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Leader sequences of murine coronavirus mRNAs can be freely reassorted: Evidence for the role of free leader RNA in transcription

(RNase H/reassortment of leader RNA sequences/*trans* acting)

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ABSTRACT **Mouse hepatitis virus (MHV), which replicates in cytoplasm of infected cells, contains an identical leader RNA sequence at the 5′ end of each of the virus-specific mRNAs. Previous studies suggested that the synthesis of these mRNAs does not involve conventional RNA splicing and may instead require priming by a free leader RNA. In this communication, we demonstrate that, during a mixed infection with two different MHVs, the leader RNA sequences from one virus could be detected on the mRNAs of the coinfecting virus at a high frequency, as if the leader sequence and mRNAs were joined together from two randomly segregating RNA segments. This finding demonstrates that MHV mRNA transcription utilizes independently transcribed leader RNA species that possess the *trans*-acting property. This study thus provides further evidence in support of the unique model of "leader-primed transcription" for coronavirus mRNA synthesis.**

Mouse hepatitis virus (MHV), a prototype of the Coronaviridae, contains a nonsegmented single-stranded and plus-sense RNA of M_r 5.4 × 10^6 (1). In infected cells, the virion RNA is first transcribed into a genomic-sized, negative-stranded RNA (2), which, in turn, is transcribed into a positive-sense genomic RNA and six species of subgenomic mRNAs (3, 4). The mRNAs, ranging from M_r 0.6 × 10^6 to M_r 5.4 × 10^6 (3, 4), 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 (4). Furthermore, all of them contain an identical leader sequence of ≈72 nucleotides at their 5′ end (5, 6). Since the leader sequences are encoded only at the 5′ end of the genomic RNA, the presence of leader RNA common to all viral mRNAs suggests that mRNAs are synthesized by joining two noncontiguous RNA segments (5–7). UV transcriptional mapping studies suggested that mRNAs are not derived from cleavage of larger precursor RNAs (8). Also, the replication cycle of MHV does not include a nuclear phase (9, 10), suggesting that coronavirus mRNA synthesis does not involve conventional eukaryotic RNA splicing. Further studies suggest that the leader RNA is joined to mRNAs during transcription, but not posttranscriptionally, and that the leader RNA joining mechanism most likely involves a free leader RNA species participating in mRNA transcription (11). This transcription model proposes that a free leader RNA is synthesized initially, dissociates from the negative-stranded template, and rebinds to the template at the initiation sites of the various mRNAs, serving as a primer for transcription (11). In support of this model, distinct leader sequence-containing small viral RNA species in MHV-infected cells have been detected recently (12). At least part of these RNAs was dissociated from the RNA template on the membrane-bound transcription complex (12). We have also isolated a temperature-sensitive mutant that synthesizes only small leader RNAs, but not mRNAs, at nonpermissive temperature (12). These data suggest that MHV mRNAs are synthesized discontinuously and may involve a free leader RNA for transcription. There is, however, no direct evidence that these free leader-sequence-containing small RNA species detected are utilized for MHV RNA transcription and are not merely abortive transcription products.

In this investigation, we examined whether the free leader RNA species are true intermediates of MHV RNA transcription by studying the leader sequences of mRNAs synthesized in cells coinfected with two different strains of MHV. If MHV transcription uses free leader RNA species from a cytoplasmic pool as a primer, the leader sequences can be expected to be reassorted freely between the mRNAs of the two different MHVs. We have indeed detected such a reassortment of the leader sequences. This study clearly demonstrates that a separate and free leader RNA species is involved in the transcription of MHV mRNAs, providing a solid support for the model of "leader-primed transcription" (11).

MATERIALS AND METHODS

Viruses and Cells. Two MHVs, B1 (13) and CA21 (14), were used. They are recombinant viruses between the A59 and JHM strains. The B1 genome contains JHM-derived sequences at the 5′ end, the rest being derived from A59. Thus, all of the subgenomic mRNAs have A59 body sequences and JHM leader sequences (13). CA21 has the opposite structure (14). These two viruses grow to the same titer (data not shown). Viruses were propagated in DBT cells as described (15). For mixed infection, a multiplicity of infection of four was used for each virus.

Preparation of Virus-Specific Intracellular RNAs. ^{32}P-labeled intracellular MHV-specific RNA was extracted from infected cells as described (4) and separated by electrophoresis on 1% or 1.5% agarose gels after denaturation with 1 M glyoxal (16). Preparative gel electrophoresis was performed on 1% or 1.3% agarose gels containing 6 M urea according to published procedures (15). The RNA was eluted from gel slices by the method of Langridge *et al.* (17).

Cleavage of MHV RNA with RNase H. The use of RNase H to cleave RNA at specific sites was a modification of the procedures described by Donis-Keller (18). Two synthetic oligodeoxyribonucleotides, 5′ G-A-G-C-C-T-G-T-C-T-A-C-G-C-C-C-T 3′ (termed oligomer B1) and 5′ T-T-T-G-C-T-T-T-G-T-C-C-C-T-C-T-C 3′ (termed oligomer CA21) were used. They were complementary to the stretch of sequences described in Fig. 1 (see *Results*). Purified MHV mRNA derived from infected DBT cells (5 × 10^6 cells) was hybrid-

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Abbreviation: MHV, mouse hepatitis virus.

ized with 4 μg of one of the synthetic oligomers for 1 hr at 50°C in a 30-μl solution of 70% formamide/0.4 M NaCl/0.01 M Pipes buffer (pH 7.0)/2 mM EDTA. After ethanol precipitation and several washings with 75% ethanol, the hybrids were incubated in a 10-μl solution of 40 mM Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.03 μg of bovine serum albumin per μl, and 1 unit of *Escherichia coli* RNase H (Pharmacia) for 30 min at 37°C. After incubation, the samples were extracted with phenol/chloroform, and the RNA was precipitated with ethanol. The cleavage products were separated by electrophoresis on 1.3% urea/agarose gels (15) and, in some cases, further purified by oligo(dT)-cellulose column chromatography.

Oligonucleotide Fingerprinting. RNAs were digested with RNase T1 and the resistant oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis as described (15).

RESULTS

Strategy for the Detection of Possible Leader Reassortment. The leader-primed transcription model proposes that a leader RNA is initially transcribed, dissociates from the negative-stranded template, and rebinds to the template at the initiation sites of various mRNAs to serve as the primer for transcription (11). If MHV transcription indeed utilizes free leader RNA as a primer, one might predict that, during a mixed infection, the leader RNA from the different viruses would be mixed and randomly exchangeable between the mRNAs of the coinfecting viruses. The detection of such leader-reassorted mRNAs will establish that the free leader RNA is involved in the transcription of MHV mRNAs.

We selected two viruses, B1 and CA21, with similar growth kinetics and rates of RNA synthesis for this study. The subgenomic mRNAs of these two viruses have distinguishable leader and body sequences, allowing detection of the possible reassortment of the leader sequences in these mRNAs by oligonucleotide fingerprinting (13, 14). We first examined the smallest mRNA, no. 7, for the origins of leader and body sequences in the cells coinfected with B1 and CA21. We have shown previously (13, 14) that the sequences of subgenomic mRNAs of B1 and CA21 are almost identical to the published sequences of A59 (19) and JHM (20) strains of MHV, respectively, except that the leader sequences of B1 and CA21 were, conversely, derived from those of JHM and A59, respectively. To separate the mRNAs of these two viruses in the doubly infected cells, we designed the following procedure to take advantage of the slight sequence divergence between these two viruses. It has been shown that JHM and A59 share 94% homology within the coding sequences of mRNA 7; however, there is <40% homology between nucleotides 1271–1287 from the 5′ end of mRNA 7 of JHM and the corresponding nucleotides of A59 (Fig. 1) (19, 20). Since B1 and CA21 share the mRNA 7 body sequences with A59 and JHM, respectively, we prepared two oligomers that are complementary to this highly divergent region within mRNA 7 (the underlined sequences in Fig. 1). These two oligomers, referred to as oligomer B1 and oligomer CA21, respectively, were used as specific tools to separate B1 and CA21 mRNAs by the strategy depicted in Fig. 2. If free leader RNA is utilized for the transcription of MHV mRNAs, each mRNA species in B1- and CA21-coinfected cells would consist of four structures (Fig. 2). Purified mRNA 7 from coinfected cells was hybridized with one of the synthetic oligomers, treated with RNase H, which specifically digests RNA of RNA·DNA hybrids, and then separated by agarose gel electrophoresis. Three fragments should be generated by such treatment. One fraction (fragment A in Fig. 2) represents the mRNA species that did not bind the oligomer. The mRNA species with sequence complementary to the oligomer would be digested with RNase H, yielding two fragments of different sizes, one containing the 5′ end with the leader sequence (fragment B) and the other containing the 3′ end (fragment C). After electrophoretic separation and purification, each fragment was examined by oligonucleotide fingerprinting. The presence of the leader oligonucleotides derived from both viruses in fragments A and B of either B1- or CA21-specific mRNAs should indicate that the leader RNA sequence can be exchanged between coinfecting viruses.

Evidence for the Presence of Leader-Reassorted RNA. The specificity of such an approach was established by hybridizing oligomer B1 or oligomer CA21 to ^{32}P-labeled mRNA 7 purified from cells singly infected with B1 or CA21. The hybrids were treated with RNase H and the fragments were separated by gel electrophoresis. Fig. 3 *Left* shows that CA21 mRNA 7 was separated into two fragments, B and C, after hybridization with oligomer CA21 but not with oligomer B1. Reciprocal results were obtained when B1 mRNA 7 was used instead of CA21 mRNA 7. These data indicate that this approach can distinguish B1- and CA21-specific mRNAs in mixedly infected cells. RNase T1-resistant oligonucleotide fingerprints of each purified fragment are shown in Fig. 3 *Right*. The fingerprints of both A fragments were identical to those of the intact mRNA 7 of each individual virus (data not shown; ref. 13). The leader-specific oligonucleotides of CA21 (indicated by arrowheads) and those of B1 (indicated by arrows) were distinguishable (4, 5, 13, 14, 21, 22). The rest of the mRNA sequences of these two viruses could also be distinguished. The B fragment contained the respective leader-specific oligonucleotides of B1 and CA21, but they lacked the poly(A) and several oligonucleotides that were present at the 3′ end of the A fragments. C fragments contained the poly(A) and several small T1 oligonucleotides found in A fragments but not in B. These data demonstrated that B and C fragments represent the 5′-end and the 3′-end parts of mRNA 7, respectively.

To determine if the leader RNA sequences could be exchanged between mRNAs of two different MHVs in coinfected cells, the ^{32}P-labeled mRNA 7 derived from B1- and CA21-coinfected cells was purified by urea/agarose gel electrophoresis and hybridized with oligomer CA21 or oligomer B1. The hybrids were treated with RNase H and the resultant fragments were separated by urea/agarose gel electrophoresis (Fig. 4 *Left*). To eliminate contamination from other RNA fragments, each fragment was further purified by selection with oligo(dT)-cellulose column chromatography. Oligo(dT)-binding portions of A and C fragments and the nonbinding part of B fragment were collected and further purified by gel electrophoresis. The oligonucleotide fingerprints of each fragment are shown in Fig. 4 *Right*. It is evident that the oligonucleotide fingerprints of all the B1-specific fragments were similar to those obtained from the

FIG. 1. Sequences of the divergent region within mRNA 7 of JHM and A59 (19, 20). Sequences complementary to the underlined regions were used to prepare the B1- and CA21-specific oligomers.

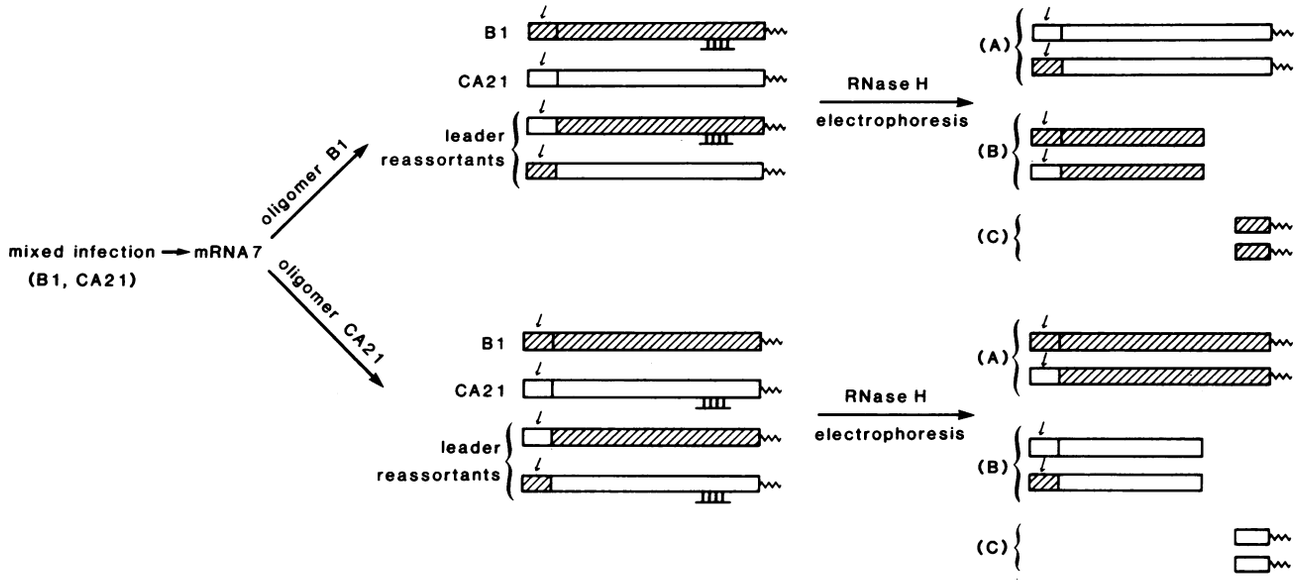

FIG. 2. Strategy for the detection of the leader reassortment. The ^{32}P-labeled purified mRNA 7 from B1- and CA21-coinfected cells was hybridized with oligomer B1 or oligomer CA21 and treated with RNase H. Three RNA fragments (A, B, and C) were obtained after gel electrophoresis. The mRNA 7 of B1 and CA21 is represented as the hatched and open bars, respectively. The leader sequences are indicated by *l* and the poly(A) sequences are represented by zigzagged lines.

single infection (Fig. 3). However, fragments A and B contained two additional oligonucleotides (indicated by arrowheads), which represent CA21-leader-specific T1 oligonucleotides. No other CA21-specific oligonucleotides were detected. The intensity of CA21- and B1-leader-specific oligonucleotides is roughly the same, suggesting that a significant portion of the B1 mRNA 7 contained CA21-specific leader RNA sequence. Reciprocal observations were obtained when the fragments of CA21-specific mRNA 7 were examined, using the opposite oligomer. B1-leader-specific T1 oligonucleotides (indicated by arrows) were detected reproducibly, though in much lower molar yield, in the fingerprints of fragments A and B of CA21 mRNA 7. No other B1 oligonucleotides were present. These studies demonstrated that the leader sequences could be joined to the body sequences of mRNAs of a different virus during mixed infection, although the frequency of leader exchange is not reciprocal and varies with different viruses.

To ensure that the phenomenon of leader sequence exchange is not specific for only the mRNA 7, we also studied mRNA no. 6. Since MHV mRNAs have a nested set structure (4), mRNA 6 includes the sequences of mRNA 7. Thus, the same experimental approach using the same set of oligomers could be used for the study of mRNA 6. Fig. 5 shows the oligonucleotide fingerprints of mRNA 6 of B1 and CA21 derived from single infection and also the B1-specific mRNA

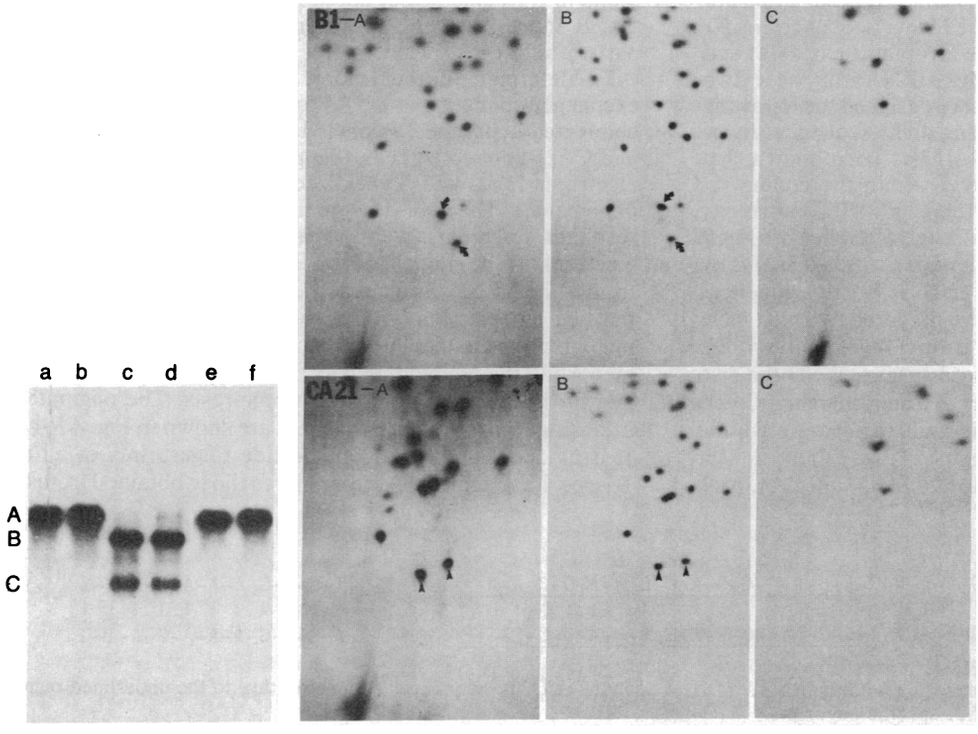

FIG. 3. RNA fragments of mRNA 7 of B1 and CA21 obtained after RNase H digestion. (*Left*) The ^{32}P-labeled purified mRNA 7 of CA21 or B1 was hybridized with oligomer CA21 or oligomer B1 and treated with RNase H. Resultant fragments (A, B, and C) were separated by agarose gel electrophoresis after denaturation with glyoxal. Lane a, B1 RNA only; lane b, B1 RNA hybridized with oligomer CA21; lane c, B1 RNA hybridized with oligomer B1; lane d, CA21 RNA hybridized with oligomer CA21; lane e, CA21 RNA hybridized with oligomer B1; lane f, CA21 RNA only. (*Right*) RNase T1-resistant oligonucleotide fingerprints of the RNA fragments. A, B, and C represent the RNA fragments A, B, and C, respectively, of B1 and CA21. The spots marked by arrows and arrowheads represent the leader-specific oligonucleotides of B1 and CA21, respectively.

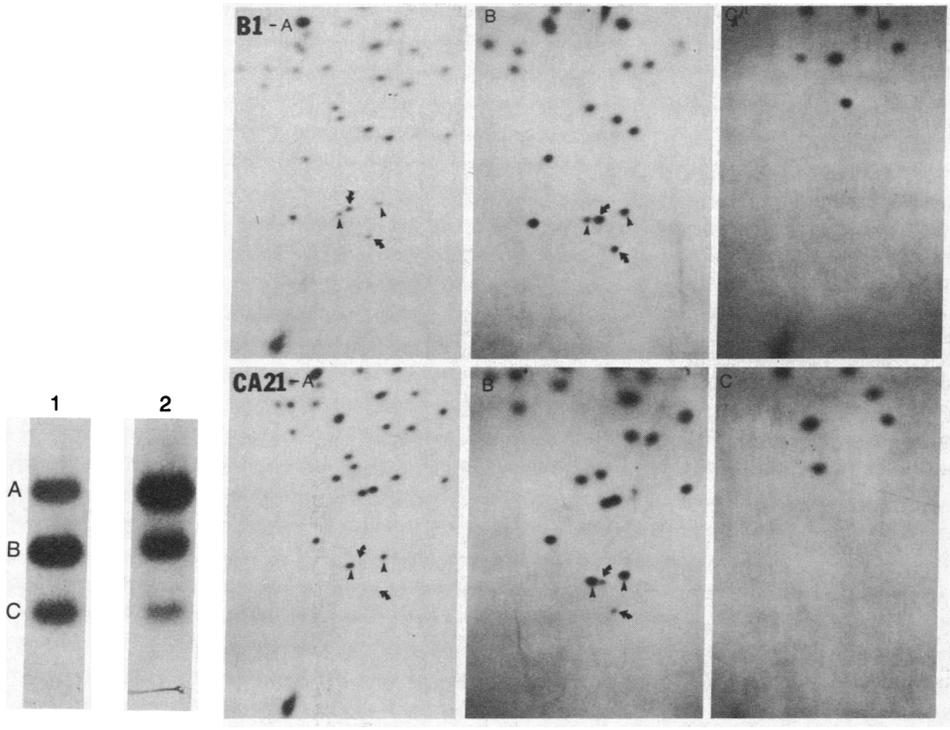

FIG. 4. RNA fragments (A, B, and C) of mRNA 7 from mix-infected cells obtained after RNase H digestion. (*Left*) The ^{32}P-labeled mRNA 7 derived from B1- and CA21-coinfected cells was hybridized with oligomer CA21 (lane 1) and oligomer B1 (lane 2), treated with RNase H, and separated by urea/agarose gel electrophoresis. (*Right*) Oligonucleotide fingerprints of RNA fragments shown in *Left*. A, B, and C represent the RNA fragments A, B, and C, respectively, of B1 and CA21 mRNAs. The spots denoted by arrows and arrowheads represent the leader-specific oligonucleotides of B1 and CA21, respectively.

6 derived from B1- and CA21-coinfected cells. It is clear that, similar to mRNA 7, a significant amount of the CA21-specific leader sequences was joined to the mRNA 6 of the coinfecting B1 virus. These results suggest that the reassortment of leader sequences of mRNAs might be a general property common to all MHV mRNAs.

DISCUSSION

Previous studies suggested that MHV utilizes a unique mechanism for the synthesis of subgenomic mRNAs. Each mRNA contains a stretch of 72-nucleotide leader sequences that is found only at the 5′ ends of genomic RNA and mRNAs of MHV (5–7). The studies of replicative form and replicative intermediate RNAs of MHV led us to propose a leader-primed transcription model (11). This model is supported by the recent demonstration of free leader-containing RNAs in the cytoplasm of MHV-infected cells and by the isolation of a temperature-sensitive mutant that synthesizes only the small leader RNAs without concomitant synthesis of mRNAs (12). These results suggest a discontinuous transcription of the leader RNA and of the mRNAs (11, 12). The data presented in this communication further showed that the leader sequences could be exchanged between the mRNAs of two coinfecting viruses at a relatively high frequency. This high frequency of leader exchange is most consistent with the interpretation that the leader RNA segment functions as a free and separate transcriptional unit that participates in mRNA transcription in *trans*. Therefore, the leader-containing RNA species detected previously in MHV-infected cells (12) are likely to represent *bona fide* RNA transcription intermediates rather than abortive transcription products.

It should be pointed out that only the leader sequences appeared to be involved in the reassortment during mixed infection. However, since the B1 and CA21 viruses share essentially identical sequences at the 5′ ends of coding region of mRNA 7 (19, 20) and since the T1-oligonucleotide fingerprinting method would not detect the entire sequences, it cannot be determined exactly how many nucleotides are involved in the leader reassortment. It has been shown that several small free RNA species containing the leader sequences were present in the MHV-infected cells (12). It would be interesting to know whether all of these RNAs are involved in mRNA transcription. It is conceivable that multiple leader-containing RNA species of different lengths are utilized for mRNA transcription.

The demonstration of the involvement of a free leader RNA in MHV RNA transcription provides solid support for

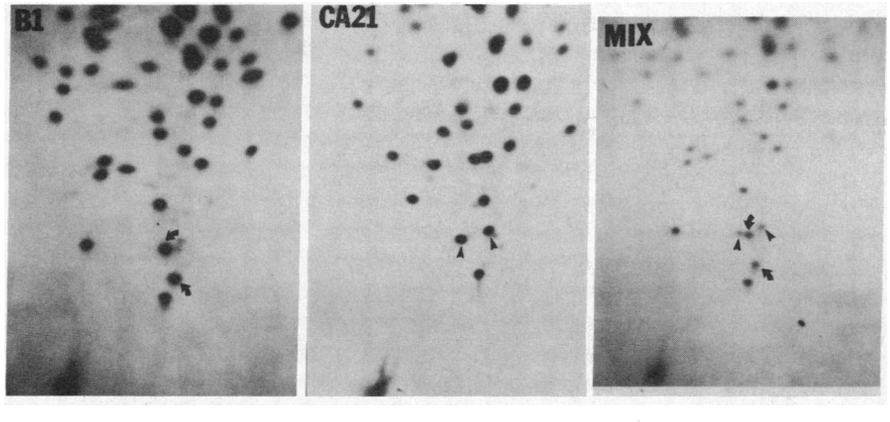

FIG. 5. Oligonucleotide fingerprints of mRNA 6 of B1 and CA21 derived from single and mixed infections. The mRNA 6 from B1- and CA21-coinfected cells was hybridized with oligomer CA21 and treated with RNase H. The fingerprint of the fragment A that was resistant to RNase H digestion is shown. Arrows and arrowheads represent the leader-specific oligonucleotides of B1 and CA-21, respectively.

the model of leader-primed transcription (11). Nevertheless, direct evidence that these free leader RNAs actually serve as a primer for transcription, rather than being spliced to the 5′ end of the elongating mRNA chains, is still lacking. However, preliminary data in our laboratory indicate that the expression of anti-sense leader RNA inhibits the RNA transcription and viral replication of MHV in L cells, suggesting that the removal of the leader RNA would inhibit the transcription of the subgenomic mRNAs of MHV (L. Soe, personal communication). Also, we have not detected any partial mRNA transcription intermediates shorter than the size of the complete leader RNA (72 nucleotides), suggesting that the transcription of MHV mRNAs cannot be initiated without the leader RNA segment (R. Baric, personal communication). These preliminary data suggest that the leader RNA is indeed utilized as a primer for transcription. At the present time, it is not clear whether the transcription of the full-length genomic RNA also requires the participation of a free leader RNA species.

It is interesting that the CA21-specific leader RNA was reassorted to B1 mRNAs at a higher frequency than the reciprocal exchange. Since the leader sequence of CA21 is identical to that of A59 and that of B1 is identical to JHM leader (13, 14), we have performed a similar leader reassortment experiment using A59 and JHM as the coinfecting viruses. Although this approach is complicated by the fact that A59 grows to a much higher titer than JHM, the preliminary data again suggest that the JHM leader RNA is reassorted to the coinfecting viral mRNAs less frequently than the A59 leader (data not shown). The reason for this differential exchange is not clear. Since B1 and CA21 synthesized the same amount of viral RNA and yielded similar amount of infectious virus (data not shown), the advantage of A59 leader is probably not due to differences in the quantity of viral RNA. The understanding of the molecular basis of the selective advantage in leader RNA reassortment for different leader RNAs would be very useful for our understanding of the mechanism of initiation of MHV RNA transcription.

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