VIROLOGY **156,** 331–341 (1987)

Multiple Recombination Sites at the 5′-End of Murine Coronavirus RNA

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Received August 11, 1986; accepted October 31, 1986

Mouse hepatitis virus (MHV), a murine coronavirus, contains a nonsegmented RNA genome. We have previously shown that MHV could undergo RNA–RNA recombination in crosses between temperature-sensitive mutants and wild-type viruses at a very high frequency (S. Makino, J. G. Keck, S. A. Stohlman, and M. M. C. Lai (1986) *J. Virol.* 57, 729–737). To better define the mechanism of RNA recombination, we have performed additional crosses involving different sets of MHV strains. Three or possibly four classes of recombinants were isolated. Recombinants in the first class, which are similar to the ones previously reported, contain a single crossover in either gene A or B, which are the 5′-most genes. The second class of recombinants contain double crossovers in gene A. The third class of recombinants have crossovers within the leader sequence located at the 5′-end of the genome. The crossover sites of the third class have been located between 35 and 60 nucleotides from the 5′-end of the leader RNA. One of these recombinants has double crossovers within the short region comprising the leader sequences. Finally, we describe one recombinant which may contain a triple crossover. The presence of so many recombination sites within the 5′-end of the genome of murine coronaviruses confirms that RNA recombination is a frequent event during MHV replication and is consistent with our proposed model of "copy-choice" recombination in which RNA replication occurs in a discontinuous and nonprocessive manner.

INTRODUCTION

Mouse hepatitis virus (MHV), the prototype of the Coronaviridae family, is an enveloped, single-stranded, nonsegmented, and positive-sense RNA virus. The viral genome contains a 5′-cap and a 3′-polyadenylated tail, and has a mol wt of 5.4×10^6 Da (Lai and Stohlman, 1978, 1981; Wege *et al.,* 1978). Upon infection, the virus produces a full-length genomic RNA and six subgenomic mRNAs arranged in a 3′-coterminal nested-set structure (Lai *et al.,* 1981). In addition, each mRNA appears to contain an identical 72-nucleotide leader sequence at the 5′-end (Lai *et al.,* 1983, 1984; Spaan *et al.,* 1983). The leader RNA is added to the mRNAs by a unique mechanism of "leader-primed transcription"; i.e., a free leader RNA is synthesized, dissociates from the template, and then rebinds to the RNA template downstream at the initiation sites for the various mRNAs to serve as the primer for transcription (Baric *et al.,* 1983, 1985). This mechanism predicts that leader RNA regulates the transcription of subgenomic mRNAs.

Recently, our laboratory has also demonstrated that MHV could undergo RNA–RNA recombination. Initially, RNA recombinants were obtained from crosses between temperature-sensitive (ts) mutants of the A59 and JHM strains (Lai *et al.,* 1985). Additional recombinants with various crossover points located in the 5′-most genes of the RNA genome have subsequently been isolated (Makino *et al.,* 1986). All of the viruses described previously have single crossovers (Lai *et al.,* 1985; Makino *et al.,* 1986). Crosses between a ts mutant of A59 and a wild-type JHM strain suggested that MHV RNA recombination occurs at a very high frequency (Makino *et al.,* 1986). The high frequency can best be explained by the involvement of free RNA intermediates during RNA replication. In support of this interpretation, RNA intermediates of various sizes, derived from the 5′-end of the genome and thus containing the leader sequences, have been detected in MHV-infected cells (Baric *et al.,* 1987). These intermediates could conceivably participate in RNA recombination via a copy-choice mechanism. We have described this mechanism as discontinuous and nonprocessive RNA replication (Makino *et al.,* 1986); 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.

To better understand the mechanism of recombination and determine the role these leader-containing RNA intermediates may play in recombination, we expanded our panel of MHV recombinants. We performed crosses between several different pairs of ts mutants and wild-type MHVs and have isolated several new classes of RNA recombinants. Among these are viruses with multiple crossovers, including recombinants with crossovers within the leader sequences. The isolation of these recombinants reaffirms our model of discon-

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0042-6822/87 $3.00

tinuous and nonprocessive RNA transcription in coronaviruses.

MATERIALS AND METHODS

Viruses and cells

The MHV wild-type strains JHM and A59 and ts mutants derived from these strains were used. The ts mutants LA16, LA8, and LA10 of A59 were isolated on L2 cells after mutagenesis of parental A59 (J. Egbert *et al.*, unpublished observation). These mutants do not synthesize mRNAs at the nonpermissive temperature. The ts mutants of JHM have previously been described (Leibowitz *et al.*, 1982). The viruses were grown on the DBT or L2 cell lines according to published procedures (Lai *et al.*, 1978, 1981).

Isolation of recombinants

Recombinants were isolated by two alternative methods as described by Lai *et al.* (1985) and Makino *et al.* (1986) with slight modifications. Coinfection involving ts mutants of both A59 and JHM was carried out at 32° for 90 min, and was then shifted to 39° (nonpermissive). Virus was harvested at 16 hr postinfection (p.i.). Coinfection between wild-type JHM and ts LA10 was performed at 37° for 1 hr and was then shifted to 39°. Medium was changed at 2 hr p.i. and was harvested at 14 hr p.i. After both sets of infections, viral isolates were plaque-purified at 39° at least three times before further studies.

Radiolabeling and isolation of virion genomic and intracellular RNAs

The radiolabeling of genomic and intracellular viral RNAs with [^{32}P]orthophosphate was performed as previously reported (Lai *et al.*, 1985). The ^{32}P-labeled virions were purified by sedimentation on sucrose gradients according to the published procedures (Lai and Stohlman, 1978). Genomic RNA was extracted from purified virion using phenol and was further purified by sucrose gradient sedimentation (Lai *et al.*, 1981; Lai and Stohlman, 1978). The ^{32}P-labeled intracellular RNAs were extracted from the infected cells at 6 hr p.i. and were separated by electrophoresis on 1% agarose gels. The mRNAs were identified by autoradiography and were extracted from the gels by Dounce homogenization in a buffer containing 0.4 *M* ammonium acetate, 0.1 *M* Tris–HCl, pH 7.4, and 1 m*M* EDTA, and incubated at 37° with constant shaking overnight. The elution buffer was clarified of gel pieces by centrifugation at 15,000 *g* for 15 min, and the RNA was precipitated with ethanol.

One-dimensional fingerprinting analysis

Screening of recombinants by one-dimensional oligonucleotide fingerprinting of virus-specific mRNA 7 followed our published procedure (Makino *et al.*, 1986). Briefly, ^{32}P-labeled mRNA 7 was eluted from the gel and digested with RNase T_1 (200 U/ml) in 5 μl of buffer containing 10 m*M* Tris–HCl, pH 7.4, and 1 m*M* EDTA for 1 hr at 37°. After addition of 5 μl of formamide containing 0.1% bromphenol blue and 0.1% xylene cyanol FF, the samples were loaded on a 22% polyacrylamide gel in 50 m*M* Tris–borate buffer, pH 8.2. Electrophoresis was carried out for 16 hr at 600 V.

Two-dimensional fingerprinting analysis

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

Primer extension and sequence analysis

The synthetic oligodeoxyribonucleotide (15-mer) 5′-AGGAACAAAAGACAT-3′, prepared by Dr. Minnie McMillan, University of Southern California Cancer Center, was used as the primer for primer extension studies. This oligomer is complementary to the 5′-end of the coding sequences (nucleotides 84–98 from the 5′-end of mRNA 7) of the A59 strain (Armstrong *et al.*, 1983). Conditions for 5′-end-labeling of the primer, hybridization to MHV intracellular RNA, and primer extension with reverse transcriptase were as previously described (Lai *et al.*, 1984). Reaction products were separated by electrophoresis in 12% polyacrylamide gels containing 6 *M* urea and were eluted from the gel according to published procedures (Maxam and Gilbert, 1980). DNA sequence analysis of the primer-extended products was performed by the Maxam–Gilbert procedure (Friedmann, 1979; Maxam and Gilbert, 1980).

RESULTS

Recombination between temperature-sensitive mutants of MHV

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 crossovers clustered within the 5′-half of the RNA genome.

To further understand the mechanism of RNA recombination, we performed two additional crosses between different pairs of ts mutants, one using the ts mutants LA16 of A59 and 129 of JHM and the other

using LA8 of A59 and 203 of JHM. 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 mRNA 7. Since the leader and mRNA 7 sequences are derived from the opposite ends of the genomic RNA, any recombinants with an odd number of crossovers 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 nonhybrid 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 crossovers. By using these two approaches, we have isolated recombinants arising from both single and double crossover events. From the first cross between LA16 and 129, we detected several recombinants with a single crossover. One of these, C1, has an oligonucleotide fingerprint very similar to that of the independently isolated recombinant B1 which was reported previously (Lai *et al.,* 1985) (Fig. 1). Thus, this crossover site appears to be favored in several crosses. It is noteworthy that the mRNA 7 of C1 contains the JHM-specific leader oligonucleotides 8 and 8a (Makino *et al.,* 1984) instead of the corresponding A59 oligonucleotides. Additionally, it contains a novel oligonucleotide 14a, while the oligonucleotide 34 of A59 is missing. This additional change has previously been described for B1 recombinant (Lai *et al.,* 1985).

The second cross between ts mutant LA8 of A59 and ts mutant 203 of JHM yielded three recombinant viruses. The first, A4, has a mRNA 7 identical to the parental A59 (Fig. 2). However, longer exposure of the oligonucleotide fingerprints revealed a faint oligonucleotide derived from JHM, while the A59 oligonucleotide corresponding to the same genetic region is missing (data not shown). This result suggests that A4 could be a recombinant with a double crossover. This conclusion was supported by the fingerprint of the A4 genomic RNA, which revealed the presence of JHM-specific oligonucleotide 3 and the absence of the corresponding A59-specific oligonucleotide 5 (Fig. 3). Thus, this recombinant has a short double crossover at the 5′-end of gene A since these two oligonucleotides have been mapped in that location (Lai *et al.,* 1981; Makino *et al.,* 1984). The genomic fingerprint of A4 is very faint because of difficulty in obtaining sufficient quantity of viral RNA. It appears that several additional A59 oligonucleotides are missing. However, no corresponding JHM oligonucleotides are present. Thus, it is unlikely that there are additional crossovers.

The second recombinant isolate, A5, has a mRNA 7

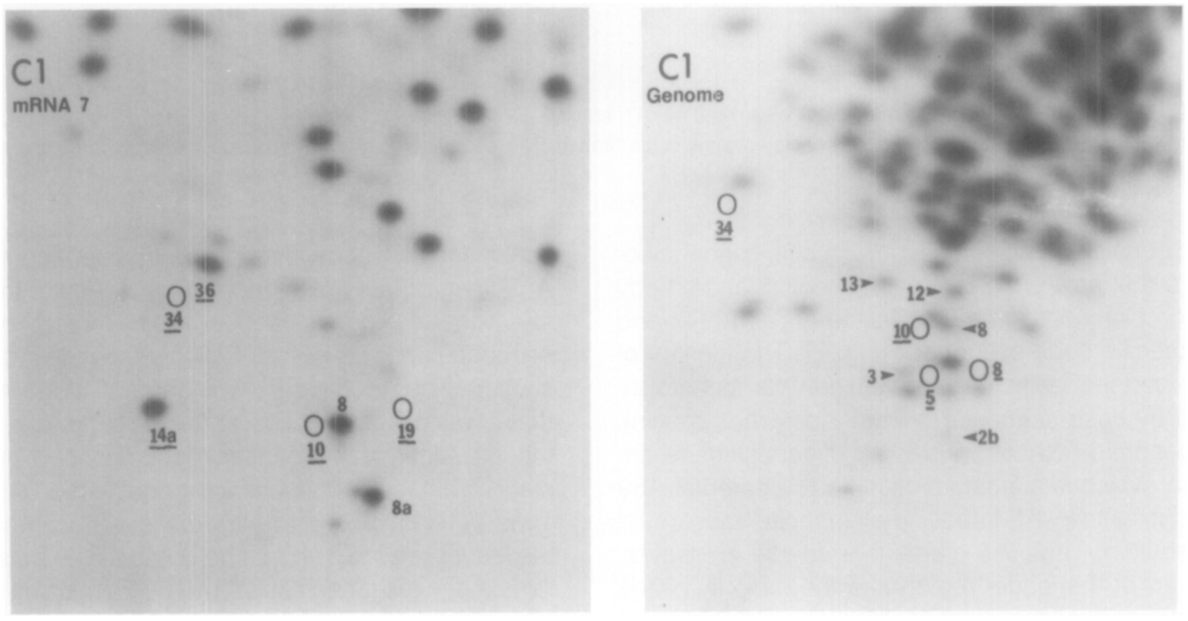

FIG. 1. Oligonucleotide fingerprints of mRNA 7 and genomic RNA of isolate C1. ^{32}P-labeled mRNA 7 and genomic RNA were digested with RNase T_1 and analyzed by two-dimensional polyacrylamide gel electrophoresis. The numbering system of A59 is according to Lai *et al.* (1981), and JHM is according to Makino *et al.* (1984). The underlined spots are A59 specific and the circles represent missing spots. The spots denoted by arrowheads are JHM specific. The oligonucleotides 10 and 19 are the leader-specific and leader-body junction oligonucleotides of A59, respectively. The oligonucleotides 8 and 8a are the corresponding oligonucleotides of JHM.

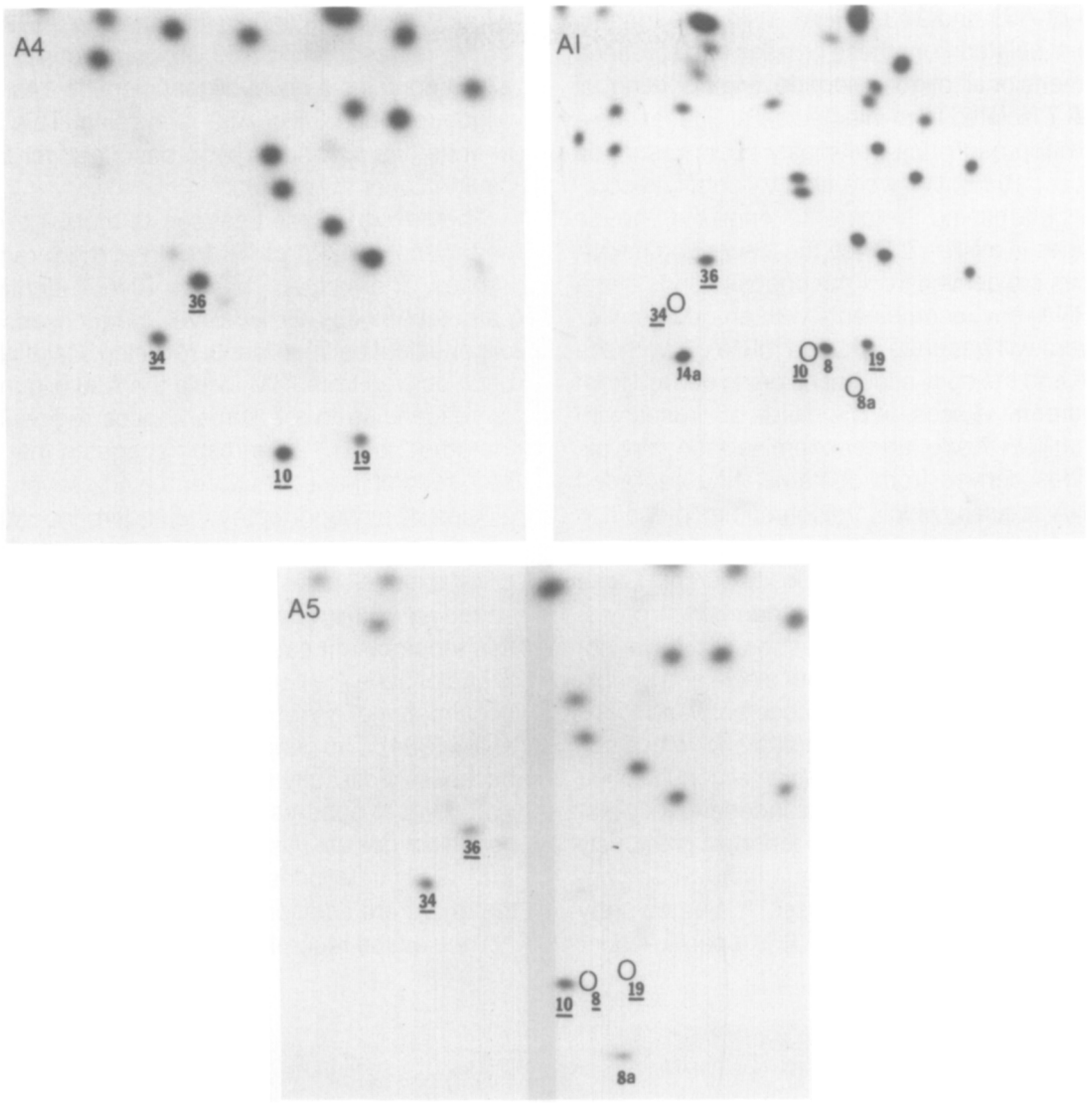

FIG. 2. Oligonucleotide fingerprints of mRNA 7 of isolates A4, A1, and A5. ^{32}P-labeled mRNA 7 was purified by agarose gel electrophoresis, digested with RNase T_1, and analyzed by two-dimensional fingerprinting. The underlined spots are A59 specific and the circles represent missing spots. The numbering system is the same as for Fig. 1.

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. 2). 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. 3). No JHM-specific oligonucleotides could be identified. Thus, A5 contains a double crossover, 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 third isolate, A1, has a mRNA 7 which contains the JHM-specific leader oligonucleotide 8, the A59-specific leader-body junction oligonucleotide 19, and A59-specific body sequences (Fig. 2). Again, the identity of these oligonucleotides was confirmed by base sequence analysis of these oligonucleotides (data not shown). 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. 3). Thus, A1 contains a single crossover located within the leader region. It should be noted that A1 also contains a new oligonucleotide 14a but is missing the oligonucleotide 34. The same genetic change has been observed in C1 recombinant and the previously described B1 (Lai *et al.,* 1985). The significance of this genetic change is not clear.

The crossover site of A1 is within the same area as

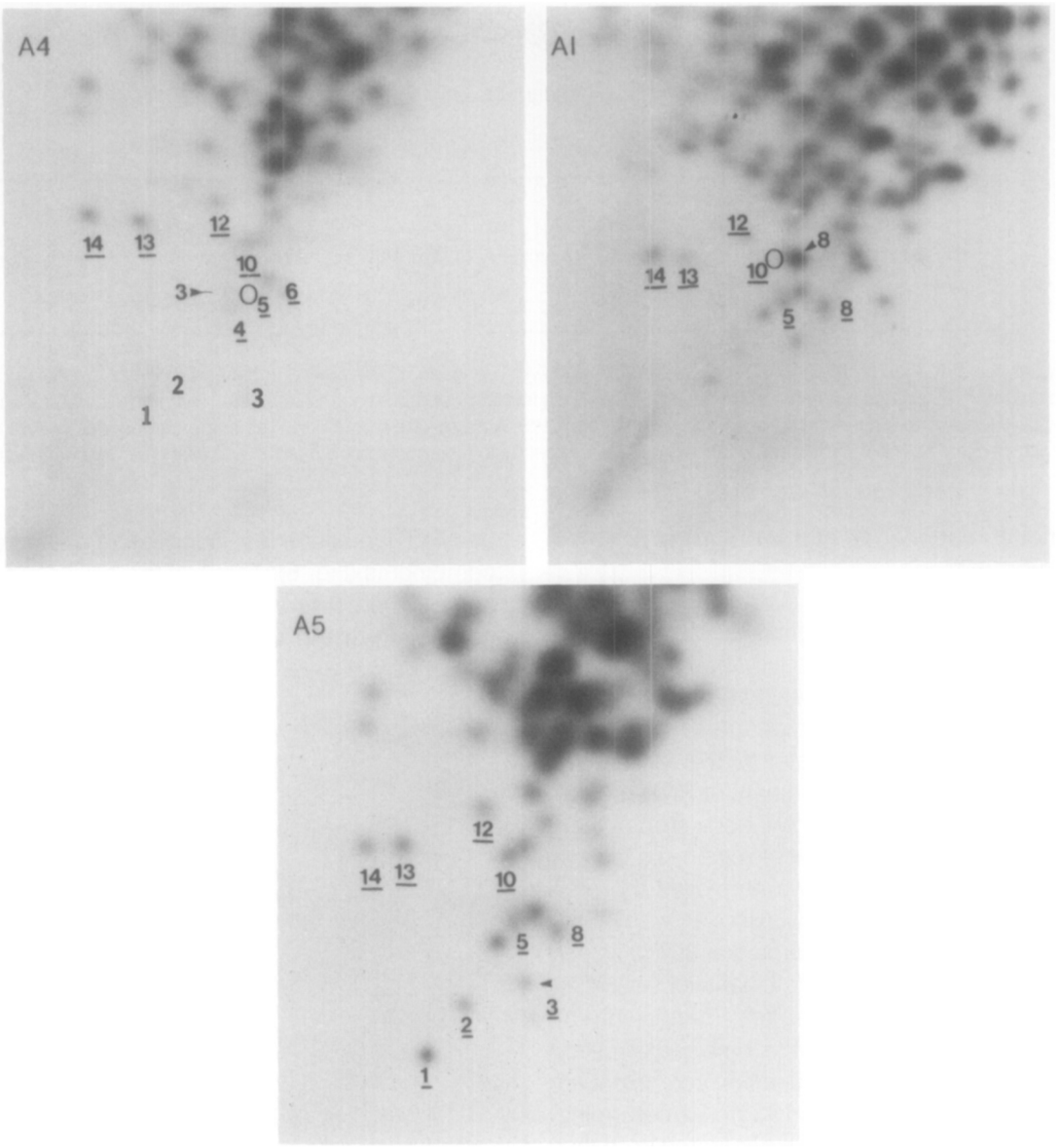

FIG. 3. Oligonucleotide fingerprints of genomic RNA of isolates A4, A1, and A5. ^{32}P-labeled virion genomic RNA was digested with RNase T_1 and analyzed by two-dimensional fingerprinting. The arrows in A4 and A1 represent JHM-specific spots. Circles represent missing A59 spots. Underlined spots are A59 specific. The arrowhead in A5 indicates a new spot, the origin of which is not unclear.

the one in A5, and thus, the crossovers in these two recombinants are almost reciprocal. To confirm the recombination site in the leader region of viruses A5 and A1, we carried out sequence analysis of the leader region. A 5′-end-labeled synthetic 15-mer complementary to the 5′-end of the coding region of mRNA 7 (see Materials and Methods) of A59 was hybridized to intracellular mRNA and was primer-extended with reverse transcriptase. Since mRNA 7 is the predominant virus-specific RNA species in MHV-infected cells, the major primer-extended products should represent the leader RNA derived from mRNA 7. The products were separated by electrophoresis, eluted, and sequenced by the method of Maxam and Gilbert. Recombinant A5 contains a single base mutation at position 35 corresponding to A59 while the remainder of the leader was JHM-specific in sequence (Fig. 4). In contrast, recombinant A1 contains the opposite structure in which the first base mutation is JHM-specific and the rest of the leader corresponds to the sequences of A59. Thus, the crossover sites in these recombinants should be between nucleotides 35 and 60 from the 5′-end. We cannot define the exact crossover point in these recombinants because of the high homology of bases in this region. The position of the second crossover in A5 has not been determined precisely.

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                30        40        50        60        70        80        90
                                                                    oligo #3
JHM     GUACGUACCC UCUCUACUCU AAAACUCUUG UAGUUUAAA UCUAA UCUAA UCUAA ACUUUA AGGAUGUCUU UUGUUCCU

A59     ---------- ----A----- ---------- ---------       UCUAA UCUAA ------ ---------- --------

A5      ---------- ----A----- ---------- --------- UCUAA UCUAA UCUAA ------ ---------- --------

A1      ---------- ----U----- ---------- ---------       UCUAA UCUAA ------ ---------- --------
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FIG. 4. Sequences of the leader regions of recombinants A5 and A1. The leader cDNAs were obtained by primer extension of a synthetic primer complementary to the coding region of mRNA 7 of A59, using intracellular mRNA as the template. The primer-extended products were separated by polyacrylamide gel electrophoresis and sequenced by the Maxam–Gilbert method. The leader sequences of A59 and JHM are included for comparison. Numbers at the top of the figure refer to the nucleotide position from the 5′-end of the leader sequence.

Recombination between ts mutant LA10 of A59 and wild-type JHM

The second strategy for isolating recombinants involved coinfection with wild-type JHM and a ts mutant of A59, LA10, which accumulates leader RNA but not mRNAs at the nonpermissive temperature (Baric *et al.*, 1985). If free leader RNA participates in RNA replication, it might be expected that the majority of RNA recombinants isolated from this cross will have recombined at or within the 3′-end of the leader region. To isolate recombinants from this cross, we employed the procedures previously described (Makino *et al.*, 1986), namely, mixed infection was performed at 39° to select only wild-type JHM virus and recombinant viruses. After three successive undiluted passages, the majority of the progeny virus would consist of recombinant viruses, probably as a result of multiple rounds of recombination or amplification (Makino *et al.*, 1986). To determine whether recombinant viruses were generated from this cross, we analyzed the oligonucleotide fingerprints of mRNA 7 derived from the cells infected with the uncloned progeny virus. Any recombinant viruses with an odd number of crossovers will yield a mRNA 7 with a leader sequence derived from one parent and the body sequences derived from the other parent. Figure 5 shows the oligonucleotide fingerprint of mRNA 7 from such a mixed population. It is similar to the fingerprint of JHM mRNA 7, except that it contains the A59-specific, instead of JHM-specific, leader and leader-body junction oligonucleotides. These results suggest that this virus population contained some viruses that had a mRNA 7 containing an A59 leader and a JHM body.

The plaque-purified isolates from this mixed infection were screened by one-dimensional fingerprinting of mRNA 7 of each isolate (Makino *et al.*, 1986). Four of the six isolates (D2, D3, D6, and D8) contained A59-specific leader oligonucleotides (10 and 19) and JHM body oligonucleotides, suggesting that they are recombinants (Fig. 6). Three of the six isolates (D6, D5, and D2) were further examined by two-dimensional fingerprinting of the same mRNA. Figure 7 shows that

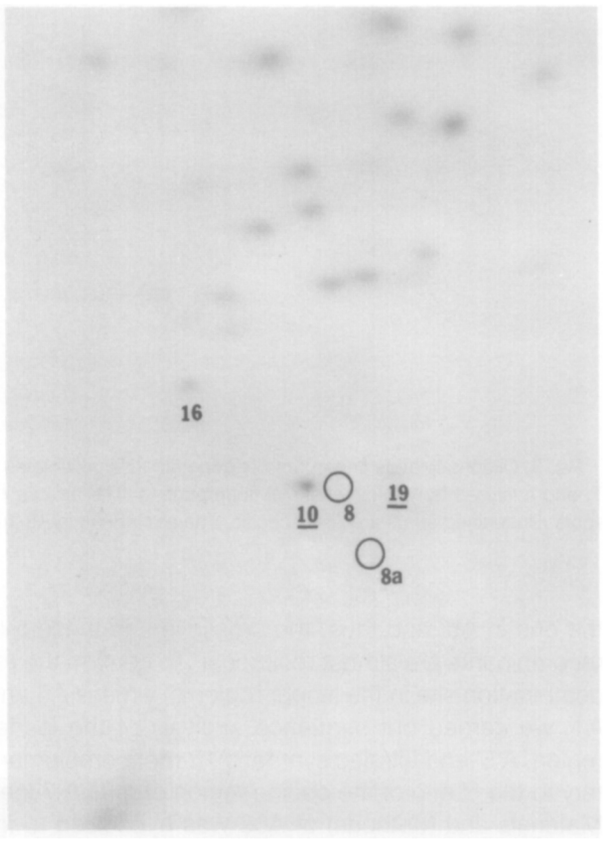

FIG. 5. Oligonucleotide fingerprint of mRNA 7 of the mixed virus population obtained after three serial passages of the progeny of the cross between LA10 and JHM. The underlined spots 10 and 19 indicate A59 leader-specific oligonucleotide and A59 leader-body fusion oligonucleotide, respectively. Oligonucleotide spot 16 is a JHM-specific spot. Note that the JHM-specific leader (8) and leader-body fusion oligonucleotides (8a) are barely detectable.

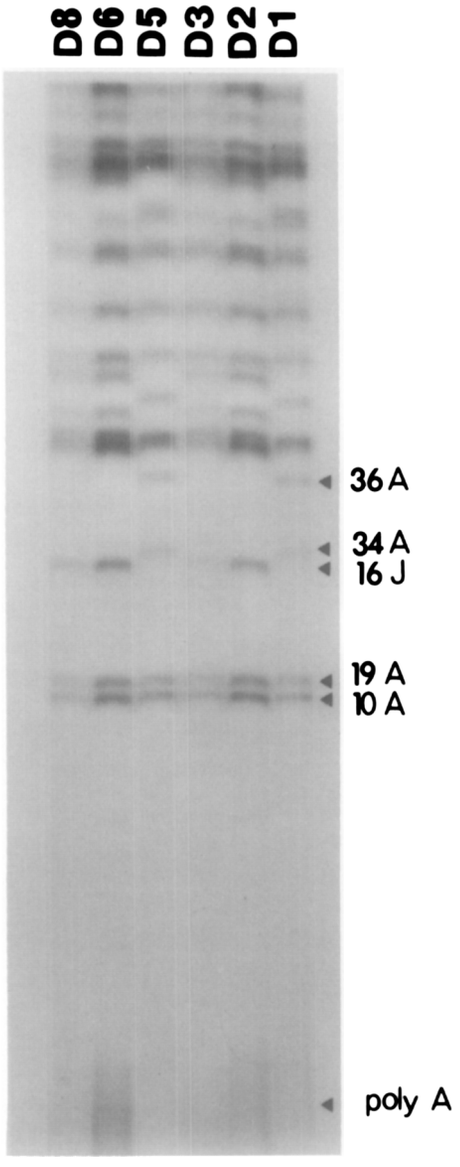

FIG. 6. One-dimensional oligonucleotide fingerprints of mRNA 7 of various plaque isolates from the cross between LA10 and JHM. ^{32}P-labeled mRNA 7 of each isolate was digested with RNase T_1 and applied to a 22% polyacrylamide slab gel (pH 8.2). Oligonucleotides 10 and 19 are A59 leader and leader-body fusion oligonucleotides, respectively. Oligonucleotide 16 is specific for mRNA 7 of JHM. Oligonucleotides 34 and 36 are the coding sequences of mRNA 7 of A59.

D2 and D6 are recombinants, with A59-specific leader and leader-body junction sequences and JHM body sequences. To determine the crossover sites of these recombinants, the genomic RNA of these isolates was examined by two-dimensional oligonucleotide fingerprinting (Fig. 8). Isolate D6 has a fingerprint identical to that of JHM except that the leader oligonucleotide of JHM is replaced with the A59-specific leader oligonucleotide 10. The leader-body junction oligonucleotide could not be identified in the genomic fingerprint. No other A59-specific oligonucleotides were found. This observation, together with its mRNA 7 structure, suggested that the crossover site of this recombinant is close to the 3′-side of the leader sequences at the 5′-end of the genome.

Another isolate D5 has a mRNA 7 identical to parental A59, while some of the faint oligonucleotides, which are derived from the degraded larger mRNAs, appear to be JHM in origin. It suggests that isolate D5 is a recombinant virus with two or more even-numbered crossovers. The fingerprint of the genomic RNA of isolate D5 contains mostly A59 oligonucleotides but is missing two internal A59-specific oligonucleotides, 16 and 4 (Fig. 8). 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 crossover within gene A. However, the precise locations of the crossovers could not be determined since the D5 genome contains several new oligonucleotides, the origins of which are not clear.

A third isolate, D2, contains the 5′-end of A59, as evidenced by the presence of oligonucleotides 10, 20, 5, and 16, which correspond to the leader and 5′-end of the gene A sequences of A59 (Fig. 8). The corresponding JHM spots 8, 3, and 12 are missing. The remaining oligonucleotides are all JHM. Thus, the crossover point is close to the 3′-end of the gene A. However, two JHM-specific spots (40 and 44), which were located close to the junction of genes A and B (Makino *et al.*, 1984), are missing. The corresponding A59 spot 6, which maps in the same location (Lai *et al.*, 1981), is present. Thus, there are probably two additional crossovers in this region. Therefore, the isolate D2 may represent the progeny of a triple crossover. However, since there is only one A59-specific oligonucleotide identified in this region, it cannot be firmly established that this region contains genetic crossovers, instead of base mutation. We have attempted to examine the genome structure of the other three isolates; however, they are very poor virus producers. Therefore, we have not been able to obtain sufficient quantity of radiolabeled RNA for structural analysis. The genetic structures of the recombinants described in this report are summarized in Fig. 9.

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

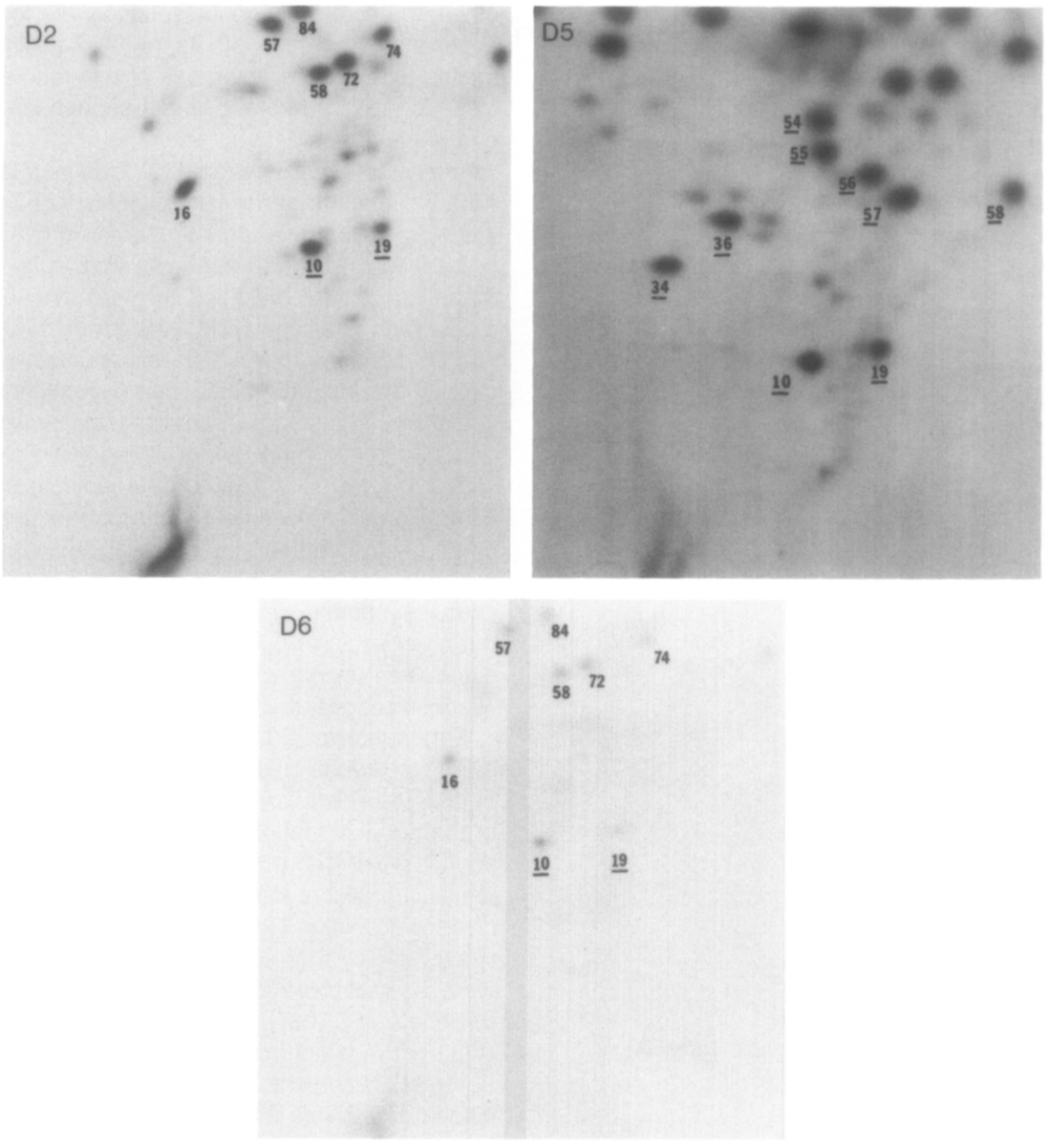

FIG. 7. Two-dimensional fingerprints of mRNA 7 of isolates D2, D5, and D6. The underlined spots are A59 specific. Those not underlined are JHM specific. The numbering system is the same as in Fig. 1.

crossovers in the coding region of the RNA genome. The data presented in this report show at least two new classes of murine coronavirus recombinants. We have demonstrated for the first time that recombination can occur within the leader region itself. Furthermore, we have found recombination at multiple sites on a single viral genome, suggesting that it is possible to have double and possibly multiple crossover events during replication. It should be noted that the recombinational nature of the virus isolates studied in this report was established by the detection of new T_1-oligonucleotides derived from the other parent and the loss of the corresponding oligonucleotides in the original parent. In most cases, two or more contiguous T_1-oligonucleotides in the same region are substituted. Thus, they represent true genetic crossovers involving long stretches of sequences within RNA genomes, rather than base mutations.

The isolation of the double recombinants D5, A5, and A4 reemphasizes our previous contention that recombination occurs at a high frequency (Makino *et al.*, 1986). Prior to this study, high frequency of recombination was shown by the number of recombinants in a population. These three isolates demonstrate that recombination can occur several times within the same viral genome. The possibility that recombinant D2 is a

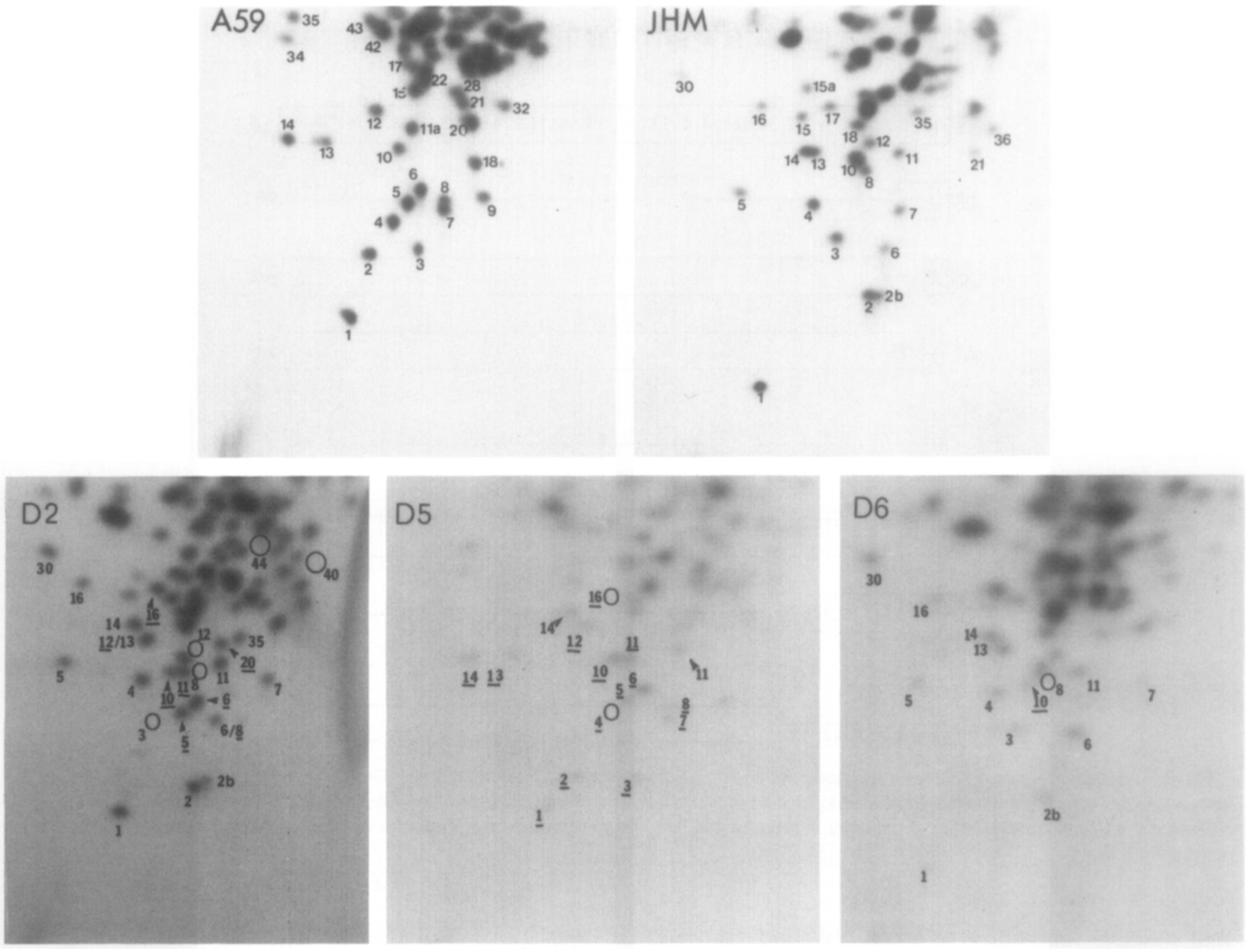

FIG. 8. Two-dimensional fingerprints of genomic RNA of various recombinants. The underlined spots are A59 specific and those not underlined are JHM specific. Circles represent missing spots.

triple cross makes this argument even stronger. Whether they represent crossing-over during single or multiple rounds of RNA replication is not clear.

Recombinant isolates A1 and A5 were shown to have crossovers within the leader region by both oligonucleotide fingerprinting and sequence analysis. The crossover points in these viruses are between 35 and 60 nucleotides from the 5′-end of the genome. Due to the high degree of sequence homology between JHM and A59 in this region, the exact site of recombination cannot be more precisely defined. It should be noted that the recombinational nature of these two viruses could not be unequivocally established. Since A59 and JHM differ in the leader region only by a single base change at the nucleotide 35 and an insertion of 5 nucleotides (UCUAA) at nucleotide 60 in the JHM genome (Fig. 4), the intraleader recombinant A1 is different from the parental A59 by only a single base. Thus, it cannot be ruled out that A1 arose by base mutation rather than by recombination. However, the findings that this virus was detected at a high frequency, that nucleotide 35 was converted to a nucleotide identical to JHM, and that a reciprocal change (in recombinant A5) was detected at the same site, suggests that this virus is more likely to be a true RNA recombinant. It is intriguing to note that analysis of the leader sequence demonstrates a potential hairpin structure near this site of recombination (Shieh *et al.*, 1987; Baric *et al.*, 1987). Three leader-containing RNA intermediates between 35 and 60 nucleotides long have also been detected in MHV-infected cells (Baric *et al.*, 1987). 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 recombinants obtained by coinfection with LA10 and JHM were somewhat unexpected. LA10 accumulates only leader RNAs but not mRNAs at the nonpermissive temperature (Baric *et al.*, 1985). The fact

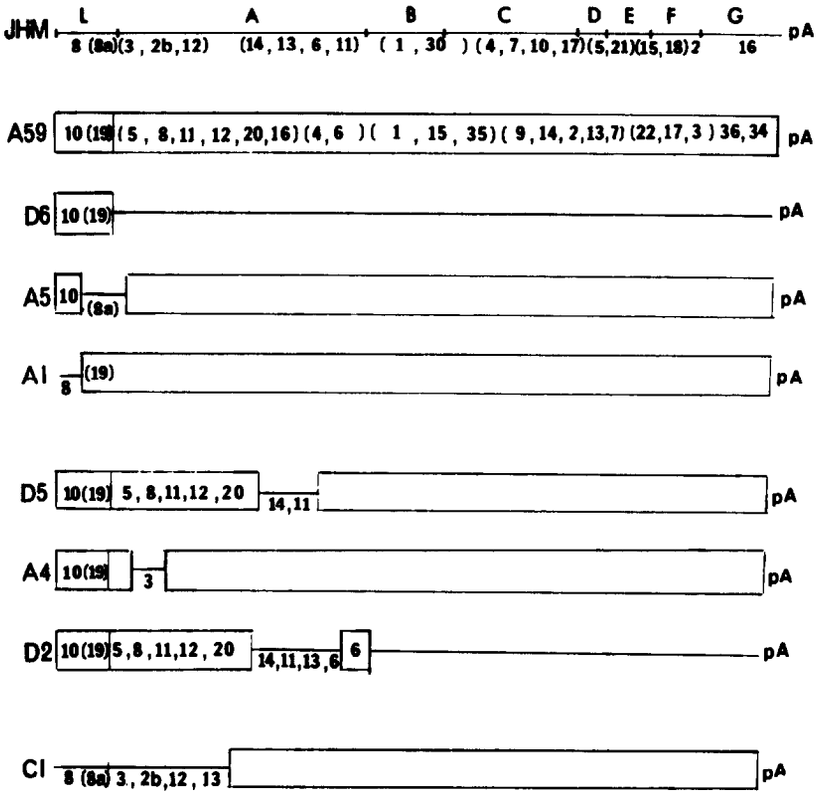

FIG. 9. Schematic representation of the oligonucleotide maps of wild-type and recombinant viruses. The maps of A59 and JHM are derived from previously published data (Lai *et al.*, 1981; Makino *et al.*, 1984). The orders of oligonucleotides in the brackets are arbitrary. A through G represent the seven known genes. The leader is represented by L. The oligonucleotide numbers in the boxes are A59 specific. The map is not drawn to scale.

that multiple recombinants were obtained from this cross suggests that recombination took place either between negative-stranded RNAs or between the small amounts of positive-stranded RNA synthesized due to leakiness of the ts defect. This interpretation is also applicable to the recombination between other ts mutants which fail to synthesize mRNAs. The recombinant D6 is particularly interesting: it contains the A59 leader sequence and the JHM sequences in the remaining portion of the genomic RNA. This recombinant could have resulted from the use of free leader RNAs accompanied by LA10 at the nonpermissive temperature. Whether this type of recombinants constitute a majority of recombinants in this genetic cross would require analysis of additional recombinants.

The high frequency of RNA recombination detected previously (Makino *et al.*, 1986), and confirmed in this report, has led us to propose that coronavirus RNA replication proceeds in a discontinuous and nonprocessive manner, generating free RNA intermediates (Makino *et al.*, 1986). These RNA species could serve as intermediates in normal RNA replication and in RNA recombination during a mixed infection. The isolation of recombinants with multiple crossovers is compatible with the idea of frequent transcriptional pausing. The RNA intermediates generated by such transcriptional pausing have now been characterized in detail in an accompanying paper (Baric *et al.*, 1987). Several recombinants, e.g., A5 and A1, have apparently recombined at sites corresponding to these intermediates. It would now be important to examine whether all of the recombination sites map to areas on the genomic RNA which give rise to these intermediates.

ACKNOWLEDGMENTS

We thank Monica Mueller and Gary Nelson for excellent technical assistance and Carol Flores for typing the manuscript. This work was supported by the U.S. Public Health Research Grants AI 19244 and NS 18146, National Multiple Sclerosis Society Research Grant 1449, and National Science Foundation Grant DMB-9332.

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