

High-Frequency RNA Recombination of Murine Coronaviruses

SHINJI MAKINO,* JAMES G. KECK, STEPHEN A. STOHLMAN, AND MICHAEL M. C. LAI

Departments of Microbiology and Neurology, University of Southern California, School of Medicine, Los Angeles, California 90033*

Received 19 July 1985/Accepted 1 November 1985

The RNA genome of coronaviruses consists of a single species of nonsegmented RNA. In this communication, we demonstrate that the RNA genomes of different strains of murine coronaviruses recombine during mixed infection at a very high frequency. Susceptible cells were coinfecting with a temperature-sensitive mutant of one strain of mouse hepatitis virus (MHV) and a wild-type virus of a different strain. Of 21 randomly isolated viruses released from the coinfecting cells at the nonpermissive temperature, 2 were recombinants which differed in the site of recombination. After three serial passages of the original virus pool derived from the mixed infection, the majority of the progeny viruses were recombinants. These recombinant viruses represented at least five different recombination sites between the two parental MHV strains. Such a high-frequency recombination between nonsegmented RNA genomes of MHV suggests that segmented RNA intermediates might be generated during MHV replication. We propose that the RNA replication of MHV proceeds in a discontinuous and nonprocessive manner, thus generating free segmented RNA intermediates, which could be used in RNA recombination via a copy-choice mechanism.

Mouse hepatitis virus (MHV), a member of the *Coronaviridae*, contains a nonsegmented, single-stranded, and positive-sensed RNA of molecular weight 5.4×10^6 (19, 32). After MHV infection, the virion RNA codes for an early polymerase (5, 6) which is responsible for the synthesis of a genome-length negative-stranded RNA (18). The latter is then transcribed by the late polymerases into a positive-sensed genomic RNA and six species of subgenomic mRNAs (5, 6). These mRNAs, ranging in size from 0.6×10^6 to 5.4×10^6 molecular weight, have a nested-set structure containing sequences starting from the 3' end of the genomic RNA that extend for various distances toward the 5' end (15, 17, 22). Furthermore, each mRNA and also the genomic RNA contain an identical sequence of approximately 72 nucleotides at their 5' end (14, 16, 30). These nucleotides, the leader sequences, are encoded only at the 5' end of the genomic RNA. UV transcriptional mapping studies suggest that the viral mRNAs are not derived from cleavage of larger precursor RNA (9). Also, since the virus replicates in enucleated cells, no nuclear phase is necessary for replication (4, 34). Therefore, coronavirus mRNA synthesis does not involve conventional eucaryotic RNA splicing. Studies of replicative-intermediate RNA and double-stranded replicative-form RNA further suggest that the leader RNA is joined to mRNAs during transcription but not post-transcriptionally and that the leader RNA joining mechanism does not involve "looping out" of intervening sequences in the negative-stranded RNA template (1). In addition, we have recently detected distinct virus-specific small RNA species containing the leader sequences in MHV-infected cells (2). These leader-containing RNAs of discrete lengths were present in both the cytosol and membrane fractions of infected cells, suggesting that at least some of these RNAs were dissociated from the RNA template on the membrane-bound transcription complex (5). Whether these free leader-containing RNAs were products of abortive RNA transcription or were normal intermediates of transcription and

utilized for subsequent transcription is not known. We have also isolated a temperature-sensitive (ts) mutant which synthesizes only small leader RNAs, but not mRNAs, at the nonpermissive temperature (2). These data suggest that MHV mRNAs are synthesized discontinuously and may utilize the free leader RNA as a primer for transcription.

We have recently reported the isolation of a recombinant virus between two different strains of MHV, A59 and JHM (14a). This recombinant virus contains about 3 kilobases of JHM-derived sequences at the 5' end, while the remainder of the genomic RNA is derived from the A59 strain. Each intracellular subgenomic mRNA of this recombinant virus contains hybrid sequences consisting of the coding sequences of A59 joined to the leader sequences of JHM (14a). This type of true RNA recombination has not been reported in other RNA viruses except picornaviruses (12). The mechanism for the generation of recombinant MHV is not clear. One possible mechanism involves the large free MHV-specific leader-sequence-containing RNA species detected in the MHV-infected cells (2). In a mixed infection, these free RNA species could conceivably bind to the RNA minus-strand template of heterologous MHV strain and reinitiate RNA transcription. If these free leader-containing RNA fragments are true intermediates of RNA transcription, it might be expected that the frequency of RNA recombination in MHV could be very high as a result of exchange of free RNA intermediates between two different viruses during RNA replication. In this communication, we report that recombinant viruses were detected at very high frequency after mixed infection of two different strains of MHV. This observation supports a model of discontinuous and nonprocessive RNA replication in which free intermediate-sized RNA products are involved. The significance of this replication model will be discussed.

MATERIALS AND METHODS

Viruses and cells. The JHM and A59 strains of MHV and ts mutants of A59 were used. The ts mutants of A59 (LA12 and LA7) were isolated on L2 cells after mutagenesis of virus stock with 20 μ g of 5-azacytidine per ml. The detailed

* Corresponding author.

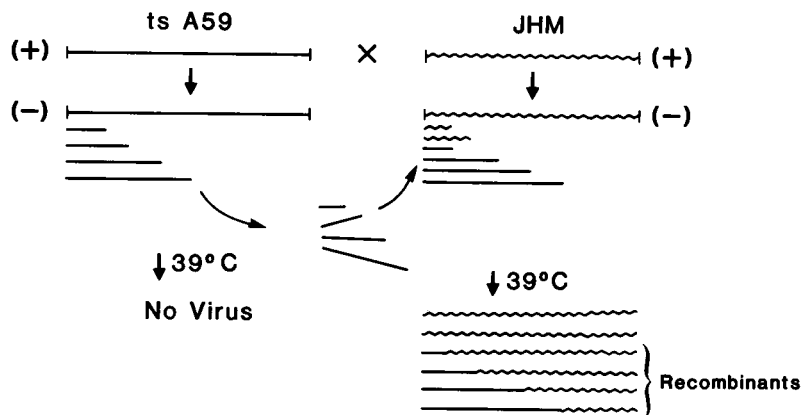


FIG. 1. Schematic drawing of the experimental protocol for isolation of recombinant viruses and the possible mechanism of RNA recombination in murine coronavirus.

protocol of the isolation and characterization of MHV ts mutants will be published elsewhere (J. Egbert, T. Wei, R. S. Baric, M. M. C. Lai, and S. A. Stohman, unpublished data). The viruses were grown in the DBT cell line as described previously (24).

Isolation of recombinant viruses. The protocol for the isolation of the potential recombinant viruses is outlined in Fig. 1. Briefly, DBT cells were grown in a 24-well plate (Falcon) and coinfecting with one of the ts mutants of A59 and wild-type (wt) JHM at a multiplicity of infection of 10 and 2, respectively. Virus adsorption was performed at room temperature for 1 h, and unadsorbed virus was removed by washing the cells with phosphate-buffered saline. The infected cells were incubated at 39°C for 16 h, and the media were harvested as virus stocks. The released viruses were plaque purified three times at 37°C on DBT cells, since MHV frequently did not produce distinct plaques at 39°C. Each isolate was assayed at 32 and 39°C, and those which induced cytopathic effect at 39°C were used for further experiments. For experiments involving serial passages of potential recombinant viruses, the supernatant from the mixed infection was passaged on DBT cells once at 39°C and twice at 37°C. The viruses were then plaque purified as described above.

Radiolabeling and isolation of virion genomic and intracellular RNAs. ³²P labeling of the genomic and intracellular RNAs followed the published procedures (24, 25). The genomic RNA and intracellular RNAs were separated by electrophoresis on 1% urea-agarose gels (24). The RNA was eluted from gel slices by the method of Langridge et al. (20).

One-dimensional (1-D) fingerprinting analysis. The ³²P-labeled mRNA 7 eluted from the gel was digested exhaustively with RNase T₁ (200 U/ml) in 5 μl of buffer containing 0.01 M Tris hydrochloride (pH 7.4) and 1 mM EDTA at 37°C for 1 h. After digestion, the solution was mixed with 5 μl of formamide containing 0.1% bromophenol blue and 0.1% xylene cyanol FF and loaded onto 22% polyacrylamide slab gels (40 by 35 by 0.02 cm) made in 0.05 M Tris borate buffer (pH 8.2). The electrophoresis was performed at 650 V for 16 h. After electrophoresis, the gel was exposed to Kodak XAR-5 film with an intensifying screen at 4°C.

Two-dimensional oligonucleotide fingerprinting and base composition analysis. Two-dimensional polyacrylamide gel electrophoresis was performed as previously described (24). The isolated T₁ oligonucleotides were digested with RNase A and separated by DEAE paper electrophoresis for base composition analysis as previously described (3).

RESULTS

Development of screening methods for recombinant MHV.

To determine whether MHV strains recombine at high frequency, we designed an experiment in which we randomly isolated viruses produced from cells coinfecting with two different strains of MHV and determined the percentage of recombinant viruses in the progeny. To reduce the number of viruses to be isolated, we designed a protocol as outlined in Fig. 1. DBT cells were coinfecting with an RNA⁺ ts mutant of A59 (LA12) and wt JHM, and propagated at the nonpermissive temperature (39°C). The progeny were plaque purified and randomly selected for further study. The rationale behind this approach is that, at the nonpermissive temperature, mRNA synthesis of both JHM and ts A59 proceeds normally but no infectious A59 virus particles are produced. Therefore, only the wt JHM and possible recombinants or revertant A59 virus would be detected. Since mRNA synthesis of the ts A59 occurs normally, it would contribute to the formation of recombinant viruses, perhaps by producing free leader-containing RNA intermediates (2) or by some other mechanism. The leader-containing RNAs from the ts A59 could bind to JHM minus-strand RNA template and continue transcription. As a result, recombinant viruses would be produced. Since no selection pressure against the wt JHM virus was used, the percentage of recombinant progeny recombinant viruses should reflect the recombination frequency between these two viruses under the infection conditions used.

To detect the presence of recombinant viruses among the progeny, we employed a simplified screening method. Since all the subgenomic mRNAs of MHV consist of nested-set sequences derived from the 3' end and a 72-nucleotide leader derived from the 5' end of the genomic RNA (14), a recombinant virus with one or more odd numbers of cross-overs should have subgenomic mRNAs which contain A59 leader sequences and JHM body sequences or vice versa. Since mRNA 7 is the smallest, most abundant mRNA species and is closest to the 3' end of MHV genome, the structure of mRNA 7 in each progeny virus provided an easy and sensitive screening tool for detecting recombinant viruses. We have previously shown that the leader sequences and leader-body junction sequences of A59 and JHM can be distinguished by conventional two-dimensional oligonucleotide fingerprinting (14a). To further simplify the screening procedures, we determined whether the leader sequences

and body sequences of the mRNA 7 of these two viruses could be distinguished by 1-D fingerprinting. The mRNA 7 from these two viruses was digested with RNase T₁ and separated by polyacrylamide gel electrophoresis at pH 8.2, which separates oligonucleotides purely by size. The leader-body fusion oligonucleotide 8a of JHM (25) could be distinguished from the leader-body fusion oligonucleotide 19 of A59 (17) (Fig. 2). However, the leader-specific oligonucleotides of both viruses (oligonucleotides 10 and 8, respectively) (17, 25) could not be separated by 1-D fingerprinting. The body sequences of these two viruses could also be distinguished by the presence of oligonucleotide 36 in A59 and the lack of a corresponding oligonucleotide in JHM. The identities of the leader-specific oligonucleotides were confirmed by base-composition analysis (data not shown), in agreement with the published sequence data (17, 30). Thus, 1-D fingerprinting can clearly distinguish the viral origins of the leader and body sequences of mRNA 7 of each virus. We therefore adopted this method for screening recombinant viruses.

Detection of recombinant viruses between A59 and JHM strains. Plaque-purified viruses isolated from the progeny of the mixed infection with ts A59 (LA12) and wt JHM were analyzed by the 1-D fingerprinting of their mRNA 7. Two virus clones, CA13 and CA21, showed altered fingerprints from those of either JHM or A59, while the rest of the clones were identical to JHM (Fig. 3). Both CA13 and CA21 contained A59-specific leader-body junction oligonucleotide 19 but lacked JHM-specific junction oligonucleotide 8a. Other oligonucleotides were indistinguishable from those of JHM. Two-dimensional fingerprinting of mRNA 7 of these two virus clones confirmed that they did contain A59-specific leader and leader-body fusion oligonucleotides (10 and 19), while the rest of the oligonucleotides were derived from the JHM parent (data not shown). These data strongly suggest that these two virus clones represent true recombinants which contain A59 leader sequence at their 5' terminus but JHM sequence at the 3' part of the genome. A total of 21 virus clones isolated from the mixed infection with ts A59 (LA12) and wt JHM were examined. Only these two clones appeared likely candidates for RNA recombinants. The finding of two recombinant viruses out of 21 randomly isolated progeny viruses suggests that recombination occurs at an extremely high frequency during MHV infection.

Structure of genomic RNA of recombinant viruses. To confirm the recombinant nature of these two viruses, CA13 and CA21, and to localize the possible crossover sites within the genomic RNAs, we examined their genomic RNAs by two-dimensional T₁-oligonucleotide fingerprinting. The ³²P-labeled 60S RNA from purified viruses was digested with RNase T₁ and analyzed by two-dimensional polyacrylamide gel electrophoresis (Fig. 4). CA13 and CA21 contained most of the large T₁-oligonucleotide spots present in the genome of JHM. However, they both lacked JHM-specific oligonucleotides 2b, 3, 8, 12, and 14. CA13 lacked two additional oligonucleotides, 1 and 11. Both viruses also contained several oligonucleotides not present in JHM, which correspond to A59-specific T₁ oligonucleotides. CA13 contained A59 T₁ oligonucleotides 1, 4, 5, 6, 8, 10, 20, 21, and 28, while CA21 contained A59 T₁ oligonucleotides 5, 8, 10, 21, and 28. A59 specificity of these oligonucleotides was established by fingerprinting mixtures of RNA from the recombinant and parental viruses and, in some cases, also by base composition analysis (data not shown). It is noteworthy that all the JHM-specific oligonucleotides missing in CA13 and CA21 are localized at the 5' end of the JHM genome (25, 31) and that all the A59-specific oligonucleotides present in these

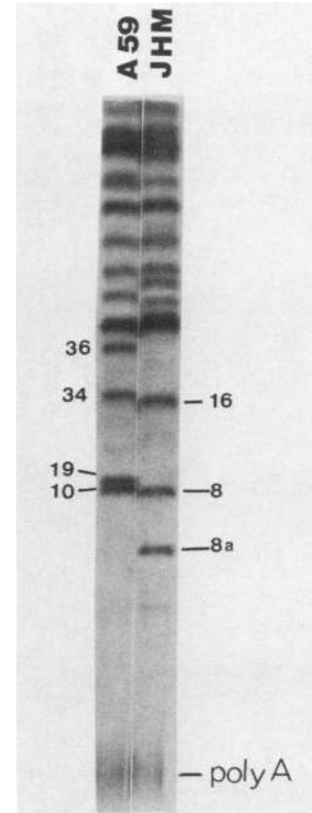


FIG. 2. 1-D oligonucleotide fingerprints of mRNA 7 of A59 and JHM. ³²P-labeled intracellular mRNAs of A59 and JHM were separated by urea-agarose gel electrophoresis, extracted from the gels, digested with RNase T₁, and applied to 22% polyacrylamide slab gels (pH 8.2). Electrophoresis was from bottom to top. Numbers show specific oligonucleotides. Oligonucleotides 10 and 8 are the leader-specific oligonucleotides of A59 and JHM, respectively (17, 25). Oligonucleotides 19 and 8a are the leader-body fusion oligonucleotides of A59 and JHM, respectively.

two viruses are also localized at the corresponding positions of the 5' end of the A59 genomic RNA (15). Thus, these two viruses are indeed recombinant viruses derived by true exchange of genetic material between A59 and JHM. Furthermore, CA21 and CA13 represent the progeny of two independent recombination events. The genetic structure of these two viruses is summarized in Fig. 6.

Recombinant viruses derived from serial passages. If RNA recombination between MHV strains indeed occurs at a very high frequency, it is conceivable that by serially passaging the unpurified progeny viruses derived from the mixed infection, further recombinational events could take place between wt JHM and the recombinants or between the recombinants themselves. As a result, new recombinant viruses with new crossover points might be detectable. We therefore passaged the unpurified progeny virus from the mixed infection three times on DBT cells and randomly plaque purified eight virus clones. These clones were analyzed by 1-D fingerprinting analysis of purified mRNA 7. Surprisingly, six (designated CA40 to CA45) of the eight virus clones showed 1-D fingerprinting patterns identical to that of CA13 and CA21, suggesting that they were also recombinant viruses between A59 and JHM (data not shown). The two remaining clones were identical to JHM and were not analyzed further.

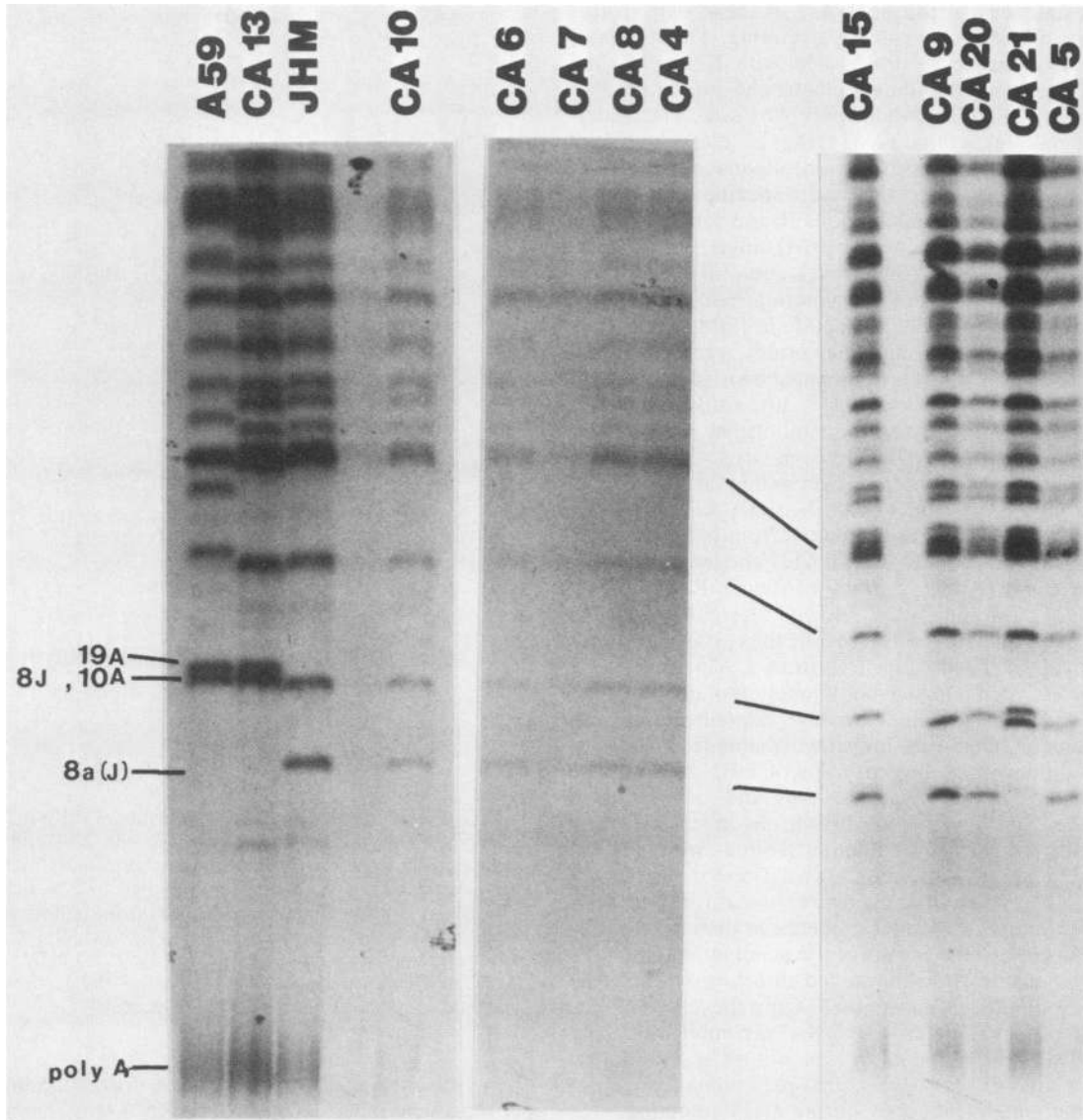


FIG. 3. 1-D oligonucleotide fingerprints of mRNA 7 of virus clones isolated from mixed infection of ts A59 (LA12) and wt JHM. The conditions of separation were the same as described in the legend to Fig. 2. A59 and JHM are the parental viruses.

To determine whether these viruses were derived from amplification of the original recombinant viruses CA13 and CA21 or were derived from additional recombinational events, we examined the two-dimensional oligonucleotide fingerprints of their genomic RNA (Fig. 5). The fingerprint of CA43 was similar to that of CA13 except that CA43 contained JHM-specific oligonucleotide 1 instead of A59-specified oligonucleotide 1. The fingerprints of genomic RNA of CA40, CA41, CA42, CA44, and CA45 also showed patterns very similar to that of CA21, with some minor variations. They contained the same A59-specific oligonucleotides, namely, spots 5, 8, 10, 21, and 28, except that CA44 did not have oligonucleotide 21. Furthermore, all these viruses lacked JHM-specific oligonucleotides 3, 8, 12, and 2b, while CA40, CA41, and CA44 lacked an additional JHM-specific oligonucleotide 15a. The genetic structure of these recombinant viruses is summarized in Fig. 6. It appears that most of these eight recombinant viruses contain

different recombination sites. CA44 contained the shortest A59 sequence, which maps within the first 1.5 kilobases of the 5' end of the genome. The recombination sites of CA45, CA40, and CA41 were located at similar or identical sites, while CA21 and CA42 recombined at a site located at the 3' side of the crossover points of the above three viruses. CA13 contained the largest A59 sequence at the 5' end of the genome, including the entire A59 gene A and at least part of gene B. The crossover point of CA43 was close to but at the 5' side of that of CA13.

There were several oligonucleotides which could not be unequivocally accounted for, such as oligonucleotide 15a of JHM which was not present in CA40, CA41, and CA44. The oligonucleotide varies in amount among different JHM stocks and is not present in some of the JHM variants (25). The map location of this oligonucleotide is not clear. Several other oligonucleotides (marked by arrows in Fig. 5) also correspond to other JHM oligonucleotides which are ex-

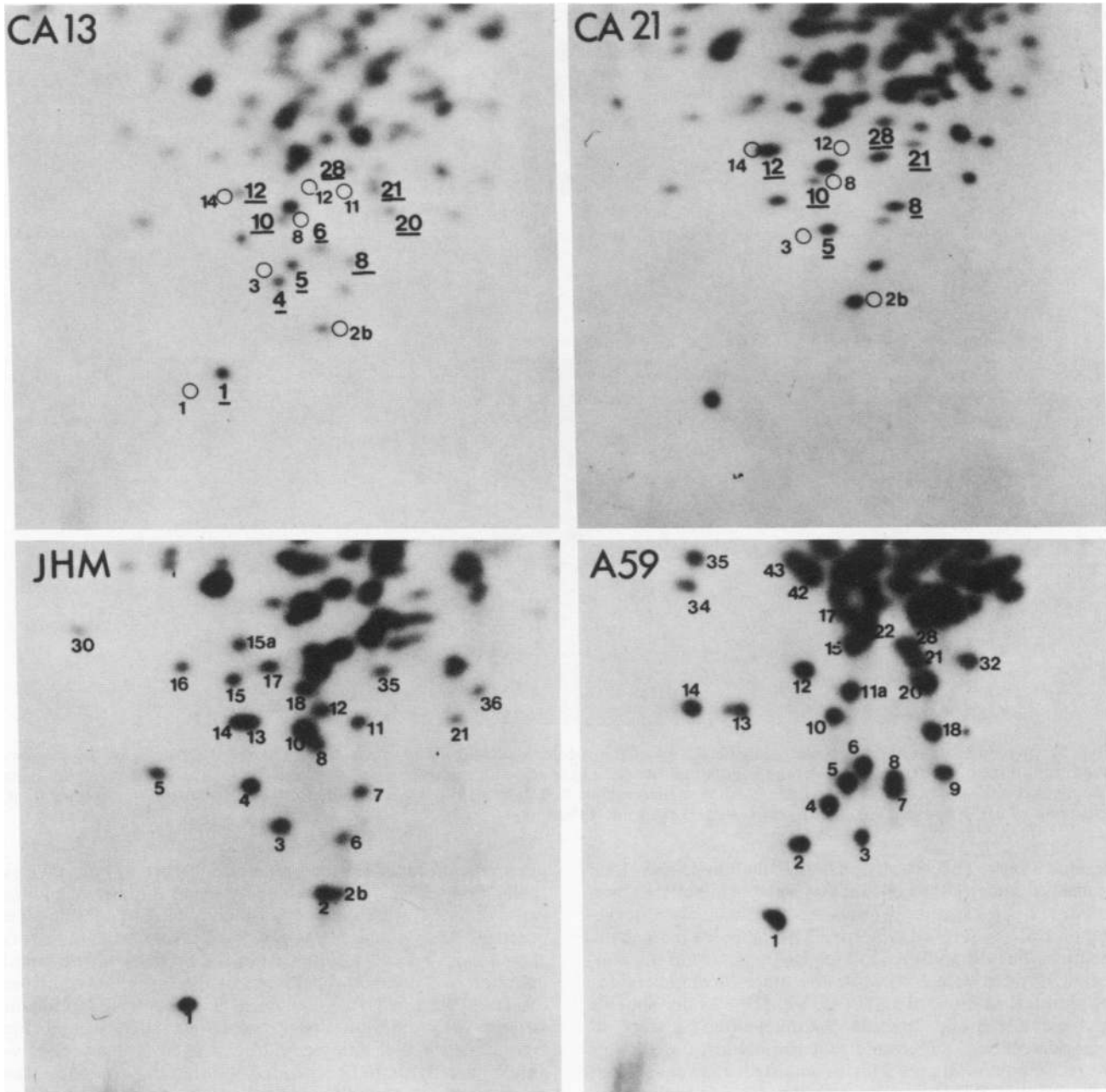


FIG. 4. Oligonucleotide fingerprints of the genomic RNAs of two different recombinant viruses, CA13 and CA21, and the parental viruses, A59 and JHM. ³²P-labeled purified genomic RNA of each virus was digested with RNase T₁ and analyzed by two-dimensional fingerprinting. The numbering system for A59 is according to Lai et al. (15), and that for JHM is according to Stohlman et al. (31) and Makino et al. (25). The underlined spots in CA13 and CA21 are those derived from A59, and circles represent the JHM spots missing in CA13 and CA21.

tremely variable in JHM virus stocks (25). Also, since oligonucleotide 12 of A59 was identical to oligonucleotide 13 of JHM, we could not determine whether there was genetic exchange between A59 and JHM within this region. Therefore, the crossover points for recombinants CA21 and CA42 were only approximate. The recombinant viruses isolated after three serial passages of the uncloned progeny virus from the original mixed infection have, in general, fewer A59-derived sequences than those (CA13 and CA21) isolated directly. These data suggest that they may have been derived from additional recombination between earlier recombinants

and the wt JHM virus. However, the possibility that these new recombinants were derived from amplification of the recombinants present in the original stock cannot be rigorously ruled out.

RNA recombination with another ts mutant virus. The isolation of recombinant viruses described above was achieved with the LA12 ts mutant of A59. To examine whether high-frequency RNA recombination is specific for this ts mutant, another RNA⁺ ts mutant of A59 (LA7) was examined. DBT cells were coinfectd with LA7 and wt JHM, and the progeny virus was passaged three times as

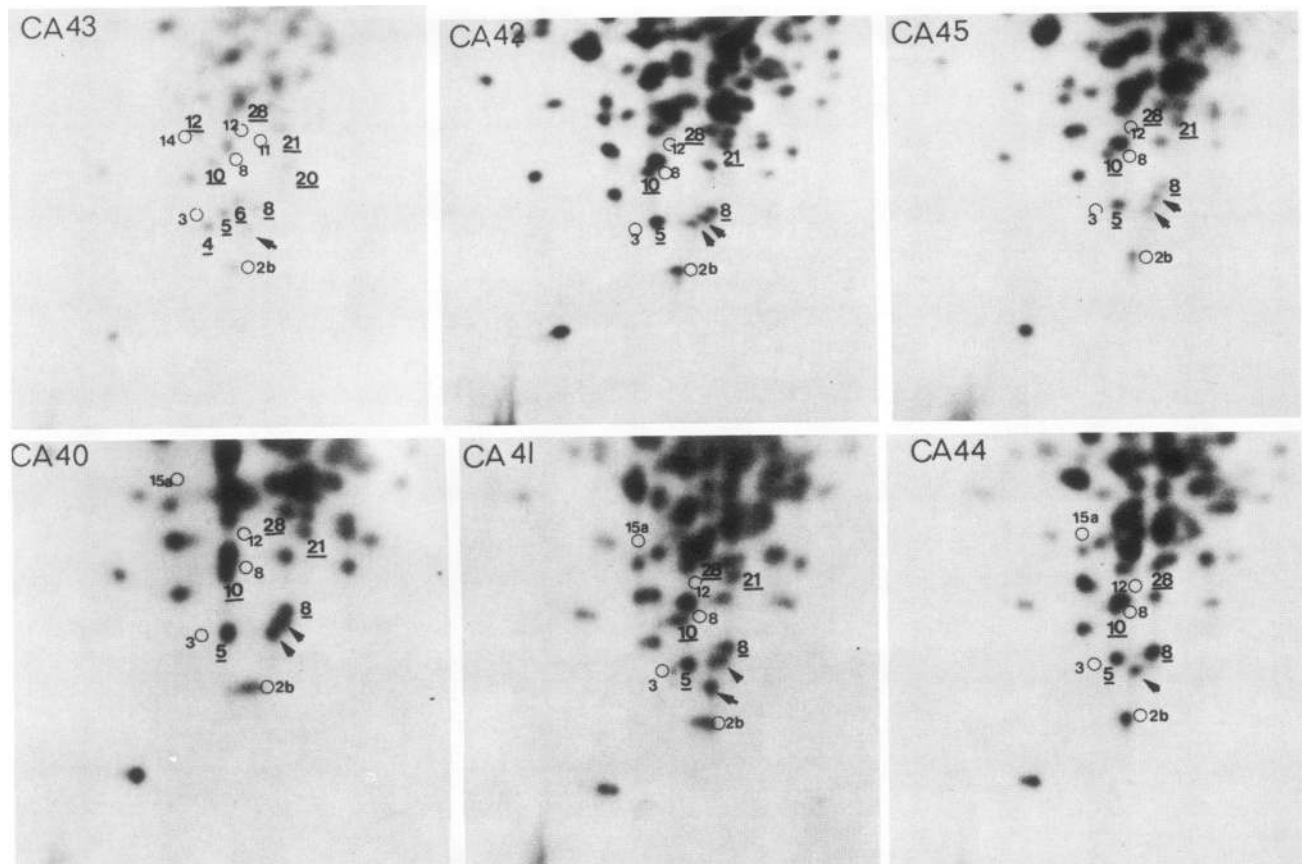


FIG. 5. Oligonucleotide fingerprints of genomic RNAs of the recombinant viruses isolated after three serial passages of the progeny derived from mixed infection. The underlined spots indicate the oligonucleotides derived from A59, and circles represent the JHM spots missing in recombinant viruses. The spots marked by arrows are derived from JHM but are variable from virus preparation to preparation, even among wt JHM viruses. The numbering system is the same as in Fig. 4.

described above. This serially passaged uncloned virus stock was used to infect DBT cells, and virus-specific mRNA was labeled with $^{32}\text{P}_i$. Figure 7 shows the oligonucleotide fingerprint of mRNA 7 of this virus. The A59 leader-specific oligonucleotide 10 and the A59 leader-body fusion oligonucleotide 19 were detected, while the other oligonucleotides were identical to those of JHM (23, 25). JHM-leader-specific oligonucleotide 8 and junction oligonucleotide 8a were almost undetectable, suggesting that the majority of viruses were recombinant viruses which contained A59 sequence at the 5' end and JHM sequences at the 3' end. Preliminary data indicated that five of eight viruses from this infection were recombinants, suggesting that high-frequency RNA recombination is a general property of MHV.

DISCUSSION

The data presented in this report demonstrate that RNA recombination occurs with high frequency between two different strains of MHV. The genomic RNAs of all recombinant viruses examined to date contained 5' sequences of A59 joined to the 3' end of JHM. However, the crossover site on the genome was different among most of the recombinant viruses. Four of the eight recombinant viruses had the same or very similar genomic structure, suggesting that there could be a favored recombination site(s). High frequency of recombination between wt JHM and a ts mutant of A59 was observed with at least two different ts mutants. Thus, high-frequency RNA recombination may represent a general

phenomenon for MHV and a unique feature of coronavirus replication. Since only a relatively small number of clones was examined, the frequency of recombination could not be precisely determined. However, the facts that recombinants accounted for 2 of 21 clones from the progeny of the initial infection and that serial passages of the virus derived from the coinfecting cells resulted in a majority of recombinant viruses with different crossover points suggest that the recombination frequency could be at least 10% or conceivably higher. It should be pointed out that this infection was performed under conditions in which the ts A59 was used at a fivefold-higher multiplicity of infection than that of JHM. This, coupled with the observation that A59 grows to higher titers than JHM (unpublished observation), might favor the generation of these recombinants and might also explain the findings that all the recombinants contained 5' A59 sequences and 3' JHM sequences and that no reciprocal recombinants were obtained. An additional possibility is that the ts lesions of the A59 mutants are close to the extreme 3' end of the genome, thus precluding the generation of the reciprocal recombinants. In any case, since the selection method used did not favor the recombinants over JHM, the high recombination frequency detected in this study should represent a true characteristic of coronaviruses. It should also be pointed out that the 1-D fingerprinting screening procedure used in this study will not detect most recombinants with double crossovers. The type of recombinants obtained was also limited by the genetic location of the ts

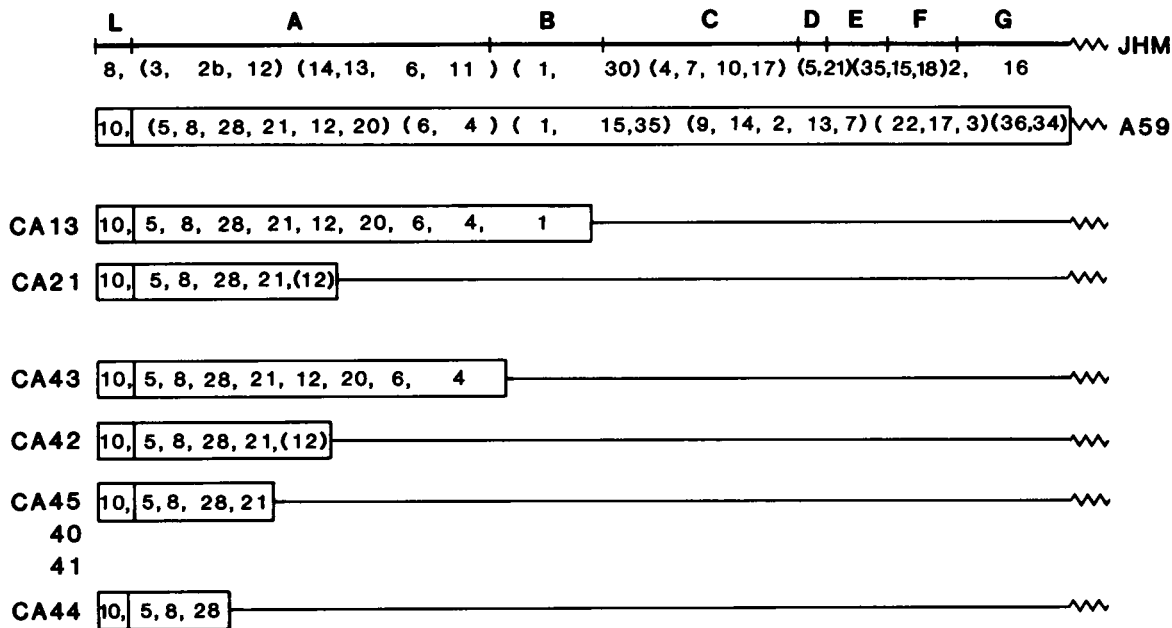


FIG. 6. Schematic representation of the oligonucleotide maps of recombinant viruses. The maps of A59 and JHM were derived from published data (15, 25, 31). The order of oligonucleotides within each bracket is arbitrary. The seven genetic regions (from A to G) are based on the data described previously (15, 29). L indicates 5'-end leader sequence (16, 30). The oligonucleotides within the boxes are the spots derived from A59. Since oligonucleotide 12 of A59 has a corresponding spot in JHM (oligonucleotide 13) (Fig. 4), the parental origin of this oligonucleotide in CA21 and CA42 is not clear and is therefore indicated with parentheses.

lesions of the mutants used. Therefore, actual recombination frequency might in fact be higher than that observed in this study. The reason the recombinants containing 5' A59 sequences and 3' JHM sequences predominated after serial passages of the virus mixture is not clear at this time. Preliminary data suggest that these recombinants do not grow better than the wt JHM virus (unpublished observation), indicating that recombinants were not preferentially amplified. It is possible that the 5'-end sequences of A59 might have some advantages over the corresponding sequences of JHM in promoting RNA recombination and initiating transcription. We have recently found that the A59 leader RNA indeed has such an advantage (S. Makino, S. A. Stohlman, and M. M. C. Lai, manuscript in preparation).

Such an extraordinarily high frequency of recombination is reminiscent of RNA reassortment in viruses with segmented genomes, e.g., influenza virus and reovirus (7, 28). Is it possible that, at some stages during coronavirus replication, either nascent or parental virus RNA also exists in segmented forms? If this is the case, these segmented RNAs might provide a mechanism for free reassortment of different RNA, resulting in high-frequency "recombination." This possibility has recently been suggested by the finding that discrete RNA species containing the MHV leader sequences are present in the MHV-infected cells (2). These 5' RNA species range from 50 nucleotides to 1 kilobase and are dissociated from the RNA template. Thus, they probably represent an incomplete RNA transcription product(s). Larger leader-containing RNA species, not corresponding to mRNA species, have also been found (14). It is conceivable that these free RNA species are normal nascent intermediates of RNA replication. For example, RNA replication starts from the 3' end of the minus-strand template and "pauses" at certain specific sites on the RNA template. As a result of transcriptional pausing, some of the nascent RNA intermediates might fall off the template. These RNAs could

then reassociate with the template and reinitiate RNA replication. During a mixed infection, these free RNA intermediates could bind to the template RNA of a heterologous virus, thus generating recombinant viruses by a copy-choice mechanism (Fig. 1). The high recombination frequency suggests that the generation of free RNA intermediates normally occurs during MHV RNA replication. Therefore, the generation of recombinant viruses might result from the reassortment of free RNA intermediates between two different viruses. Pausing during RNA transcription has been demonstrated in bacteriophages T7 and Q β (11, 26) and has been correlated with the secondary structure of the RNA template. Preliminary data suggest that the sizes of the leader-containing RNA intermediates correspond to the distances between the 5' ends and the sites for potential hairpin loop structures in MHV RNA (R. S. Baric, unpublished observation). Whether this is indeed the mechanism of RNA recombination requires further study. Nevertheless, high-frequency RNA recombination of coronaviruses suggests that this virus might utilize a mechanism of discontinuous and nonprocessive mode of RNA replication. True RNA recombination has also been observed in other non-segmented RNA viruses, including retroviruses and picornaviruses. Retroviruses probably recombine during reverse transcription via a possible heterozygote (10). The mechanism of RNA recombination in picornaviruses, however, has not been addressed. It would be interesting to investigate whether picornaviruses might also utilize a replication mechanism similar to that of coronaviruses.

We have previously suggested that the transcription of MHV mRNAs requires a free leader RNA as a primer (1, 2). Whether the free leader RNA is also utilized for the replication of the genomic RNA of MHV is not certain. If so, recombinants with the A59 leader and the other sequences from JHM would have been detected. Thus, the absence of such recombinants suggests that the replication of MHV

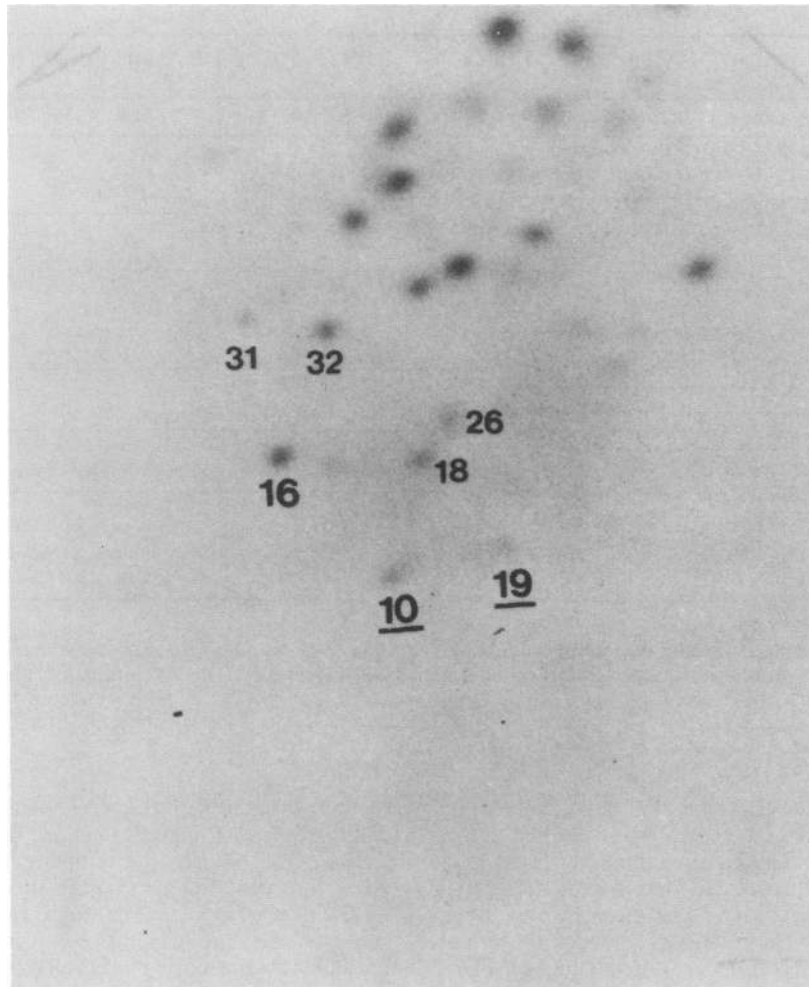


FIG. 7. Oligonucleotide fingerprints of mRNA 7 of the virus obtained after three serial passages of the progeny from the mixed infection with LA7 and JHM. Underlined spots 10 and 19 indicate A59 leader-specific oligonucleotide and A59 leader-body fusion oligonucleotide, respectively. Oligonucleotide spots 18, 26, 31, and 32 are oligonucleotides derived from the body sequences of mRNA 6 of JHM (25). The spots derived from JHM mRNA 6 are probably due to contamination from degraded larger-size mRNA species. Note that the JHM-specific leader and leader-body fusion oligonucleotides are not detectable.

genomic RNA does not utilize the free leader RNA. However, we have recently detected a recombinant which has a crossover site within the leader sequences (unpublished observation), suggesting that there are pausing sites within the leader region. Such a recombinant is currently being studied.

Recently, the structure of the intracellular defective interfering RNAs of MHV was reported (23). This proposed mechanism of MHV RNA replication might also explain the generation of defective interfering RNA of MHV. Every defective interfering-specific RNA species contains leader sequences and has multiple deletions in the internal sequences. It is conceivable that MHV leader-containing RNA species are dissociated from the negative template of standard virus during transcription and reassociate to incorrect sites on the template. Reinitiation of RNA transcription at the alternative sites would result in the generation of defective interfering RNA.

The study of MHV recombinant viruses will be useful for understanding the mechanism of RNA transcription of coronaviruses. The recombinant viruses isolated in the pre-

sent studies and previous study (14a) clearly showed that the A59 leader RNA can fuse to the body sequence of JHM mRNA, and vice versa. This indicates a flexibility in the leader/body fusion process. If we can isolate recombinant viruses with parents containing a more diverse leader sequence, they might provide insight into the sequence requirement for the leader-body fusion process during MHV mRNA transcription. Recombinants will also provide valuable information concerning the roles of various viral genes in pathogenesis. It should be noted that the JHM strain of MHV causes demyelination in rats and mice (8, 13, 27) and is a model for studying demyelinating diseases such as multiple sclerosis (33). Furthermore, recombinants will also provide a genetic tool for localizing the lesions of ts mutants. In this study, one recombinant virus (CA13) was found to contain A59 sequences upstream from gene *B*, which codes for a nonstructural protein, p30 (21, 29). Thus, the ts lesion of the mutant LA12 used in this study is probably localized within genes downstream from gene *B*. Isolation of additional recombinants utilizing this ts mutant will permit precise mapping of its ts lesion.

ACKNOWLEDGMENTS

We thank Ralph S. Baric for his critical comments, Anne Holmquist for editorial assistance, and Toni Baric and Madelene Graves for assistance in manuscript preparation.

This work was supported in part by National Multiple Sclerosis Society research grant RG 1449-A-1, National Science Foundation grant PCM-4507, and Public Health Service grant AI 19244 from the National Institutes of Health.

LITERATURE CITED

1. Baric, R. S., S. A. Stohlman, and M. M. C. Lai. 1983. Characterization of replicative intermediate RNA of mouse hepatitis virus: presence of leader RNA sequences on nascent chains. *J. Virol.* **48**:633-640.
2. Baric, R. S., S. A. Stohlman, M. K. Razavi, and M. M. C. Lai. 1985. Characterization of leader-related small RNAs in coronavirus-infected cells: further evidence for leader-primed mechanism of transcription. *Virus Res.* **3**:19-33.
3. Barrell, B. G. 1971. Fractionation and sequence analysis of radioactive nucleotides, p. 751-779. *In* G. L. Cantoni and D. R. Davies (ed.), *Procedures in nucleic acid research*, vol. 2. Harper & Row, Publishers, Inc., New York.
4. Brayton, P. R., R. G. Ganges, and S. A. Stohlman. 1981. Host cell nuclear function and murine hepatitis virus replication. *J. Gen. Virol.* **50**:457-460.
5. Brayton, P. R., M. M. C. Lai, C. D. Patton, and S. A. Stohlman. 1982. Characterization of two RNA polymerase activities induced by mouse hepatitis virus. *J. Virol.* **42**:847-853.
6. Brayton, P. R., S. A. Stohlman, and M. M. C. Lai. 1984. Further characterization of mouse hepatitis virus RNA dependent RNA polymerases. *Virology* **133**:197-201.
7. Fields, B. N. 1981. Genetics of reovirus. *Curr. Top. Microbiol. Immunol.* **91**:1-24.
8. Herndon, R. M., D. E. Griffin, U. McCormick, and L. P. Weiner. 1975. Mouse hepatitis virus-induced recurrent demyelination. *Arch. Neurol.* **33**:32-35.
9. Jacobs, L., W. J. M. Spaan, M. C. Horzinek, and B. A. M. van der Zeijst. 1981. The synthesis of the subgenomic mRNAs of mouse hepatitis virus is initiated independently: evidence from UV transcription mapping. *J. Virol.* **39**:401-406.
10. Junghans, R. P., L. R. Boone, and A. M. Skalka. 1982. Retroviral DNA H structure: displacement-assimilation model of recombination. *Cell* **30**:53-62.
11. Kassavetis, G. A., and M. J. Chamberlin. 1981. Pausing and termination of transcription within the early region of bacteriophage T7 DNA in vitro. *J. Biol. Chem.* **256**:2777-2786.
12. King, A. M. Q., D. McCahon, W. R. Slade, and J. W. I. Newman. 1982. Recombination in RNA. *Cell* **29**:921-928.
13. Knobler, R. L., M. Dubois-Dalq, M. V. Haspel, A. P. Claysmith, P. W. Lampert, and M. B. A. Oldstone. 1981. Selective localization of wild type and mutant mouse hepatitis virus (JHM stain) antigens in CNS tissue by fluorescence, light and electron microscopy. *J. Neuroimmunol.* **1**:81-92.
14. Lai, M. M. C., R. S. Baric, P. R. Brayton, and S. A. Stohlman. 1984. Characterization of leader RNA sequence on the virion and mRNAs of mouse hepatitis virus, a cytoplasmic virus. *Proc. Natl. Acad. Sci. USA* **81**:3626-3630.
- 14a. Lai, M. M. C., R. S. Baric, S. Makino, J. G. Keck, J. Egbert, J. L. Leibowitz, and S. A. Stohlman. 1985. Recombination between nonsegmented RNA genomes of murine coronaviruses. *J. Virol.* **56**:449-456.
15. Lai, M. M. C., P. R. Brayton, R. C. Armen, C. D. Patton, C. Pugh, and S. A. Stohlman. 1981. Mouse hepatitis virus A59: mRNA structure and genetic localization of the sequence divergence from hepatotropic strain MHV-3. *J. Virol.* **39**:823-834.
16. Lai, M. M. C., C. D. Patton, R. S. Baric, and S. A. Stohlman. 1983. Presence of leader sequences in the mRNA of mouse hepatitis virus. *J. Virol.* **46**:1027-1033.
17. Lai, M. M. C., C. D. Patton, and S. A. Stohlman. 1982. Further characterization of mRNAs of mouse hepatitis virus: presence of common 5'-end nucleotides. *J. Virol.* **41**:557-565.
18. Lai, M. M. C., C. D. Patton, and S. A. Stohlman. 1982. Replication of mouse hepatitis virus: negative-stranded RNA and replicative-form RNA are of genome length. *J. Virol.* **44**:487-492.
19. Lai, M. M. C., and S. A. Stohlman. 1978. RNA of mouse hepatitis virus. *J. Virol.* **26**:235-242.
20. Langridge, L., P. Langridge, and P. L. Berquist. 1980. Extraction of nucleic acids from agarose gels. *Anal. Biochem.* **103**:264-271.
21. Leibowitz, J. L., S. R. Weiss, E. Paavola, and C. W. Bond. 1982. Cell-free translation of murine coronavirus RNA. *J. Virol.* **43**:905-913.
22. Leibowitz, J. L., K. C. Wilhelmson, and C. W. Bond. 1981. The virus-specific intracellular RNA species of two murine coronaviruses: MHV-A59 and MHV-JHM. *Virology* **114**:39-51.
23. Makino, S., N. Fujioka, and K. Fujiwara. 1985. Structure of the intracellular defective viral RNAs of defective interfering particles of mouse hepatitis virus. *J. Virol.* **54**:329-336.
24. Makino, S., F. Taguchi, and K. Fujiwara. 1984. Defective interfering particles of mouse hepatitis virus. *Virology* **133**:9-17.
25. Makino, S., F. Taguchi, N. Hirano, and K. Fujiwara. 1984. Analysis of genomic and intracellular viral RNAs of small plaque mutants of mouse hepatitis virus, JHM strain. *Virology* **139**:138-151.
26. Mills, D. R., C. Dabkin, and F. R. Kramer. 1978. Template-determined, variable rate of RNA chain elongation. *Cell* **15**:541-550.
27. Nagashima, K., H. Wege, P. Meyermann, and V. ter Meulen. 1978. Coronavirus induced subacute demyelinating encephalitis in rats: a morphological analysis. *Acta Neuropathol.* **44**:63-70.
28. Scholtissek, C., W. Rhode, V. V. von Hoyningen, and R. Rott. 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* **87**:13-20.
29. Siddell, S. 1983. Coronavirus JHM: coding assignments of subgenomic mRNAs. *J. Gen. Virol.* **64**:113-125.
30. Spaan, W., H. Delius, M. Skinner, J. Armstrong, P. Rottier, S. Smeekens, B. A. M. van der Zeijst, and S. G. Siddell. 1983. Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. *EMBO J.* **2**:1939-1944.
31. Stohlman, S. A., P. R. Brayton, J. O. Fleming, L. P. Weiner, and M. M. C. Lai. 1982. Murine coronaviruses: isolation and characterization of two plaque morphology variants of the JHM neurotrophic strains. *J. Gen. Virol.* **63**:265-275.
32. Wege, H., A. Müller, and V. ter Meulen. 1978. Genomic RNA of the murine coronavirus JHM. *J. Gen. Virol.* **41**:217-227.
33. Wege, H., S. Siddell, and V. ter Meulen. 1982. The biology and pathogenesis of coronaviruses. *Curr. Top. Microbiol. Immunol.* **99**:165-200.
34. Wilhelmson, K. C., J. L. Leibowitz, C. W. Bond, and J. A. Robb. 1981. The replication of murine coronaviruses in enucleated cells. *Virology* **110**:225-230.