In Vivo RNA-RNA Recombination of Coronavirus in Mouse Brain

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RNA-RNA recombination between different strains of the murine coronavirus mouse hepatitis virus (MHV) occurs at a very high frequency in tissue culture. To demonstrate that RNA recombination may play a role in the evolution and pathogenesis of coronaviruses, we sought to determine whether MHV recombination could occur during replication in the animal host of the virus. By using two selectable markers, i.e., temperature sensitivity and monoclonal antibody neutralization, we isolated several recombinant viruses from the brains of mice infected with two different strains of MHV. The recombination frequency was very high, and recombination occurred at multiple sites on the viral RNA genome. This finding suggests that RNA-RNA recombination may play a significant role in natural evolution and neuropathogenesis of coronaviruses.

RNA-RNA recombination between RNA viruses has been recognized as an increasingly frequent event *in vitro*. Picornavirus (12), coronavirus (14), and, more recently, brome mosaic virus (3) have been found to undergo RNA recombination at various frequencies. Coronavirus provides a particularly striking example of RNA recombination, since the recombination frequency between two coronaviruses can be extremely high, approaching 10% in some cases (21). Many recombinant coronaviruses of various genetic structures, resulting from multiple crossovers between different coronavirus strains, have been isolated (10, 14, 20, 21). Although most of the RNA recombination studies thus far have been performed with tissue culture, RNA recombination has also been demonstrated in the natural host of picornaviruses. For instance, one case of poliovirus vaccine-associated poliomyelitis in a human host has been shown to be caused by recombination between two poliovirus vaccine strains (11). More recently, poliovirus recombinants were also observed in a child immunized with Sabin vaccines (23). Since coronavirus RNA recombination occurs readily *in vitro*, we sought to determine whether coronaviruses could also recombine *in vivo*. Our study indicates that such recombination does occur at a very high frequency in the central nervous systems (CNS) of infected mice. Since coronaviruses cause demyelination in animals (5, 27) and may be associated with multiple sclerosis (4), *in vivo* RNA recombination may have important implications in coronavirus neurovirulence.

Coronaviruses are enveloped RNA viruses that infect many vertebrates. These viruses are responsible for more than 20% of common colds in humans (19) and cause diseases involving the respiratory and gastrointestinal tracts of livestock (19). They also cause hepatitis and CNS demyelination in rodents (5, 27). The prototype coronavirus, mouse hepatitis virus (MHV), has a single-stranded RNA genome of positive sense, with a molecular weight of more than 5×10^6 to 6×10^6 (17, 26). In virus-infected cells, six subgenomic mRNAs, each containing an identical 72-nucleotide leader sequence, are synthesized (13, 15, 24). The leader sequence is derived from the 5' end of the genome and added to the mRNAs by a unique mechanism of leader

RNA-primed transcription (2). Workers in our laboratory have further suggested that the replication of coronavirus RNA proceeds in a discontinuous and nonprocessive manner, leading to a high frequency of RNA recombination, probably via a copy choice mechanism (1, 21).

To determine whether RNA-RNA recombination of coronavirus is a phenomenon unique to tissue culture or may occur in the natural host of the virus, we investigated whether RNA recombinants could be isolated from the CNS of mice simultaneously infected with two different strains of MHV. This approach was taken because the neuropathogenicity of MHV is the most extensively studied biological property of the virus. Five-week-old C57BL/6J mice were inoculated intracerebrally in opposite hemispheres with either the JHM-DL strain (25) or a temperature-sensitive (*ts*) mutant, LA9 (J. Egbert, unpublished observation), of the A59 strain of MHV. This *ts* mutant was chosen because preliminary studies showed that it would grow to a significant titer in mouse brain, thus enhancing chances for recombination. A total of 1.1×10^5 PFU of JHM-DL and 2.8×10^5 PFU of LA9 were inoculated per mouse. Two days postinfection, the mice that had received JHM-DL alone or both JHM-DL and LA9 appeared dehydrated and showed signs of mild encephalitis. In contrast, the mice which had received only LA9 appeared normal. Mice were sacrificed, the brains were removed aseptically and manually disrupted in Tenbrock homogenizers after the addition of 2 ml of phosphate-buffered saline, and the viruses present in the brains were analyzed by plaque assays. Homogenates were centrifuged at $800 \times g$ for 5 min to remove cell debris, and the supernatant was analyzed by plaque assays on L2 cells. Assay mixtures were incubated at 35°C (permissive temperature) to determine total virus yields. Mixtures for duplicate assays were incubated at 39°C (nonpermissive temperature) to determine the titer of the parental JHM-DL. The virus was also incubated with neutralizing monoclonal antibodies J.2.2 and J.7.2 (6) specific for JHM-DL and then assayed at 39°C to detect the possible recombinants. Previous studies have shown that these antibodies, when used in conjunction, reduce JHM-DL titers by approximately 8 log₁₀ units (6). Since the growth of *ts* mutant LA9 is inhibited at 39°C, this last assay would detect recombinants between JHM-DL and LA9, possible revertants of LA9, or residual nonneutralized JHM-DL. All isolates described were obtained from widely

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TABLE 1. Plaque assay of viruses harvested from brain homogenate under different conditions

Viruses inoculated	Virus titer (PFU/ml) ^a at:		
	35°C	39°C	39°C + MAb
JHM-DL	5.5×10^4	6.7×10^4	1.4×10^1
A59-LA9	1.2×10^4	5.9×10^2	3.9×10^2
JHM-DL + A59-LA9	2.3×10^5	2.1×10^4	1.1×10^4

^a The recombination frequency was determined by using the following equation: recombination frequency = $100 \times \{ \text{titer of mix population (MAb) at } 39^\circ\text{C} - [\text{JHM-DL (MAb) titer at } 39^\circ\text{C} + \text{LA9 (MAb) titer at } 39^\circ\text{C}] / \text{titer of mix population at } 35^\circ\text{C} \}$.

separated plaques to minimize possible in vitro recombination.

Table 1 shows the results of plaque assays of the virus obtained from mice infected with JHM-DL or LA9 only and those infected with both viruses. The JHM-DL titer from brain tissue was reduced by more than 3 log₁₀ units by the antibody treatment, suggesting that these antibodies could effectively remove most, but not all, of the parental JHM-DL virus. The presence of a low level of surviving viruses was probably caused by the inhibition of monoclonal antibodies by the brain homogenate. The titer of LA9 was not affected by the antibody treatment and was lower by approximately 2 log₁₀ units at 39°C than at 35°C. The relatively poor retention of the *ts* phenotype suggests a higher reversion frequency during in vivo replication than was evident during in vitro replication. The virus harvested from mice inoculated with both viruses showed a significantly higher titer at 39°C after the antibody treatment than did the virus obtained from the single infections, suggesting that RNA recombination has occurred during the mixed infection. The recombination frequency could be calculated from these data to be roughly 4.6%.

To provide biochemical evidence that MHV RNA recombination indeed had occurred in vivo, we subcultured 20 isolated plaques from monoclonal antibody-treated brain homogenates incubated at 39°C. Each isolate was propagated without further plaque purification to help ensure that no RNA recombination took place in vitro. To screen for possible RNA recombinants, the intracellular ³²P_i-labeled RNA from cells infected with different virus isolates was separated by agarose gel electrophoresis (21). The smallest mRNA, mRNA 7, was eluted, digested with RNase T₁, and separated by one-dimensional polyacrylamide gel electrophoresis (oligonucleotide fingerprinting) as follows. DBT cells, a mouse brain tumor cell line (7), were infected for 1 h at 37°C with various virus isolates derived from the brain homogenates of mix-infected mice at a multiplicity of infection of 1 to 10. After virus adsorption, the cells were incubated in phosphate-free medium for 3 h. At 2 h postinfection (p.i.), actinomycin D (2.5 µg/ml) was added to the medium. At 3 h p.i., ³²P_i was added to a final concentration of 200 µCi/ml. Intracellular RNA was extracted at 7 to 8 h p.i. For RNA extraction, the cell monolayers were chilled on ice and washed with phosphate-buffered saline. The cells were lysed in NTE (0.01 M Tris hydrochloride [pH 7.4], 0.1 M NaCl, 0.001 M EDTA) containing 0.5% Nonidet P-40. The nuclei were removed by centrifugation at $2,000 \times g$ for 5 min. The supernatant was adjusted to contain 1% sodium dodecyl sulfate and extracted with phenol-chloroform. The RNA was then precipitated at -20°C overnight. The intracellular RNA was suspended and electrophoresed on a 1% agarose gel containing 6 M urea (21). After electrophoresis,

the gel was exposed to a Kodak XAR-5 film. The RNA band corresponding to mRNA 7 was extracted with butanol (18). The RNA was treated with RNase T₁ and loaded onto 20% polyacrylamide gels made in Tris-borate buffer (pH 8.0), which is equivalent to the second dimension of the standard two-dimensional oligonucleotide fingerprinting procedure (15, 22). The rationale for this approach is that mRNA 7 is derived from the extreme 3' end of the RNA genome (15), but also contains a leader sequence derived from the 5' end (16). Thus, any RNA recombinant with an odd number of crossovers should have an mRNA 7 consisting of sequences derived from the different parental viruses. Figure 1 shows such an analysis. The oligonucleotides A19 and J8a represent the leader sequences of A59 and JHM-DL, respectively (16, 22). The remaining oligonucleotides are derived from the coding regions of mRNA 7 (21). The majority of virus isolates have a fingerprint pattern identical to that of either LA9 or JHM-DL. However, two viruses, IL-17 and IL-27, contained a JHM-DL-specific leader oligonucleotide and A59-specific mRNA 7 coding sequences. Therefore, these two viruses are most probably recombinant viruses. Using this technique, we could not rule out the possibility that

FIG. 1. One-dimensional oligonucleotide fingerprints of mRNA 7 of various virus isolates. The fingerprinting was carried out as described in the text. The oligonucleotides A19 and J8a are the A59- and JHM-DL-specific leader oligonucleotides, respectively. The oligonucleotides A10 and J8 represent additional leader-specific oligonucleotides which cannot be separated in this one-dimensional fingerprint (21). The remaining oligonucleotides are derived from the body (coding region) of mRNA 7. The different lanes represent different virus isolates. The oligonucleotides marked with A were derived from A59, and those marked with J were from JHM-DL. The fingerprints of IL-1, IL-6, IL-8, IL-13, IL-14, IL-18, and IL-21 are identical to that of A59 (21). The band indicated by an open triangle in these viruses probably represents contamination from larger mRNAs. The fingerprints of IL-5, IL-7, and IL-11 are identical to that of JHM-DL (4). IL-17 and IL-27 appear to be recombinants.

FIG. 2. Two-dimensional oligonucleotide fingerprints of genomic RNAs of recombinants and their parental viruses. The fingerprinting was carried out as described in the text. Schematic drawings of the fingerprints of the two recombinants are included. The numbering of oligonucleotides are as described for A59 (15) and JHM-DL (22).

some of the remaining viruses are also recombinants with an even number of crossovers.

To determine the recombination sites of IL-27 and IL-17 and to examine whether other isolates are recombinants with double crossovers, we studied several viruses by two-dimensional oligonucleotide fingerprinting of their genomic RNAs. DBT cells (7) were infected with different viruses at 37°C. At 5 h p.i., the medium was removed and replaced with phosphate-free medium containing $^{32}\text{P}_i$ (250 $\mu\text{Ci/ml}$). At 14 h p.i., the medium was removed and cleared of cell debris by centrifugation at $2,000 \times g$ for 10 min at 4°C. Virus was purified from the medium by sedimentation at 27,000 rpm for 4 h in an SW28 rotor at 4°C on linear 20 to 42% (wt/wt) sucrose gradients made in NTE. The virus was collected and pelleted through a 20% sucrose cushion for 4 h at 27,000 rpm in an SW28 rotor at 4°C. RNA was extracted from the pelleted virus by using phenol-chloroform, separated by agarose gel electrophoresis, and eluted as described previously (18). The virion RNA was exhaustively digested with RNase T_1 and analyzed by two-dimensional polyacrylamide gel electrophoresis (15, 21). Figure 2 shows the analysis of two viruses, IL-27 and IL-6, which clearly contain oligonucleotides derived from both A59 and JHM-DL. The crossover sites are shown in Fig. 3. It is noteworthy that the presence of recombination was established only when at least two contiguous oligonucleotides from one parent were

present or missing. Thus, the genetic changes in these viruses are probably not due to mutations. It is evident that IL-27 contained three crossovers, whereas IL-6 contained two crossovers. The finding that both the leader region and the mRNA 7 body sequences of IL-6 were derived from A59 accounts for the finding that the mRNA 7 of IL-6 was identical to that of A59 (Fig. 1). Several other viruses, including IL-1, IL-8, IL-13, and IL-14, were also examined. All contained two crossovers (data not shown), which accounts for the observations that they synthesize an mRNA 7 indistinguishable from that of A59. The recombination sites of these additional viruses have not yet been precisely determined. In addition, IL-17 was found to be a mixture of

FIG. 3. Schematic diagram of the genetic map of the recombinants. The boxed areas represent A59-derived sequences. A through G represent the seven genes identified for MHV (15). L represents the leader sequence (13). Numbers represent the oligonucleotides identified. Maps are not drawn to scale. The map locations of the oligonucleotides were derived from the previous studies (15, 22).

two recombinants, both of which had a single crossover. They are probably also *in vivo* recombinants, since no parental A59 or JHM viruses could be detected in the genomic fingerprint of the IL-17 virus. We have also studied several viruses, for example, IL-7, which have a JHM-DL-like mRNA 7. They appear to be identical to the parental JHM-DL, and are therefore most probably JHM viruses which have escaped neutralization by monoclonal antibodies. The finding that most of the viruses studied biochemically are recombinants suggests that the recombination frequency *in vivo* is very high, similar to our previous *in vitro* studies (21).

This study clearly indicates that RNA recombination can take place at a high frequency during *in vivo* replication. It is significant that recombination was demonstrated during coronavirus replication in the CNS, since some MHV strains induce encephalitis and CNS demyelination (5, 27). Thus, RNA recombination may play a role in the neuropathogenesis and persistence of coronaviruses in the CNS. In this regard, it has been recently shown that two avirulent herpes simplex virus strains could recombine to generate virulent neurotropic viruses (9). It is conceivable that RNA recombination may also contribute to neuropathogenesis of coronaviruses in a similar manner. This study and the isolation of recombinant poliovirus vaccine strains in humans (11, 23) suggest that RNA recombination may also play a significant role in the natural evolution of RNA viruses, which were thought to evolve mainly by base substitutions (8).

The frequency of coronavirus recombination *in vivo* appeared to be as high as that *in vitro*, despite the procedure used in this study, in which the two parental viruses were inoculated into different hemispheres of the brain, instead of being coinoculated into the same area. This fact, as well as the observations that several recombinants contain multiple genetic crossovers, further suggests that the *in vivo* RNA-RNA recombination frequency is very high.

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