Presence of Leader Sequences in the mRNA of Mouse Hepatitis Virus

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To determine the structure and the mechanism of synthesis of mouse hepatitis virus mRNA, the map positions of the large RNase T₁-resistant oligonucleotides of the seven mouse hepatitis virus strain A59 intracellular mRNA species were studied. We found that all but one of the oligonucleotides were mapped at the positions within each mRNA consistent with the nested-set, stairlike structure of mouse hepatitis virus mRNA (Lai et al., *J. Virol.* 39:823-834). However, one oligonucleotide, 10, was mapped near the 5' ends of every mRNA and virion genomic RNA. In other words, oligonucleotide 10 and, therefore, the sequences around the 5' ends of the mRNAs are not colinear with the genomic sequences. Because this oligonucleotide is present only once in the genomic RNA, this result indicates that oligonucleotide 10 is not transcribed from multiple sites on the genomic template, but rather represents a leader RNA sequence which is joined to the body sequences of the different mRNAs during mRNA transcription. This provides the most direct evidence thus far for the presence of leader sequences in the mRNAs of mouse hepatitis virus, which is a cytoplasmic virus. Several possible mechanisms of RNA synthesis are discussed.

Mouse hepatitis virus, a member of the coronaviruses, contains a single-stranded and positive-sensed RNA of 5.4×10^6 molecular weight (7, 8, 12). The virion RNA is transcribed into a genome-sized, negative-stranded RNA by a virus-coded RNA polymerase soon after infection (2, 6). This negative-stranded RNA then serves as the template for the synthesis of seven mRNAs (4, 9, 10). These mRNAs range from 2 to 20 kilobases in size and have a nested-set structure in which the sequences of every mRNA are completely included within the sequences of the next-larger mRNA, and all the mRNAs start from the 3' end of the genome (4). UV light inactivation studies of the synthesis of the mRNAs suggested that each is synthesized independently rather than derived from the cleavage of a precursor RNA (3). Each mRNA contains a cap structure at the 5' end (8). Furthermore, these mRNAs also contain two unusual structural features. First, several contain unique RNase T₁-resistant oligonucleotides which are not found in the genome (4, 5, 9). Second, the 5'-end sequences of these mRNAs share at least five nucleotides (5). More recently, it has also been shown that mRNA no. 7 contains two oligonucleotides which are not present in a slightly larger corresponding 3' fragment of the genomic RNA (11). These structural features suggest that the mRNA sequences

are not completely colinear with the genomic RNA and that there could be structural rearrangements, processing, or both during the synthesis of the mRNAs. One such possible rearrangement is the splicing of a leader RNA segment with the body sequences of the mRNA. This model would explain the presence of common 5'-end sequences. The internal unique T₁-resistant oligonucleotides could then represent the junction sequences between the leader and the body RNA segments. To test such a hypothesis, we examined the T₁-resistant oligonucleotide map of every mRNA. We identified an oligonucleotide present in each mRNA, which could have been derived from such a leader RNA segment. This finding lends additional support for the possible involvement of RNA rearrangement during the synthesis of mRNA by mouse hepatitis virus, a virus which replicates exclusively in the cytoplasm (1, 13).

The A59 strain of mouse hepatitis virus (MHV) was used throughout these experiments. To understand the mechanism of RNA synthesis by this virus, we examined the detailed mRNA structure by mapping the genomic locations of the large RNase T₁-resistant oligonucleotides on every mRNA. This was achieved by "fingerprinting" the various size classes of 3'-end fragments derived from each mRNA according to the strategy published previously (4). This ap-

proach would reveal whether all of the sequences in the MHV mRNAs were indeed colinear with the genomic RNA. For this purpose, the ^{32}P -labeled RNA isolated from the MHV-infected L-2 cells was separated by electrophoresis on 1% agarose gels, and the seven mRNA species were eluted from the gel by published procedures (5). The individual RNAs were heated at 100°C for 3 min to partially degrade the RNA and then were bound to and eluted from an oligodeoxythymidylate-cellulose column. The polyadenylate-containing RNA was collected and separated on a 10 to 25% sucrose gradient (4). The RNAs of different sizes, which represent the RNA segments extending from the 3' end of the genome, were pooled and analyzed by fingerprinting of T_1 -resistant oligonucleotides. The oligonucleotides contained in mRNA no. 7 were separated into three groups according to their map positions on the RNA genome (Fig. 1). (A summary of the oligonucleotide map is presented below in Fig. 3.) It is noteworthy that

oligonucleotides 10 and 19 were mapped in the 5' half of the mRNA. We could not obtain a finer map because of the small size of this RNA. Similar mapping studies were carried out for all of the mRNAs. (Some representative fingerprints are presented in Fig. 2, and schematic maps are presented in Fig. 3.) The oligonucleotides shared between mRNA no. 6 and 7 were mapped at the same corresponding locations. Furthermore, all of the oligonucleotides which are unique to mRNA no. 6, but not present in mRNA no. 7, e.g., oligonucleotides 3, 17, 37, 39, and 63, were located at the 5' half of mRNA no. 6 (3). This result confirms our previous studies on the structure of MHV mRNA (4). However, a surprising finding emerged when the oligonucleotide map of mRNA no. 3 was examined. In this mRNA, essentially all of the oligonucleotides except one were mapped to their expected map positions according to the nested-set, stairlike structure of mRNAs (4). The only exception was oligonucleotide 10, which mapped near the 5'

FIG. 1. Oligonucleotide map of mRNA no. 7. ^{32}P -labeled mRNA no. 7 was isolated from the gel after electrophoresis in 1% agarose gel (4). The RNA was boiled at 100°C for 3 min before fractionation on an oligodeoxythymidylate-cellulose column. The polyadenylate-containing RNA was run on a 10 to 25% sucrose gradient made in 0.01 M NaCl-0.01 M Tris HCl (pH 7.4)-0.05% sodium dodecyl sulfate in an SW50.1 rotor at $27,000 \times g$ for 15 h. The RNAs of different sizes were collected into three fractions as indicated in (A). Each fraction was then digested with RNase T_1 and separated by two-dimensional polyacrylamide gel electrophoresis. The entire process is termed oligonucleotide mapping. (B). Fingerprints of different RNA fractions obtained from (A).

Fig. 2 continued on next page

FIG. 2.—Continued

FIG. 2. Oligonucleotide maps of mRNA no. 6, 3, and 2. ^{32}P -labeled mRNA no. 6, 3, and 2 were processed as described in the legend to Fig. 1. The polyadenylate-containing RNAs of different sizes were digested with RNase T_1 and separated by two-dimensional polyacrylamide gel electrophoresis. (6), (3), and (2) represent corresponding full-length mRNAs. (6-A) and (6-B) represent 18S and 10S RNA fragments, respectively, derived from the 3' end of mRNA no. 6. (3-A) and (3-B) represent 30S and 25S RNA fragments, respectively, derived from the 3' end of mRNA no. 3. (2-A) and (2-B) represent 35S and 25S RNA fragments derived from the 3' end of mRNA no. 2. Note that full-length mRNA no. 6 is 18 to 19S, mRNA no. 5 is roughly 22- to 23S, and mRNA no. 2 and 3 are roughly 35 to 38S (4).

(10,19,57,56,55,51,36,34,52)50(53,54) #7
 (10(?),19a,3,17,37,39,63)(57,56,55,51,36,34,52)50(53,54) #6
 (10(?),3a,75,76,74,22)(3,17,37,39,63)(57,56,55,51,36,34,52)50(53,54) #5
 (10,43)(9,41,50,25,11,14)(21,13,2,33,31,7,75,76,74,22)(3,17,37,39,63)(57,56,55,51,36,34,52)50(53,54) #3
 (10,16,15,44,35)(24,1,43)(9,41,50,25,11,14)(21,13,2,33,31,7,75,76,74,22)(3,17,37,39,63)(57,56,55,51,36,34,52)50(53,54) #2

FIG. 3. Schematic oligonucleotide maps of different mRNAs. The oligonucleotide maps for different mRNAs were deduced from the oligonucleotide fingerprints of Fig. 1 and 2 and from fingerprints not shown here. The maps are presented from left to right in the direction 5' → 3'. The relative order of oligonucleotides in parentheses is not certain. Particularly, oligonucleotide 10 in mRNA no. 5 and 6 could not be determined with certainty because these RNAs were slightly contaminated with mRNA no. 7. However, it is likely that it is mapped at the extreme 5' end of the respective mRNA. These diagrams are not proportional to the actual scale of the mRNAs.

end of the mRNA. In other words, oligonucleotide 10 is located at the 5' side of the unique oligonucleotides, e.g., oligonucleotides 2, 9, 11, 13, 14, 31, 33, 41, and 50, which are present in mRNA no. 3 but not in mRNA no. 5 or 6. That oligonucleotide 10 was present in smaller mRNAs, including no. 5, 6, and 7, indicated that oligonucleotide 10 mapped at different positions in the different mRNAs. Since oligonucleotide 10 is not present more than once in the virion genomic RNA (4, 5), this oligonucleotide could not have been transcribed from multiple sites on the genomic RNA template. A similar conclusion was reached by the oligonucleotide mapping of mRNA no. 2. Again, oligonucleotide 10 mapped near the 5' end of this mRNA, i.e., at the 5' side of the mRNA no. 2-specific oligonucleotides 1 and 24. The oligonucleotide maps are summarized in the schematic diagram in Fig. 3. It is clear that oligonucleotide 10 is located near the 5' end of every mRNA. In other words, this oligonucleotide is not colinear with the genomic sequences.

This result thus strongly indicated that oligonucleotide 10 represents a leader RNA segment which is joined to the body sequences of every mRNA. This provides the strongest evidence so far for the involvement of RNA rearrangement during the synthesis of MHV mRNA, which was suggested by our previous studies of the mRNA structure (4, 5). Thus, there are three separate pieces of evidence, derived from the structural studies of mRNA, in support of RNA rearrangement during mRNA synthesis: (i) 5'-end sequences are identical for at least five nucleotides (5); (ii) several mRNAs contain an internal oligonucleotide (e.g., oligonucleotide 19a in mRNA no. 6 and oligonucleotide 3a in mRNA no. 5) not present in the genome (4, 5, 9); (iii) one oligonucleotide has been mapped near the 5' end and is not colinear with the genomic sequences. The RNA rearrangement involved in MHV mRNA synthesis has to be different from conventional RNA splicing, because MHV replicates exclusively in the cytoplasm (1, 13), and UV light

inactivation studies suggest that MHV mRNA is not derived from a large precursor RNA (3). Several models are compatible with these results. First, the RNA polymerase could jump from the leader RNA region to the body sequences of mRNA. This could be facilitated by the presumable secondary structure of the RNA, bringing these two regions together. Second, the leader RNA could be synthesized independently and then used as a primer for mRNA synthesis. Third, the leader RNA could be synthesized independently and added to the mRNA after completion of its synthesis. Experiments are in progress to determine which model is correct.

The leader RNA sequences of MHV probably consist of, at least, 5'-cap-containing oligonucleotide 82 (5 nucleotides) and oligonucleotide 10 (23 nucleotides) (5). Thus, the minimum length of the MHV leader RNA is 28 nucleotides. As we discussed previously (5), part of oligonucleotide 19 (23 nucleotides) could also be included in this leader region, as this oligonucleotide might represent the junction region between the leader and body sequences for the following reasons: it was mapped close to the 5' end of the mRNAs; it is not present in some of the mRNAs, but oligonucleotides of related sequences are present in its place in other mRNAs (e.g., oligonucleotide 19a in mRNA no. 6) (5, 9). Thus, the MHV leader RNA sequences could be considerably longer.

It should be pointed out that in previous oligonucleotide mapping of genomic RNA (4), we mapped oligonucleotides 10 and 19 to the 3' end of the MHV genome. This conclusion was not consistent with all of the fingerprints, as these two oligonucleotides were detected only in the 3' RNA fragments of roughly 18S and full-length genomic RNA but not in 3' RNA fragments of intermediate size (4). In that report, we attributed this contradiction to artifacts of fingerprinting. However, in light of the results reported here, it is likely that the presence of oligonucleotides 10 and 19 in the small fragments of genomic RNA isolated from purified virion

FIG. 4. Oligonucleotide maps of mRNA no. 1 and genomic RNA. The polyadenylate-containing RNAs derived from mRNA no. 1 and from virion genomic RNA were prepared and fingerprinted as described in the legend to Fig. 1. (V and V-A) Full-length (60S) and 3' fragment (50S), respectively, of virion genomic RNA. (1 and 1-A) Full-length (60S) and 3' fragment (50S), respectively, of mRNA no. 1.

was probably due to the presence of the small amount of mRNA no. 7 in the virion. To map these oligonucleotides more precisely on the genome-sized RNA, we performed (Fig. 4) the oligonucleotide mapping of mRNA no. 1 and viral 60S genomic RNA, with particular emphasis on the larger 3' RNA fragments. Oligonucleotide 10 was detected only in full-length RNA but not in smaller 3' RNA fragments, proving that this oligonucleotide indeed represents the 5'-end sequences and that it is not present more than once in the genomic RNA. The map position of oligonucleotide 19 can not be precisely determined, owing to the complexity of the fingerprints in the vicinity of this oligonucleotide. Spaan et al. (11) recently also reported that two oligonucleotides in mRNA no. 7 (very possibly, oligonucleotides 10 and 19) were not present in the 3' end of the viral genomic RNA. However, the map positions of these oligonucleotides were not determined in that study. Our present finding here thus strongly suggests that a segment of leader RNA exists at the 5' end of every mRNA of MHV.

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