.

Recently, our laboratory has demonstrated that MHV can undergo RNA-RNA recombination at a very high frequency (Makino et al., 1986). The frequency of recombination is reminiscent of RNA segment reassortment in viruses with segmented RNA genomes, such as reovirus (Fields, 1981). This phenomenon is very unique and can best be explained by the generation of free segmented RNA intermediates during RNA replication. In support of this interpretation, RNA intermediates of various sizes, derived from the 5'-end of the genome have been detected in MHV-infected cells (Baric et al., this volume). These intermediates could conceivably participate in RNA recombination via a

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copy-choice mechanism. We have described this mechanism as discontinuous and nonprocessive RNA replication (Makino et al., 1986; Baric et al., this volume), i.e. RNA synthesis stops at various places on the template and falls off the template, creating free RNA intermediates, which would reassociate with the template to continue transcription.

The availability of RNA recombinants provides a powerful genetic tool for studying the functions of various viral genes. We have now performed additional series of RNA recombination. These recombinants have offered additional insights into the mechanism of RNA recombination, and further confirmed the high frequency of RNA recombination. They also provide significant information concerning the structure-function relationship of viral genes.

Materials and Methods

Viruses and cells

The MHV wild-type strains JHM and A59, temperative sensitive (ts) mutants derived from these strains and wild-type MHV2 were used. The ts mutants of A59 were isolated on L2 cells after mutagenesis of parental A59 (Egbert, J. et al., unpublished observation). The ts mutants of JHM have previously been described (Leibowitz et al., 1982). The viruses were grown on the DBT or L2 cells lines according to published procedures (Lai et al., 1978, 1981).

Isolation of recombinants

Recombinants were isolated by three methods: The first method was as described by Lai et al., (1985) and Makino et al., (1986) with slight modifications. Co-infection involving ts mutants of A59 or JHM was carried out at 32° for 90 min, and shifted to 39° (nonpermissive). Virus was harvested at 16 hrs (p.i.) and plaque-purified at 39° at least three times before further studies. The second method was used for the isolation of recombinants between wild-type MHV2 and a ts mutant of A59, LA7. MHV2 does not cause fusion and A59 does. The virus was harvested from cells co-infected with MHV2 and LA7 at 39°C. After three serial passages, viruses were isolated by plaque assays at 39° on L2 cells. The fusion (+) viruses were isolated and further purified by plaque assays at least 4 times before biochemical studies.

The third method was used for the isolation of recombinants between wild type JHM and ts mutants of A59 by use of neutralizing monoclonal antibodies. The viruses harvested from the coinfected cells at 39°C were treated with a combination of two neutralizing monoclonal antibodies, J.7.2 and J.2.2, specific for the peplomer protein of JHM. The amount of antibodies used had previously been determined to be sufficient to reduce the virus titer by 4 $\log_{10}$ units. The surviving virus was then isolated by plaque assays at 39°C.

Two-dimensional fingerprinting analysis

Oligonucleotide fingerprinting by two-dimensional polyacrylamide gel electrophoresis was done as previously described (Lai et al., 1981).

Results

Recombinants with multiple cross-overs

Previous studies in our laboratory have demonstrated that RNA recombinants could be detected during mixed infections between two ts mutants of MHV (Lai et al., 1985) or between a ts mutant and a wild-type MHV (Makino et al., 1986). All of the recombinants isolated contain single cross-overs clustered within the 5'-half of the RNA genome.

To further understand the mechanism of RNA recombination, we performed additional crosses between different pairs of ts mutants, Wild-type (non-ts) viruses were isolated from these crosses and screened by two-dimensional oligonucleotide fingerprinting of their mRNA 7 isolated from infected cells. The rationale of such an approach has previously been described (Makino et al., 1986). We were able to identify recombinants by determining the parental origins of the oligonucleotides in mRNA7. Since the leader and mRNA 7 sequences are derived from the opposite ends of the genomic RNA, any recombinants with an odd-number of cross-overs will generate a hybrid mRNA 7, with the leader RNA and the body sequences being derived from different parental viruses. In the cases of viruses with non-hybrid mRNA 7, we further examined the faint oligonucleotides derived from contaminating degraded larger mRNA species, which are sometimes present in the oligonucleotide fingerprints, for the presence of oligonucleotides derived from the opposite parent. These recombinants would have an even number of cross-overs. By using these two approaches, we have isolated recombinants arising from both single and double cross-over events.

From several crosses involving ts mutants, we have obtained several recombinant viruses with multiple cross-overs. One of these double recombinants is D5, which was derived from the cross between ts mutant LA10 of A59 and Wild-type JHM. The fingerprint of the genomic RNA of this virus contains mostly A59 oligonucleotides but is missing two internal A59-specific oligonucleotides, 16 and 4 (Fig. 1). Instead, it contains two new oligonucleotides corresponding to the JHM-specific oligonucleotides 14 and 11. Previous data indicate that these JHM oligonucleotides are located in the same region of the gene A as that of the missing A59 spots (Lai et al., 1981; Makino et al., 1984). Thus, the isolate D5 contains a double cross-over within gene A.

The second double recombinant, A5, was derived from the cross between ts mutant LA8 of A59 and ts mutant 203 of JHM. This recombinant has a mRNA 7 which consists of an A59 leader-specific oligonucleotide 10 (Lai et al., 1983), a JHM-specific leader-body junction oligonucleotide 8a (Makino et al., 1984) and A59-specific body sequences (Fig. 2A). The identity of these leader-specific oligonucleotides has been confirmed by base sequence analysis (data not shown). The genomic RNA of A5 has an oligonucleotide fingerprint which is almost identical to the parental A59 virus (Fig. 2B). No JHM-specific oligonucleotides could be identified. Thus, A5 contains a double cross-over, with part of the leader region and the majority of the genomic sequences being derived from the parental A59 strain while the 3'-half of the leader sequences originates from the JHM strain. The cross-over point of this recombinant has been determined by primer extension studies to be localized between nucleotides 35 and 60 from the 5'-end of the genome, within the leader region (data not shown). It is interesting that another recombinant A1 derived from the same cross has a cross-over point at the same region of the leader RNA. This recombinant, A1, has a mRNA 7 which contains the JHM-specific leader oligonucleotide 8, the A59-specific

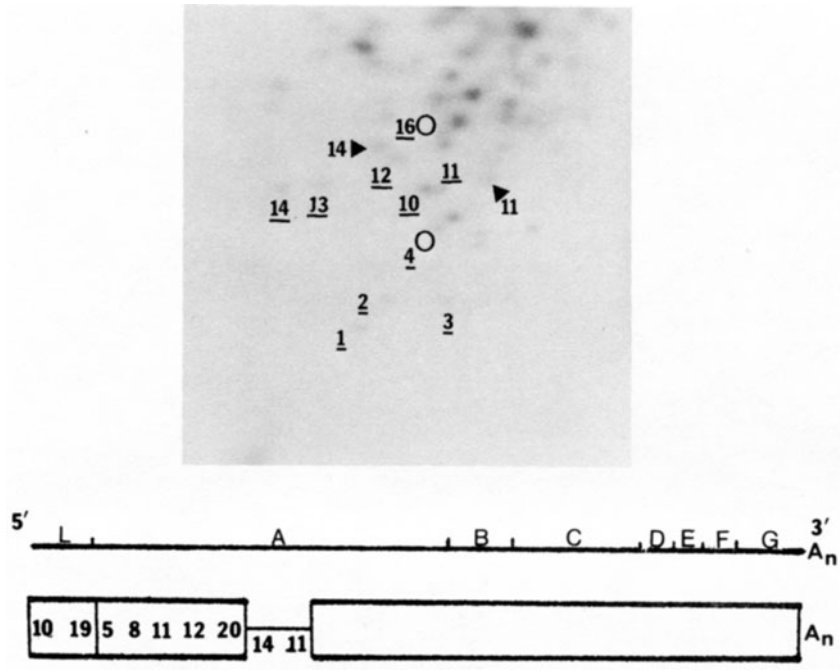

Figure 1. Oligonucleotide fingerprint of genomic RNA of isolate D5. The diagram represents the cross-over locations. The boxed sequences are A59 specific. The map is not drawn to scale.

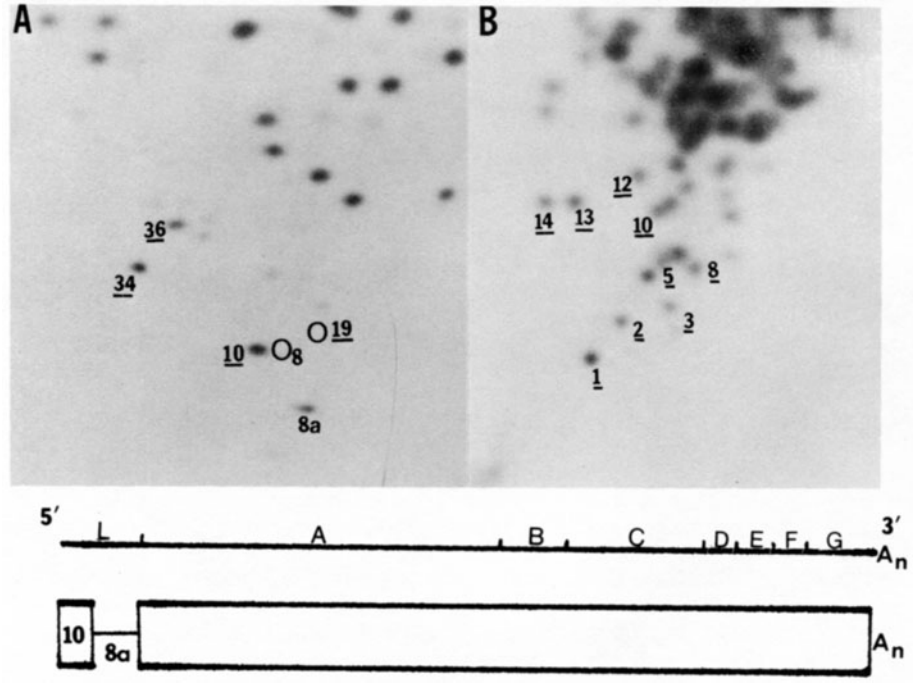

Figure 2. Oligonucleotide fingerprints of virus-specific RNA of isolate A5. A) mRNA 7; B) genome. The diagram represents the cross-over locations. The boxed sequences are A59 specific. The map is not drawn to scale.

leader-body junction oligonucleotide 19 and A59-specific body sequences (Fig. 3A). The genomic RNA fingerprint of A1 showed that it was almost identical to the parental A59, except that the A59-specific leader oligonucleotide 10 was replaced with the JHM-specific oligonucleotide 8 (Fig. 3B). Thus, A1 contains a single cross-over located in the same region within the leader region as in A5.

These studies indicate that multiple recombination can take place in the same virus genome.

Recombinants between MHV-2 and A59

The recombinants obtained by the previous approach have cross-overs localized mainly in 5'-end genes. To determine whether it is possible to have cross-overs in the 3'-end of genome, we studied the cross between MHV-2 and A59. A59 causes cell-cell fusion, while MHV-2 does not. It has been shown that induction of cell fusion is a property of E2 peplomer protein, which is the gene product of gene C. We therefore used cell-fusion as one of the selection markers for recombinants.

MHV-2 and ts mutant LA7 of A59 were used to co-infect L2 cells at an m.o.i. of approximately five at 39°C. The virus harvested were assayed by plaque formation at 39°C. Only the fusion (+) viruses were isolated, which

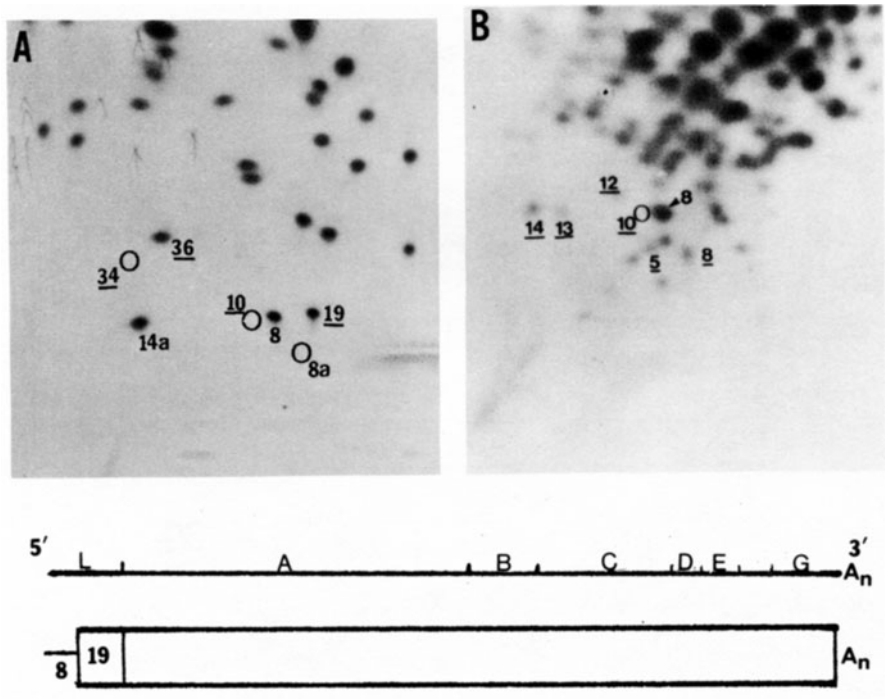

Figure 3. Oligonucleotide fingerprints of mRNA 7(A) and genomic RNA(B) of isolate A1. The diagram represents the cross-over location. The boxed sequences are A59 specific. The map is not drawn to scale.

should represent either A59 ts revertants or recombinants. The virus isolates were further plaque-purified four times before biochemical studies. During the course of plaque purification, two of the isolates, M3 and M11, were converted to fusion (-) phenotype. We have studied five of the virus isolates, three of which are fusion (+), by oligonucleotide fingerprinting of genomic RNA and subgenomic mRNAs. Their genetic maps were constructed by comparing the oligonucleotide maps of recombinants with their parental viruses (Fig.4). All of the isolates were recombinants with at least one cross-over. It is noted that the two fusion (-) recombinants contain gene C derived from MHV-2, while all of the three fusion (+) recombinants contain at least some of gene C from A59. This result is consistent with the notion that gene C is responsible for fusion activity of the virus.

Several recombinants contain multiple cross-overs. The most notable points of cross-over are those at the 3'-end and 5'-end of the genome. The cross-over at the 3'-end gene, which encodes the nucleoprotein gene, was not specifically selected. The fact that almost half of the recombinants have a cross-over at this location suggests that this is a recombinational hot spot. The 5'-end cross-over site in M10 appears to be very close to the leader termination site. This cross-over might be generated by a free leader RNA species utilized in RNA replication. Finally, all of the recombinants have an MHV-2 leader sequence, suggesting that this leader sequence might confer a growth advantage.

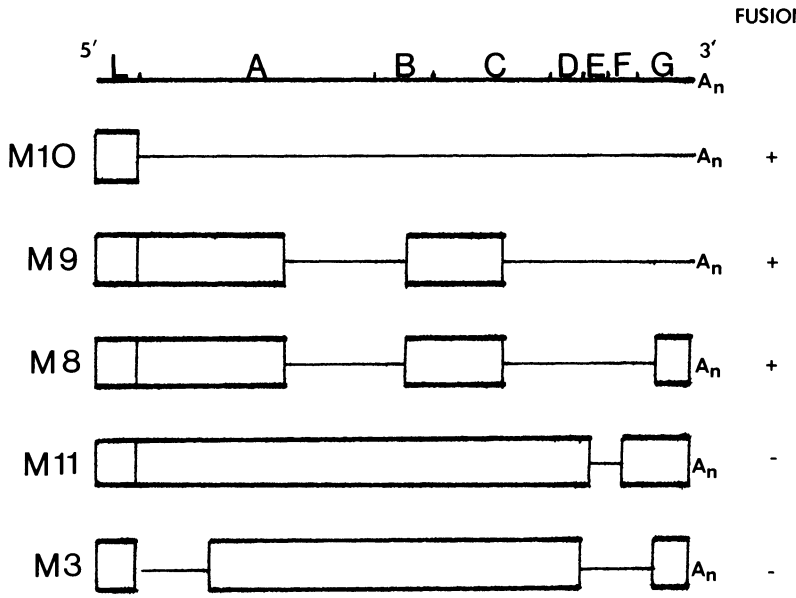

Figure 4. Schematic representation of the oligonucleotide maps of MHV-2 x A59 recombinants. The genomic sequences represented by boxes are MHV2 specific. The map is not drawn to scale.

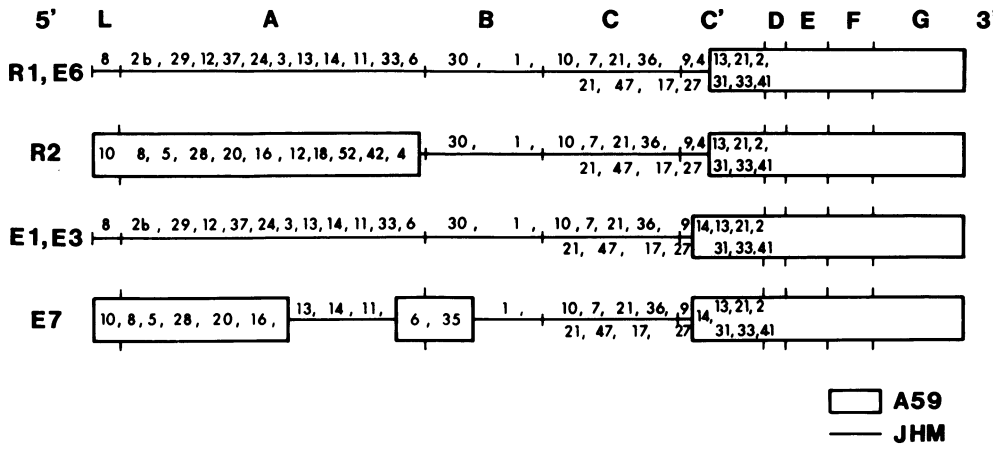

Figure 5. Schematic representation of the oligonucleotide maps of recombinants between A59 and JHM by use of neutralizing monoclonal antibodies. The map is not to scale.

Selection of Recombinants between A59 and JHM by use of neutralizing monoclonal antibodies

We have further devised a protocol taking advantage of neutralizing monoclonal antibodies specific for E2 peplomer protein, to select recombinants with cross-overs within gene C. The wild-type JHM virus and ts mutants of A59, LA7 or LA12, were used to co-infect DBT cells. The harvested virus was treated with monoclonal antibodies specific for E2 protein of JHM. The surviving viruses were isolated by plaque assays, and analyzed by oligonucleotide fingerprinting of genomic and subgenomic mRNAs. Their genetic map was summarized in Fig. 5. Four types of recombinants were obtained. Interestingly, all four types of recombinants have cross-overs within gene C. About one third of the gene at 3'-end was derived from A59, while the rest was derived from JHM. All of these recombinants fail to bind the JHM monoclonal antibodies used for selection. This result suggests that the antigenic sites for these neutralizing monoclonal antibodies are localized at the carboxyl terminal one third of the peplomer protein. Some of the recombinants have additional cross-over sites which were not selected for. This result further confirms the high frequency of RNA recombination.

Discussion

Previous reports on coronavirus recombinants have demonstrated a high frequency of recombination between two strains of MHV (Lai et al., 1985; Makino et al., 1986). All of these recombinants consist of single cross-overs in the coding region of the RNA genome. The data presented in this report show additional types of murine coronavirus recombinants. We have demonstrated that recombination can occur within the leader region itself. We have found recombination at multiple sites on a single viral genome, suggesting that it is possible to have double and multiple crossover events during replication. Furthermore, we demonstrated that recombination is not restricted to the 5'-end of the viral genome. These new types of recombinants further support the high frequency of coronavirus RNA recombination. Particularly revealing is the

cross-overs observed in many recombinants in a region where no selection pressure was applied. These results are best interpreted as suggesting the involvement of free segmented RNA intermediates in coronavirus RNA replication. These intermediates could be the results of discontinuous RNA transcription, which pauses at the sites of secondary structure on the RNA template. These free intermediates could participate in RNA recombination during mixed infection. The recombinants with cross-overs within the leader region are consistent with this interpretation. The site of recombination in these recombinants corresponds to a potential hairpin loop structure within the leader sequence. Three leader-containing RNA intermediates between 35 and 60 nucleotides long have also been detected in MHV-infected cells (Baric et al., this volume). These data suggest that RNA intermediates generated at the regions of hairpin loops on the RNA genome could be the precursor to RNA recombination.

The availability of RNA recombinants provides a powerful genetic tool to study the gene functions and pathogenesis of coronaviruses. One of such approaches is illustrated by the recombinants obtained by using neutralizing monoclonal antibodies. The genetic structure of these recombinants indicates that the neutralization epitopes and neuropathogenic determinants are mapped within the carboxyl end one-third of the peplomer proteins. This finding is surprising since the carboxyl end of the peplomer protein contains the membrane-anchoring domain of the protein. Thus, the coronavirus peplomer protein probably contains an unusual conformation. The recombinants between MHV-2 and A59 also confirmed that the fusion-inducing ability of the virus resides in the gene encoding the peplomer protein. These recombinants would be particularly useful for studies of roles of various viral genes in viral pathogenesis.

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