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Recombination between Nonsegmented RNA Genomes of Murine Coronaviruses

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We have isolated a recombinant virus between the A59 and JHM strains of mouse hepatitis virus, which contain a single species of nonsegmented RNA genome. This recombinant was derived by mixed infection of DBT cells with temperature-sensitive mutants of A59 and JHM at nonpermissive temperature. Viruses recovered at this temperature were screened by oligonucleotide fingerprinting of their genomic RNAs. One recombinant virus, B1, was found to contain mostly A59-derived sequences, but the 3 kilobases at the 5′ end of the genomic RNA was derived from JHM. Thus, the crossover point in the B1 genome is located within gene *A*, which codes for the viral RNA polymerases. The study of the intracellular RNA species of B1 virus revealed that probably all of the virus-specific subgenomic mRNA species contained the body sequences of strain A59 but the leader sequences of JHM. This result indicates that the JHM leader RNA, which differs from the A59 leader RNA, could be fused to the mRNAs of a different virus strain during RNA transcription. Furthermore, B1 virus-infected cells contain an additional subgenomic mRNA species which is transcribed from a new initiation site within gene *C*, suggesting that the leader RNA could determine the site of initiation for coronavirus mRNAs. These data represent a first report of RNA recombination between viruses, other than picornaviruses, which contain nonsegmented RNA genomes.

RNA viruses undergo rapid evolution in nature (8). Variability is primarily due to single-base mutations, resulting in either antigenic drift or accumulation of silent mutations. Viruses with segmented RNA genomes, such as reoviruses and influenza viruses, can undergo additional evolution by reassortment of various segments of the RNA, resulting in antigenic and biological changes (7, 24). Whether such an exchange of genetic material can also occur in RNA viruses with nonsegmented genomes has been debatable. Various attempts to demonstrate RNA-RNA recombination in either RNA phages or animal viruses have been largely unsuccessful (8, 9, 22). The only studies suggesting that true recombination occurs between RNA molecules of viruses with nonsegmented genomes are the genetic data of Cooper (6) and others (23) for poliovirus. More recently, biochemical studies have unequivocally established that true recombination occurs in several members of the picornavirus family (11). This phenomenon, however, has so far not been observed with other RNA viruses.

We have been studying the mechanisms of replication and transcription of the genomic RNA of mouse hepatitis virus (MHV), a murine coronavirus. This virus contains a nonsegmented single-stranded, positive-sense RNA genome of 5.4×10^6 daltons (17, 28). This genomic RNA is transcribed first into a single species of full-length negative-stranded RNA (16) which, in turn, is transcribed into seven mRNA species (13, 29). These mRNAs are arranged as a nested set from the 3′ terminus of the RNA genome (13). Furthermore, all of them contain a stretch of roughly 72-nucleotide leader sequences which are encoded from the 5′ end of the genomic RNA (12, 14, 26). The synthesis of these mRNAs probably employs a unique mechanism of leader RNA-primed transcription (2). In this report, we demonstrate that RNA recombination can occur between different strains of MHV. These data offer unequivocal evidence that true RNA recombination can take place in viruses other than picornaviruses which contain nonsegmented RNAs. This mechanism might have contributed to the genetic variability of coronaviruses (18). Furthermore, the properties of these recombinant viruses provided further insights into the mechanism of RNA transcription of MHV.

MATERIALS AND METHODS

Viruses and cells. The A59 and JHM strains of MHV and their temperature-sensitive (*ts*) mutants were used throughout the study. The *ts* mutants of the JHM strain have been described previously (19). Among these, the mutant ts203, belonging to complementation group F (19), is an RNA$^-$

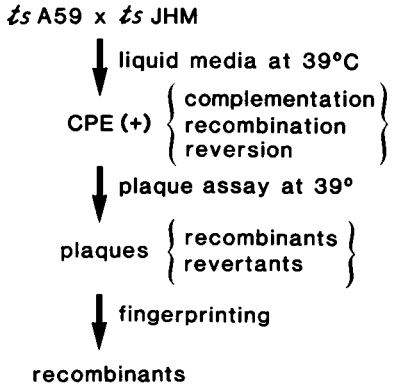

FIG. 1. Schematic protocol for the isolation of recombinant viruses of MHV. CPE, Cytopathic effect.

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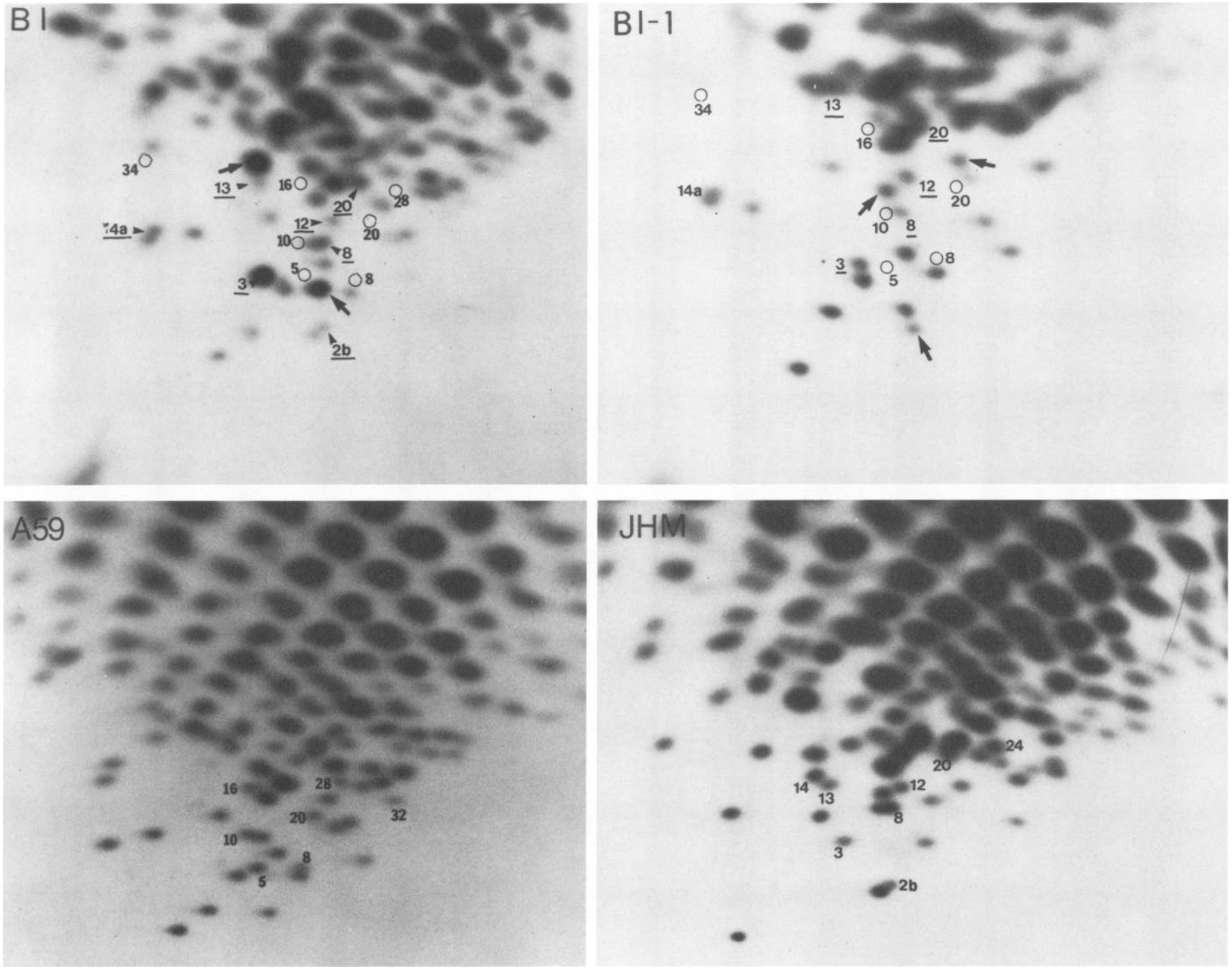
B I
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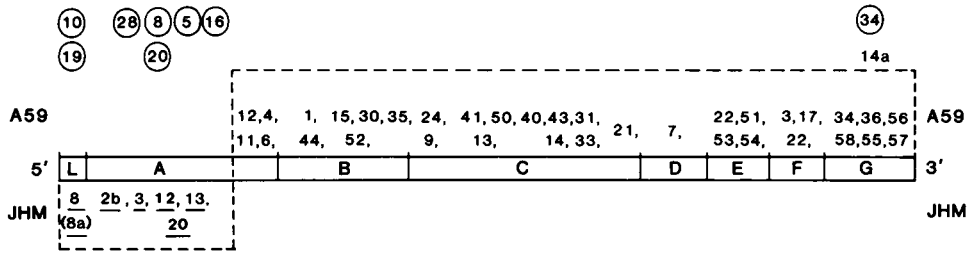


FIG. 3. Schematic representation of the oligonucleotide maps of B1 virus. The maps of strain A59 and JHM oligonucleotides were derived from published data (13, 20, 21, 27). The oligonucleotides within the dashed lines are the spots contained in the B1 virus. The circled numbers are those A59 oligonucleotides which are missing from B1. Oligonucleotide 8a is the leader-body junction oligonucleotide for mRNA 7 of JHM (21). Since most of the sequences of oligonucleotide 8a were derived from the 5'-end leader region of the genome, it was placed in the L region.

mutant (J. Leibowitz, unpublished observations). The *ts* mutants of strain A59 were isolated from 5-azacytidine- or 5-fluorouracil-mutagenized virus stocks. The isolation and characterization of these mutants will be described elsewhere (J. Egbert, T. Wei, M. M. C. Lai, and S. A. Stohlman, unpublished data). The viruses were grown in DBT cell line and maintained in Dulbecco minimum essential medium containing 10% inactivated fetal calf serum as described previously (13, 17).

Isolation of recombinant viruses. The protocol for isolation of recombinants is outlined in Fig. 1. DBT or L-2 cells were grown in a 24-well plate (Becton Dickinson Labware) and coinfected with *ts* mutants of A59 and JHM at a multiplicity of infection of 1 for each virus. The cells were kept at 32°C for 90 min and then shifted to 39°C for 16 h. The wells exhibiting cytopathic effect were marked, and the media were harvested from such wells. The virus-induced cytopathic effect should be the result of virus complementation, recombination, or reversion. The harvested viruses were plaque purified at 39°C. Individual plaques were picked and propagated as virus stocks. Each virus was further plaque assayed at both 32 and 39°C, and those viruses which showed equivalent titers at both temperatures were kept for further studies. These viruses should be either true recombinants or revertants. To distinguish between these two types of virus, these viruses were studied by oligonucleotide fingerprinting of their genomic RNA and intracellular mRNAs.

Radiolabeling and isolation of virion genomic and intracellular RNAs. ^{32}P labeling of the genomic and intracellular RNAs was by the published procedures (13, 17). The 60S genomic RNA was isolated by sucrose gradient sedimentation (17). The intracellular RNAs were separated by electrophoresis on 1% agarose gels (13). The individual RNA species were extracted from the gel by Dounce homogenization in a buffer containing 0.3 M ammonium acetate, 0.01 M Tris hydrochloride (pH 7.4), and 0.2% sodium dodecyl sulfate and incubation at 37°C for 4 h.

Oligonucleotide fingerprinting analysis. Oligonucleotide fingerprinting analysis was performed by two-dimensional polyacrylamide gel electrophoresis of RNase T_1-resistant oligonucleotides as described previously (13). The identity and origin of each oligonucleotide were determined by two methods. (i) For base composition analysis, the T_1-resistant oligonucleotides were digested with RNase A and separated by DEAE paper electrophoresis as described elsewhere (3). (ii) For oligonucleotide fingerprinting of the mixture of two RNAs, the fingerprints of such a mixture were compared with the fingerprints of the individual RNAs (18).

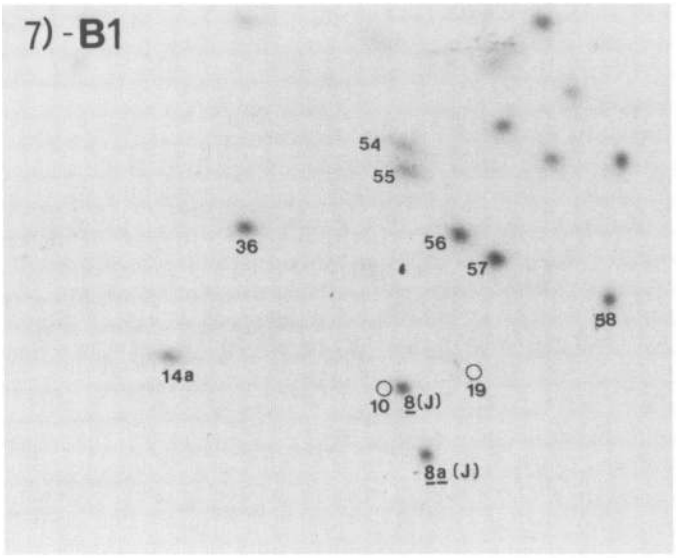
RESULTS

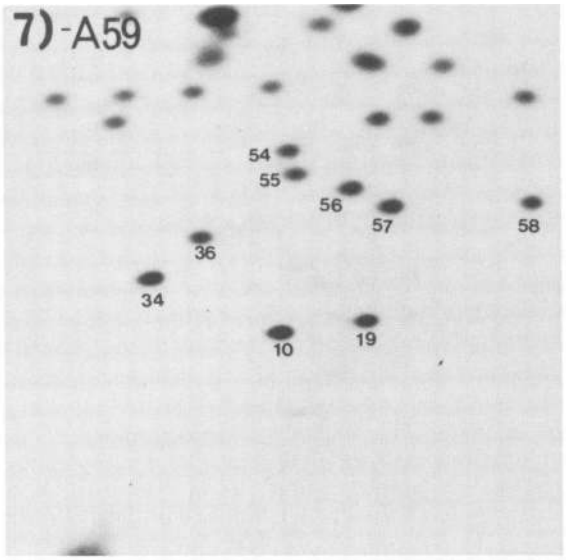
Isolation of recombinant viruses between *ts* mutants of MHV. The protocol for isolation of recombinant viruses was described in Materials and Methods and is outlined in Fig. 1. DBT or L-2 cells were coinfected with *ts* mutants of strains A59 and JHM and propagated at the nonpermissive temperature. Viruses were isolated from the cultures which had developed cytopathic effect characteristic of wild-type MHV. The rationale of this approach is that A59 and JHM strains of MHV have very distinct fingerprint patterns (18) for T_1-resistant oligonucleotides, and yet limited nucleic acid sequencing studies suggest that their genomic sequences are very homologous (1, 25). Therefore, legitimate recombination between these two strains might be possible, and the resulting recombinants containing sequences from both parental viruses can be unequivocally confirmed by fingerprinting of T_1-resistant oligonucleotides. The latter requirement is particularly important, since recombinants must be distinguished from possible revertants of the *ts* mutants. Cytopathic effect was observed in 5 of 30 different crosses

TABLE 1. Base composition of T_1-resistant oligonucleotides

Spot no.	Virus	Base composition
14a	B1	$U_3C_5(AC)_3(A_2C)_3G$
34	A59	$U_3C_5(AC)(A_2C)_3G$
8 (J)	B1, JHM	$U_9C_6(AC)_2(A_4N)G$
10	A59	$U_8C_7(AC)(A_2C)(A_4N)G$
8a (J)	B1, JHM	$U_9C_3(A_2U)_2(A_3U)(A_3C)(A_2G)$
19	A59	$U_8C_2(A_2U)(A_3U)(A_3C)(A_2G)$

FIG. 2. Oligonucleotide fingerprints of the genomic RNAs of two different passages of B1, *ts* A59, and JHM. ^{32}P-labeled 60S RNA of each virus was digested with RNase T_1 and separated by two-dimensional polyacrylamide gel elctrophoresis as previously described (13). The numbering system for A59 is according to Lai et al. (13, 15) and that for JHM is according to Stohlman et al. (27) and Makino et al. (20, 21). The underlined spots in B1 are those derived from JHM, and the circles represent the A59 spots missing in B1. The spots indicated by arrows in B1 were due to contamination from rRNAs, while the origins of the spots indicated by arrows in B1-1 are not clear.


7) - B1
54
55
36
56
57
58
14a
10
8(J)
19
8a (J)


7) - A59
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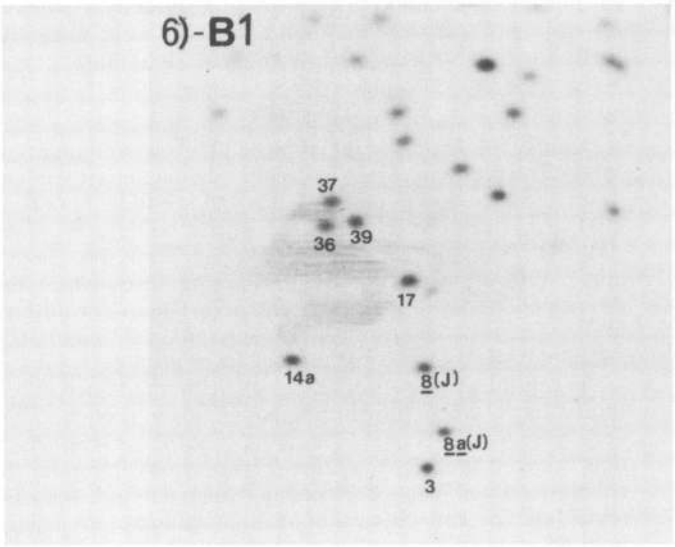
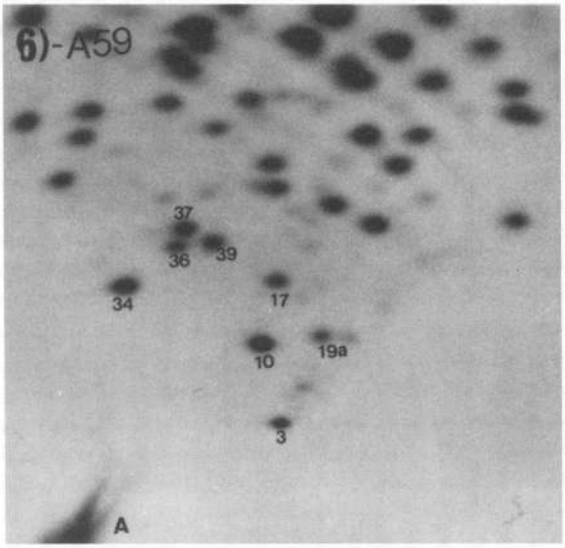

6) - B1
37
36
39
17
14a
8(J)
8a(J)
3


6) - A59
37
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34
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19a
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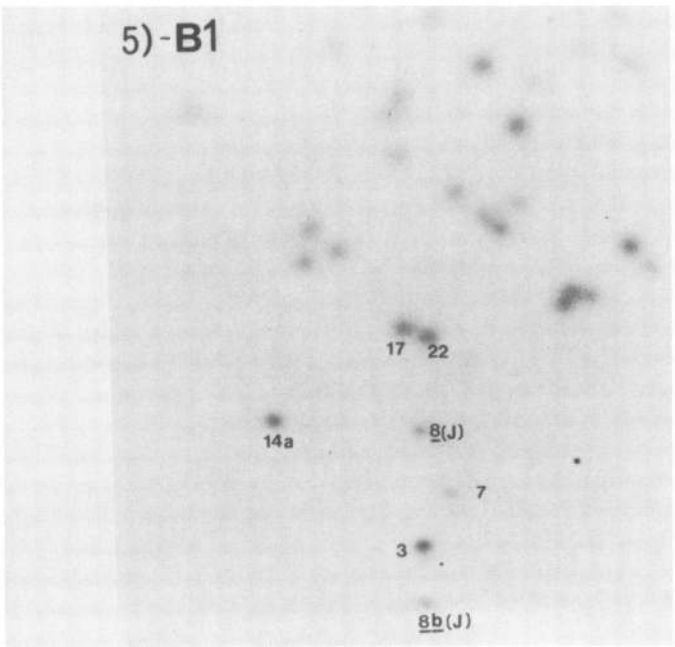
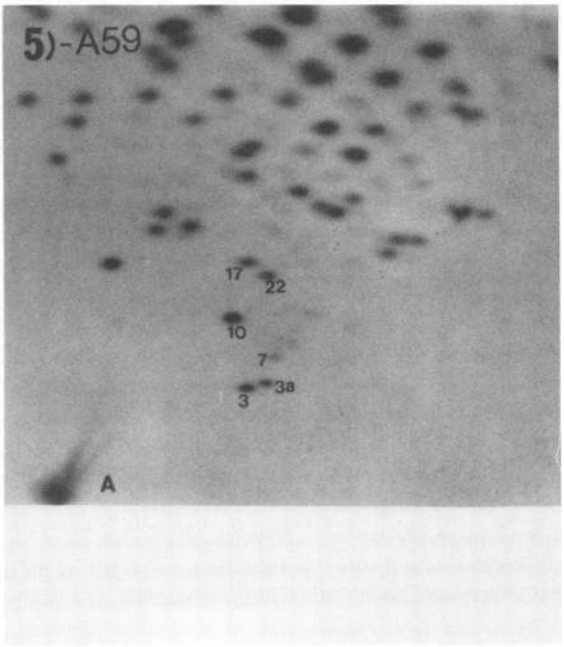
A


5) - B1
17
22
14a
8(J)
7
3
8b(J)


5) - A59
17
22
10
7
3a
3
A

involving six *ts* mutants of A59 and five *ts* mutants of JHM. The viruses from these five cultures were plaque purified at 39°C. Each virus isolate was then studied by oligonucleotide fingerprinting of the genomic RNAs. We found that most of these viruses were revertants of the *ts* mutants used. However, we have detected at least three potential recombinant viruses. The frequency of recombination cannot be determined at this time. A detailed characterization of one of these recombinants, B1, which was derived from the cross between A59 *ts* mutant A9 and JHM *ts* mutant ts203 (19), is reported below.

Characterization of the genomic RNA of the isolate B1. To determine whether the B1 virus was indeed a true recombinant between the two *ts* mutants or a revertant of one of these mutants, we studied its genomic sequence by fingerprinting of T_1-resistant oligonucleotides. The ^{32}P-labeled 60S RNA from the purified B1 virus was digested with RNase T_1 and analyzed by two-dimensional polyacrylamide gel electrophoresis. Figure 2 shows the oligonucleotide fingerprints of the B1 isolate at two different passage levels, A59 *ts* mutant A9, and JHM virus. It is clear that B1 contains most of the large T_1-resistant oligonucleotides present in the genome of strain A59. However, it lacks several A59 oligonucleotides, including no. 5, 8, 10, 16, 20, 28, and 34. Among these oligonucleotides, 5, 8, 16, 20, and 28 are mapped contiguously at the very 5′ end of the genomic RNA (13), and oligonucleotide 10 represents part of the viral leader RNA sequences which are also located at the 5′ end of the genome (14, 15). Thus, all of the detectable oligonucleotides in the 5′-end 3-kilobase regions of A59 are missing from B1 virus. Oligonucleotide 34 maps at the 3′ end of the genomic RNA (13, 15). The reason for its disappearance in B1 will be discussed below. B1 virus also contains several oligonucleotides which are not present in the A59 strain. Almost all of these oligonucleotides correspond to the JHM-specific oligonucleotides, i.e., no. 2b, 3, 8, 12, 13, and 20. The identities of these JHM-derived oligonucleotides were determined by comparison with the oligonucleotide fingerprints of the RNA mixture of A59 and JHM (18) and by base composition analysis of the oligonucleotides. All of these JHM-derived oligonucleotides in the B1 genome are localized within the first 3 kilobases from the 5′ end of JHM RNA (21, 27; Fig. 3). In addition, JHM oligonucleotide 8 has been shown to represent the leader-specific sequences of JHM (21). These results strongly indicate that B1 virus represents a true recombinant between A59 and JHM, with the crossover point roughly 3 kilobases from the 5′ end of the genome. B1 virus still contains some oligonucleotides of gene *A* of A59, for instance, oligonucleotides 4, 6, 11, 12, and 32 (13), indicating that the crossover point is within this region, which probably codes for the viral RNA polymerases (4, 5).

The fingerprints of the B1 RNA also contains several oligonucleotide spots which were not derived from either JHM or A59 parents. Among these, no. 14a has a base composition very similar to, but four nucleotides longer than, that of spot 34 of A59 (Table 1), and both spots were located at the same genetic location close to the 3′ end of the genome (Fig. 3). Therefore, spot 14a was very likely derived from a 4-nucleotide insertion of the A59 spot 34. The other unaccountable oligonucleotides (denoted by arrows in Fig. 2) were probably due to contamination of cellular RNAs because of the very low virus yield of this recombinant virus. Similar contaminating oligonucleotides have also been occasionally found in other MHVs when the virus yield is low (Lai, unpublished observation). To increase virus yield, we further passaged the B1 virus in DBT cells. B1-1 was one such late-passaged virus. Its fingerprint is essentially identical to that of early-passage B1. All of the rRNA-derived oligonucleotides were no longer present. However, B1-1 contains three additional oligonucleotides (denoted by arrows), the origins of which are not clear. They might represent minor mutations of some of the oligonucleotides of the original B1 virus.

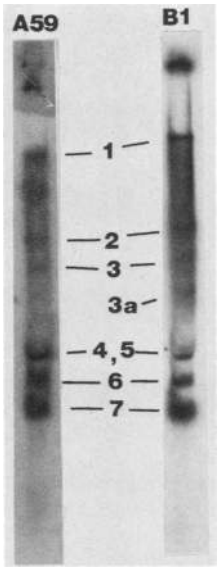


FIG. 5. Agarose gel electrophoresis of intracellular RNAs of A59 and B1. ^{32}P-labeled intracellular virus-specific RNAs of A59- and B1-infected cells were separated by electrophoresis on 1% agarose gels. The designation of mRNAs is in accordance with the published data (13). RNA 3a is the novel RNA species of B1 virus.

Intracellular RNAs of the recombinant virus B1. To further confirm that the 5′ end of B1 virus was indeed derived from JHM while the 3′ end was derived from A59 virus, we examined intracellular virus-specific mRNAs from B1 virus-infected cells. It has been shown previously that every MHV mRNA species contains leader RNA sequences derived from the 5′ end of the genome (12, 14, 26). Thus, every mRNA of B1 should contain an A59-specific body fused to the JHM-specific leader sequences. The fingerprints of B1 mRNAs (Fig. 4) show such a result. All of the T_1-resistant oligonucleotides of mRNA 7 of A59, except oligonucleotides 10, 19, and 34, were present in B1 mRNA 7. Oligonucleotide 10 is the leader-specific oligonucleotide of A59, while oligonucleotide 19 contains the leader-body junction sequences (12, 14, 26). mRNA 7 of B1 virus, however, contains three

FIG. 4. Oligonucleotide fingerprints of mRNAs 5, 6, and 7 of B1 and A59. ^{32}P-labeled intracellular mRNAs of each virus were separated by agarose gel electrophoresis, extracted from the gels, and analyzed by oligonucleotide fingerprinting. Oligonucleotides 10 and 8 (J) are the leader-specific oligonucleotides of strains A59 and JHM, respectively (15, 21). Oligonucleotides 19, 19a, and 3a are the leader-body fusion oligonucleotides of A59 (15) for mRNAs 7, 6, and 5, respectively; 8a (J) and 8b (J) are the corresponding oligonucleotides for JHM mRNAs 7 and 6; and 8b (J) is the corresponding oligonucleotide for JHM mRNA 5 (21).

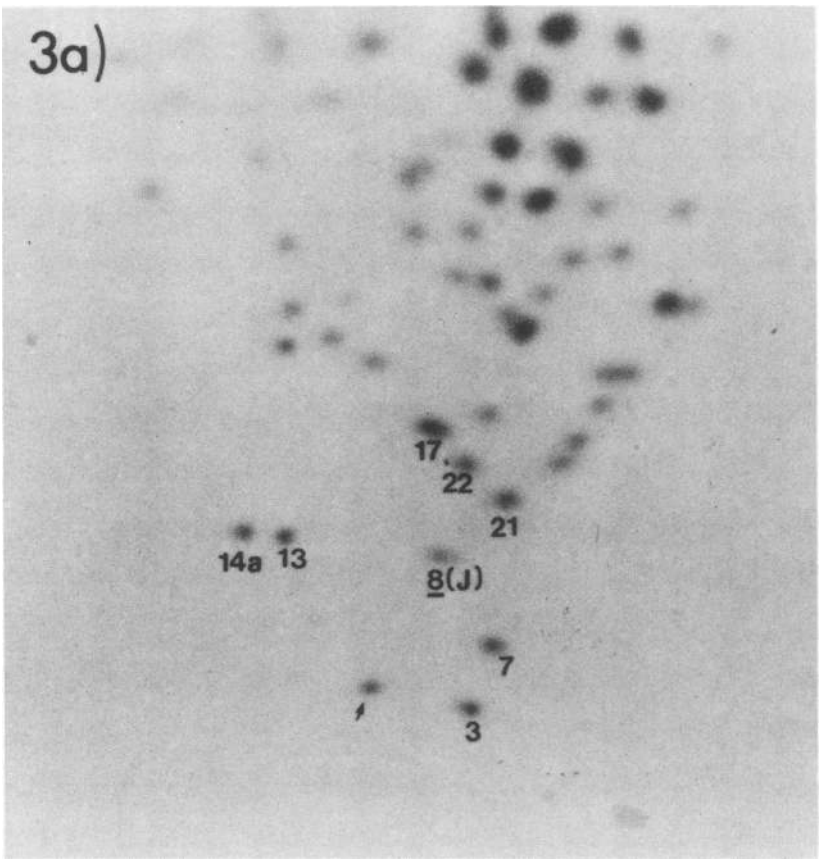


FIG. 6. Oligonucleotide fingerprint of RNA 3a of B1 virus. ^{32}P-labeled RNA was extracted from the gel as described in the legend to Fig. 5 and analyzed by oligonucleotide fingerprinting as described in the legend to Fig. 2. Spot 8 (J) is the leader-specific oligonucleotide derived from strain JHM (21). All the other oligonucleotides were derived from strain A59 except the oligonucleotide denoted by an arrow. This oligonucleotide does not belong to either A59 or JHM and, therefore, likely represents the leader-body junction oligonucleotide.

additional spots, i.e., 8, 8a, and 14a. Oligonucleotide 8 has been shown to be the leader-specific oligonucleotide of JHM, while 8a represents the leader-body junction oligonucleotide of mRNA 7 of JHM (21). The identities of these oligonucleotides were confirmed by base composition analysis (Table 1) and were in agreement with the published sequence data (25, 26). The same conclusion was reached for mRNAs 5 and 6. These results confirm that the leader sequence of B1 was derived from JHM, while the body sequences of probably all of the subgenomic mRNAs were derived from A59. The origins of spots 34 and 14a have been discussed above.

Examination of mRNA species in the B1-infected cells also revealed a very interesting phenomenon. An additional RNA species, 3a, was synthesized in the B1-infected cells (Fig. 5). By comparing the oligonucleotide fingerprint of this RNA with that of A59 mRNA 3 (13; Fig. 6), it can be concluded that B1 mRNA 3a contains the 3'-subset sequences of mRNA 3 of A59, except that it contains the JHM-specific, but not the A59-specific leader spot (Fig. 6; 13, 21). It also contains a novel oligonucleotide (denoted by an arrow in Fig. 6) which is not present in either strain A59 or JHM. It very likely represents the unique leader-body junction oligonucleotide generated by fusion between the JHM leader and A59 body sequences of this novel RNA species. Thus, this RNA has a similar nested-set sequence relationship to the other mRNA species. Therefore, it represents a real mRNA species of B1 virus, which is transcribed from a new transcriptional start site within mRNA 3.

DISCUSSION

The data presented in this report clearly established that true genetic recombination occurs between nonsegmented RNAs during the replication of murine coronaviruses. This is the first report of RNA recombination among RNA viruses, other than picornaviruses, with nonsegmented RNA genomes. RNA recombination is likely to have contributed to the diversity of coronaviruses. It is not known whether picornaviruses and coronaviruses have exceptional RNA structures or unusual mechanisms of RNA replication to permit RNA recombination. Alternatively, RNA recombination could be a general phenomenon among RNA viruses; the failure to detect recombinants in other RNA viruses could be due to the insensitivity of assay systems. The mechanism of recombination is not clear at the present time. Preliminary data on the mechanism of RNA transcription in MHV may shed light on the mechanism of RNA recombination. In the MHV-infected cells, we have detected discrete species of incompletely synthesized leader-containing RNAs which range in size from 130 to 1,000 nucleotides and are dissociated from the RNA templates (2a). These RNA products may represent intermediate transcription products of

the "stop-and-go" model of RNA transcription, as observed in T7 phage (10). These RNA intermediates could conceivably bind back to the negative-stranded RNA templates of a different virus in a mixedly infected cell and generate RNA recombinants. Thus, analysis of recombinants could possibly increase our understanding of the mechanism of RNA transcription in coronaviruses. The role of the 4-nucleotide insertion in A59 oligonucleotide 34, which is located about 1,500 nucleotides from the 3' end of the genome (1), is not clear. It is not present in either of the *ts* mutants used in this study. Interestingly, it is also present in several other recombinants we have studied (unpublished observation). Whether it represents a fortuitous mutation or is closely associated with the mechanism of recombination is not clear at this time.

Since the transcription of coronavirus mRNAs involves the fusion of the leader sequences at the 5' end of the genome and the body sequences of the various mRNA species, the recombinant viruses generated by our approach are very useful for the understanding of the mechanism of transcription. The leader sequences of strains JHM and A59 are very similar except that the JHM leader RNA contains a 5-nucleotide insertion close to the leader-body fusion site and 2 more single-base substitutions among the 72 nucleotides (25, 26). It is apparent from the studies of the intracellular RNAs of B1 virus that JHM leader sequences can be utilized to prime transcription of the A59 mRNAs efficiently and faithfully. More importantly, B1 virus synthesizes an additional species of mRNA, which represents the subset sequences of mRNA 3. Since the body sequences of this new mRNA species and its neighboring genes are entirely encoded by A59, the initiation of transcription of this new RNA species is most likely determined by sequences in the JHM leader RNA. Thus, the presence of this additional mRNA provides further evidence that the leader sequences may regulate transcription of coronavirus mRNAs (2). It should be pointed out that a minor mRNA corresponding in size to mRNA 3a has also occasionally been seen in the JHM-infected cells (29). If these two RNA species, both of which contain JHM leader sequences, have the same transcription start site, it would provide further evidence that the leader RNA determines the sites of initiation.

Complementation analysis of JHM mutants suggests that as many as six MHV genes are required for virus-specific mRNA synthesis in infected cells (19). Both the A59 and JHM mutants used in this study fail to induce MHV-specific mRNA synthesis under nonpermissive conditions (unpublished observation). In the recombinant virus B1, which has a non-ts phenotype, the 5'-most 3 kilobases of A59 RNA have been replaced by a similar length of homologous JHM sequences. This replacement strongly suggests that the genetic lesion in the A59 mutant resides in this region. The lesion in the JHM mutant cannot be localized from our data. However, the kinetics of mRNA synthesis combined with cell-free translation experiments suggests that this mutation maps in the 3' portion of genetic region A (Fig. 3). Further studies of genetic recombination should allow for the mapping of genetic defects in additional coronavirus mutants.

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