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## Random Nature of Coronavirus RNA Recombination in the Absence of Selection Pressure

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RNA–RNA recombination is thought to occur preferentially at certain selected sites and in only a few RNA viruses; the mechanism for these restrictions is unknown. In this paper we report the development of a recombination assay for coronavirus, using polymerase chain reaction, in the absence of selection pressure. Our results showed that within a 1-kb region of the peplomer gene, RNA recombination occurred at almost every potential crossover site. Thus, coronavirus RNA recombination appears to be more random than previously realized. However, after serial passages of the recombinant viruses in tissue culture, the recombination sites among the progeny viruses became clustered in the region which contains the previously reported "hot spot" for coronavirus recombination. These results suggest that RNA recombination is common and random in nature, but only certain recombinants can be selected. Thus, the presence of recombinational "hot spots" for coronavirus or other RNA viruses most likely resulted from selection of certain recombinant viruses and not restriction on the occurrence of RNA recombination. The failure to detect recombinants in other RNA viruses may therefore be due to unfavorable properties of recombinant viruses. This approach can be used to detect recombinants in these viruses. © 1991 Academic Press, Inc.

The ability to exchange genetic information may allow RNA viruses to adapt to a changing environment and to overcome potential deleterious effects caused by the high error frequency of the RNA polymerase. Viruses with segmented genomes can undergo RNA reassortment. However, the ability of RNA viruses with nonsegmented genomes to exchange genetic elements is more limited. Only a few RNA viruses, including picornaviruses, coronaviruses, and a few plant viruses have been shown to undergo RNA–RNA recombination at various efficiencies (1–7). The failure of many other RNA viruses and RNA phages to recombine has been well documented (8, 9), although recent studies have identified additional viruses which undergo nonhomologous RNA recombination under special circumstances (10, 11). Nevertheless, homologous RNA recombination remains rare despite the fact that nonhomologous RNA rearrangement, in the form of defective-interfering (DI) RNA, has been widely demonstrated for most RNA viruses.

The attempted isolation of recombinants in RNA viruses has usually been carried out by applying certain selection pressures and assaying the progeny virus harvested. This approach most likely detects only those recombinant viruses which do not lose their survival fitness under the selection pressures. By this approach, it has been shown that foot-and-mouth disease virus (FMDV) recombines in most genetic regions

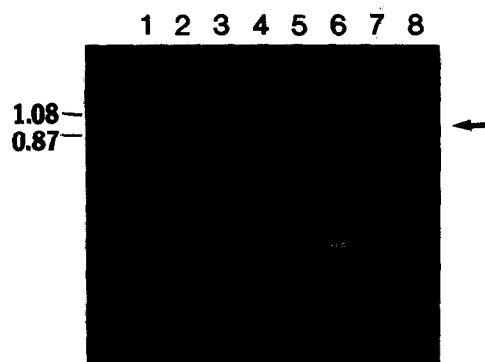
except the viral capsid protein VP1 and VP3 genes (1). Similarly, mouse hepatitis virus (MHV) and poliovirus have some apparent recombinational hot spots (12, 13). Conceivably, the limitation and clustering of recombination sites in these viruses and the failure to detect recombinants in other RNA viruses could very well be the result of selection for or against certain recombinants, and may not necessarily reflect the actual mechanism of RNA recombination or the lack thereof. To examine this issue, we initiated a study of RNA recombination in coronavirus, in the absence of selection pressure. Surprisingly, we found that the initial recombination events within a 1-kb region of the peplomer gene were almost entirely random. However, after only a few passages, the region in which recombination could be detected became restricted, such that the majority of crossover sites were localized to a small area of the region examined. Eventually, all the recombinants became undetectable. Our results suggest that RNA recombination is more common than previously recognized and that the failure to detect recombinants in certain RNA viruses may be due to a negative selection against recombinant viruses.

MHV, a prototypic member of the Coronaviridae, was used as a model system for the study of RNA recombination. MHV contains a positive-sense RNA genome of 31 kb (14, 15), which has been shown to undergo recombination both *in vitro* (4, 5, 17, 18) and *in vivo* (16) at a frequency approaching 25% for the entire genome (19). To study RNA recombination in the absence of selection pressure, we developed a polymer-

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ase chain reaction (PCR) assay using two primers specific for the potential recombinant viruses which have a crossover site between the two primers. We first attempted to detect recombinant RNA among the intracellular viral RNAs in cells coinfecting with two MHV strains. DBT cells, a murine astrocytoma cell line (20), were coinfecting with the A59 and JHM-DL (21) strains of MHV at a multiplicity of infection (m.o.i.) of 5. At 7 hr postinfection, cells were lysed with 0.5% NP-40; total RNA was extracted and treated with proteinase K, and polyA-containing RNA was selected. We chose the peplomer (S) gene for this study, since several recombinants isolated using selection markers have been shown to contain crossover sites within this gene (12). Two synthetic oligonucleotides specific for the recombinants were used as primers. A JHM-DL-specific primer (5'-CGCGATCCGTGCACATCCAAGG-3'), which bound to position 1554–1575 from the 5'-end of the peplomer gene, was used for first-strand cDNA synthesis. This primer corresponds to a region which is deleted in A59 (12, 22) and, thus, does not bind to A59. The cDNA was then used as template for amplification by PCR, along with an A59-specific second primer (5'-GGACTGAGCTCCGCTTAATGTTAATGGCTGATGCAT-3'), which bound at position 581–604 of the peplomer gene. Seven of eight nucleotides at the 3'-end of this primer differ from the corresponding region of JHM-DL. The region between these two primers spanned 950 nt and included a hypervariable area which is subject to frequent deletions (12, 22), and a previously identified recombinational "hot spot" between nucleotides 1148 and 1426 (12). Only recombinant RNAs which had a crossover between the two primers and contained A59-specific sequences on the 5'-side and JHM-DL-specific sequences on the 3'-side could be detected by this PCR approach.

PCR conditions were chosen so that these two primers would bind only to recombinant RNAs and would not generate nonspecific PCR products by reverse transcriptase or Taq polymerase jumping. RNA was amplified by a slight modification of the procedure of Fugue *et al.* (23). Briefly, 1  $\mu$ g of RNA was mixed with 50 ng of the two appropriate primers in a 100- $\mu$ l reaction mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.01% gelatin, 500  $\mu$ M each of the four deoxyribonucleoside triphosphates, and 1  $\mu$ g tRNA as a nonspecific competitor. The RNA was denatured for 2 min at 94° and chilled quickly on ice. Two units of AMV reverse transcriptase (Seikagaku, Rockville, MD) was added and the reaction incubated for 30 min at 42°. After the addition of 2 units of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), the resulting cDNA was amplified for 35 cycles, with each



**Fig. 1.** Detection of recombinant RNA using PCR. Lane 1: Detection of the parental A59 viral RNA in coinfecting cells. An 870-nt fragment was amplified using primer 196 (5'-ATATCAAGCTTGAGCACACGGGCAATAAC-3'), which bound to nucleotides 1430–1439 from the 5'-end of the peplomer gene, and primer 152, which bound to nucleotides 581–604. Both primers were specific for A59. Lane 2: Detection of the parental JHM-DL viral RNA in coinfecting cells. A 762-nt fragment was amplified using primer S2 (5'-CCCCTCGTCTTGAATAG-3'), which bound to nucleotides 1326–1343 from the 5'-end of the peplomer gene, and primer 178 (5'-GGACTGAGCTCCGTTAATGTTAATGCCCTTGGC-3'), which binds at nucleotide 581–604. Both primers were specific for JHM-DL. Lane 3: A59 RNA from a single infection was used as template for PCR with primers 109, which bound to nucleotides 1554–1575, and 152, which were specific for recombinants. Lane 4: JHM-DL RNA was used as template for PCR with primers 109 and 152. Lane 5: Equal amounts (0.5  $\mu$ g) of A59 and JHM-DL RNA were mixed together and amplified by PCR with primers 109 and 152. Lane 6: Intracellular RNA from coinfecting cells was amplified using primers 109 and 152. The lower 800-nt band was caused by incorrect binding of primer 109 at nucleotide 1401 of the S gene on the A59 RNA. Lane 7: Same as lane 5, except that the RNA mixture was from purified virions derived from single infections. Lane 8: Same as lane 6, except that purified virion RNA released from the coinfecting cells was used.

cycle consisting of 94° for 1'30", 58° for 2', and 72° for 3'.

To confirm that the two primers were specific for the recombinants, each virus was grown separately, and the RNA extracted and amplified by PCR using the pair of primers described. No product of expected size (950 bp) was detected (Fig. 1, lanes 3 and 4). However, when RNA from the coinfecting cells was used, a specific product of 950 bp (Fig. 1, lane 6), most likely derived from the recombinant RNA, was detected. Several smaller bands were observed, but these represented nonspecific PCR products since they also were seen in control lanes. The presence of each parental RNA in the coinfecting cells was confirmed by using different primer pairs specific for each parent (Fig. 1, lanes 1 and 2). To rule out the possibility that this PCR product was generated by transcriptional jumping of reverse transcriptase or Taq polymerase, RNA from separate A59 and JHM-DL infections was mixed to-

gether in equal amounts such that the total amount equaled that of the RNA from the coinfection. When the RNA mixture was used as a template for reverse transcription and PCR, the specific 950-bp product was not detected (Fig. 1, lane 5). Thus, the PCR product present in lane 6 (Fig. 1) resulted from recombination events which occurred during the coinfection. To examine the possible sites of recombination, the PCR product was purified from low-melt agarose and directionally cloned into the vector pTZ18U (U.S. Biochemicals, Cleveland, OH) through a *SacI* restriction site present on the 5'-end primer and *SmaI* on the 3'-side. DNA sequence analysis of 35 cloned PCR products showed that the crossover sites were almost randomly distributed throughout the nearly 1-kb region of the peplomer gene studied (Fig. 2A). Two additional recombinants had triple crossovers; one had three crossovers which occurred within a span of 98 nt (from 986 to 1084) and the other within 249 nt (from 897 to 1146). One recombinant RNA had a 30-nt deletion, with each parental sequence flanking the deletion (Fig. 3). It is noteworthy that the deleted site was flanked by a 6-nt repeat, but only one copy of the repeat was retained in the recombinant. The structure of this recombinant was consistent with the interpretation that RNA recombination occurs by a copy-choice mechanism as a result of polymerase jumping during transcription.

We next sought to determine whether there were any selection advantages or disadvantages for certain recombination sites once the RNA was packaged into the virion. Cells were coinfecting again with the same virus strains used above. After incubation for 13 hr, the medium containing released virus particles was harvested and the virions were pelleted through a 30% sucrose cushion at 26K in a Beckman SW 28 rotor for 3.5 hr. Viral genomic RNA isolated by proteinase K treatment and phenol/chloroform extraction was used as a template for PCR using the same primers described above. Upon applying the same controls as before, the 950-bp recombinant product was generated only from the viral RNA derived from coinfection (Fig. 1, lane 8). The recombinant PCR product was cloned into pTZ18U and DNA from individual clones was sequenced. Analysis of 53 recombinant clones revealed that, similar to the intracellular recombinants, the crossover sites in the viral recombinant RNAs were almost randomly distributed over the 1-kb region of the peplomer gene (Fig. 2B). No triple crossovers or deletions were detected.

We then determined whether the recombinant RNA population could be altered by passage in tissue culture. DBT cells were infected with the virus harvested from the coinfecting cells as described above. The su-

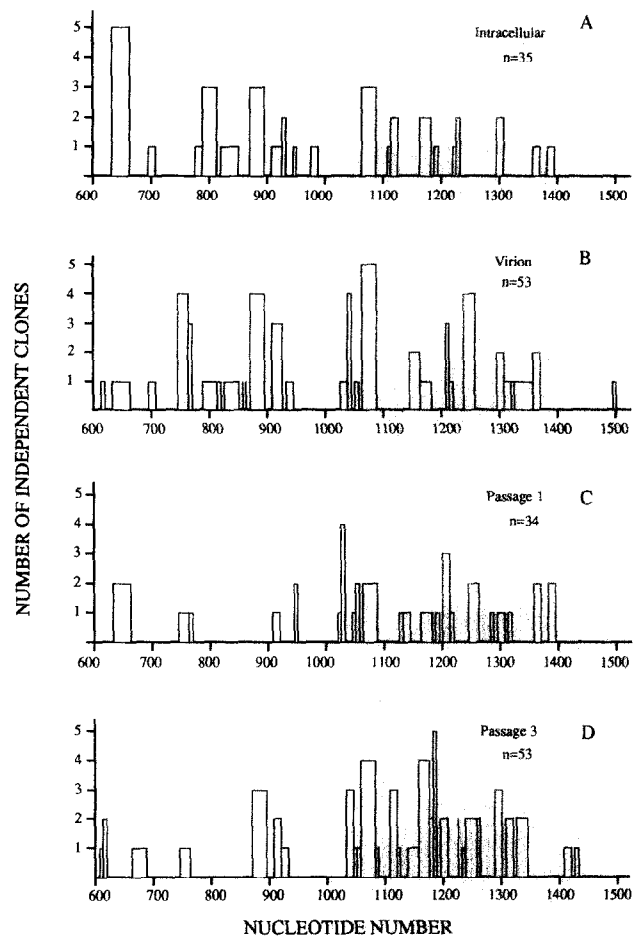


FIG. 2. Distribution of crossover sites. Each box represents the number of independent clones at each crossover site plotted against their nucleotide position from the 5'-end of the peplomer gene. The width of each box is defined by the region spanning the two nearest diverged nucleotides between A59 and JHM-DL. Thus, crossover sites could be located anywhere within the boxed area. The 3'-side of each box represents JHM-DL-specific sequences and the 5'-side represents A59-specific sequences. Crossover sites were determined by sequence analysis of individual clones. (A) Intracellular; (B) Virion; (C) Passage 1; (D) Passage 3; n, the total number of clones sequenced.

pernatant from this infection, termed Passage 1, was harvested and the progeny virions were purified. Viral genomic RNA was isolated and amplified by PCR using the same primers described previously. The specific 950-bp PCR product representing the recombinant RNA was again detected (data not shown). Sequence analysis of 34 recombinants showed that crossover sites were detected throughout the 1-kb region; however, a large proportion (67%) were clustered within nucleotides 1000–1350. This is in contrast to 40% in the same region for intracellular recombinant RNA and 47.1% for the recombinants detected in virion RNA before passage (Fig. 2C). These data suggest that some

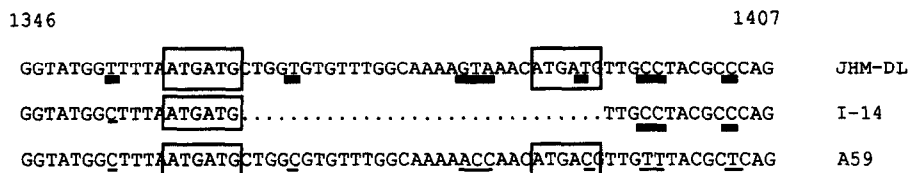


FIG. 3. Diagram of sequences around the crossover site of the recombinant I-14, which had a deletion. Both JHM-DL and A59 parental RNAs were compared with clone I-14 in the region from nucleotides 1346 to 1407 from the 5'-end of the peplomer gene. Thick lines underneath the sequences represent JHM-DL-specific sequences, and thin lines represent A59-specific sequences. Boxed areas are 6-nt repeats flanking the deletion. Placement of the boxed nucleotides in I-14 is arbitrary. Dots in I-14 denote deleted area.

recombinant RNA molecules may have selective disadvantages during subsequent infection.

After two additional passages, an even greater proportion (79.2%) of the crossover sites were localized within nucleotides 1000–1350 (Fig. 2D). By the fourth passage, no recombinant RNA molecules could be detected by PCR amplification (data not shown). This result suggests that the recombinant viral RNAs which have crossovers within the peplomer gene may have a growth disadvantage under the culture conditions. One possible reason may be that one of the parental viruses, A59, had more efficient growth properties in cell culture. The other parental virus, JHM-DL, was affected much like the recombinants, in that no JHM-DL RNA could be detected after Passage 5.

This study examined, for the first time, RNA recombination occurring in the absence of artificial selection pressures. It was surprising that the recombination sites within the 1-kb stretch of the peplomer gene appeared to be almost completely random under these conditions. Recombination was detected at the majority of the potential crossover sites; thus, there were no apparent sequence motifs or repeated nucleotides required for recombination, except in the recombinant noted above, which had a deletion accompanying the crossover. All of the recombination events examined were the result of homologous recombination, which preserved the open reading frame of the peplomer gene. One particular recombinant had a crossover between two adjoining nucleotides which differ between the two parental RNAs (Fig. 4A). A few more had crossover sites with only 1 or 2 nt separating the divergent nucleotides between the parental viruses (Figs. 4B–4D). Therefore, extensive sequence homology between the two parental RNAs at the crossover sites does not appear to be required for recombination. The same conclusion has been reached with poliovirus RNA recombination (3).

These results further support the observation that coronavirus RNA undergoes recombination at an extremely high frequency (5, 19). However, after only three virus passages, crossover sites became clustered within a small region, suggesting that certain

RNA crossover events may lead to more favorable recombinants. This is consistent with our previous finding that, when selection pressures (neutralization by monoclonal antibodies and temperature-sensitivity in replication) were used, all of the recombinants obtained had crossover sites localized between nucleotides 1148 and 1426 of the peplomer gene, even though the potential crossover region extended more than 1.5 kb (12). One possible reason for this selective localization of crossover sites is that certain regions combining to make the hybrid peplomer protein may be structurally incompatible so as to interfere with normal peplomer functions, such as binding to cellular receptors. Thus, the recombinational "hot spot" observed previously (12) most likely was the result of functional constraints on some of the recombinants, rather than a limitation of recombination events caused by RNA structure. Since the clustering site of recombination events corresponded to a hypervariable region (12, 22), it is logical to propose that this protein domain is more tolerant of structural modifications. It is interesting that recombinant RNA containing a deletion was detected only in intracellular RNA but not in RNA isolated from virions, suggesting that these recombinants could not replicate under the culture conditions. Also,

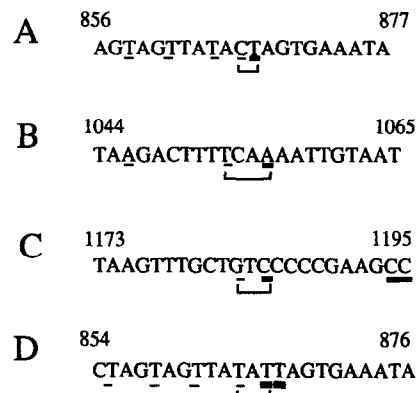


FIG. 4. Crossover sites in some of the recombinant RNAs. The brackets indicate the region in which the crossovers occurred. Thick lines underneath the sequences represent JHM-DL-specific sequences, and thin lines represent A59-specific sequences.

even at Passage 3, approximately 20% of the cross-over sites still fell outside the nucleotide 1000–1350 region. This was to be expected since recombination could still occur between the remaining parental A59 and JHM-DL RNA upon each reinfection.

It should be noted that this study examined only a small region of the MHV genome. When the entire MHV RNA is considered, it is possible that recombination may occur at different rates in different regions. Nevertheless, this study provided an interesting implication that the failure to detect homologous RNA recombination in other RNA viruses may be due to a stringent structural requirement of the viral proteins such that hybrid proteins have an evolutionary disadvantage. This may explain why nonhomologous recombination (as exemplified by DI RNA generation) can be detected readily, whereas homologous recombination has not been demonstrated for most RNA viruses. In addition, it explains why recombination events have not been detected in the capsid protein region of FMDV (7). The approach described in this report should provide a useful tool to study these possibilities.

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