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Replication of Mouse Hepatitis Virus: Negative-Stranded RNA and Replicative Form RNA Are of Genome Length

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There are seven virus-specific mRNA species in mouse hepatitis virus-infected cells (Lai et al., J. Virol. 39:823–834, 1981). In this study, we examined virus-specific negative-stranded RNA to determine whether there are corresponding multiple negative-stranded RNAs. Intracellular RNA from mouse hepatitis virus-infected cells was separated by agarose gel electrophoresis, transferred to nitrocellulose membranes, and hybridized to positive-stranded genomic 60S [^{32}P]RNA. Only a single RNA species of genomic size was detected under these conditions. This RNA was negative stranded. No negative-stranded subgenomic RNA was detected. We also studied double-stranded replicative-form RNA in the infected cells. Only one replicative-form of genomic size was detected. When the double-stranded RNA isolated without RNase treatment was analyzed, again only one RNA species of genomic size was detectable. Furthermore, most of the virus-specific mRNAs could be released from this RNA species upon heating. These results suggest that all of the mouse hepatitis virus-specific RNAs are transcribed from a single species of negative-stranded RNA template of genomic size.

Mouse hepatitis virus (MHV), a coronavirus, contains a single, positive-stranded genomic RNA with a molecular weight of 5.4×10^6 (7, 8). This RNA can be divided into at least seven genetic regions (5) which encode three structural proteins, pp50, gp23, and gp90/180, and possibly several nonstructural proteins (11). The infected cells contain six virus-specific subgenomic mRNAs and a genomic mRNA (2, 5, 9, 13). These mRNAs have a nested-set structure in which the sequence of each mRNA is included within the next-larger mRNA. Furthermore, the sequence of each mRNA corresponds to the 3'-end of the genomic RNA and extends into the 5'-end for a distance corresponding to the size of each individual mRNA species (5). Thus, all of the mRNAs share identical sequences at the 3'-end but presumably contain different 5'-half sequences. We have also found that all of the mRNAs share at least five nucleotides at the 5'-ends and that some mRNAs contain an oligonucleotide which is not present in the genomic RNA (6). These data suggest that the synthesis of MHV mRNAs involves a complex mechanism, possibly an unusual form of RNA splicing which takes place in the cytoplasm (6).

Recently, we have detected two RNA polymerase activities in MHV-infected cells, one detected early (1 h postinfection [p.i.]) and the other detected late (6 h p.i.). These two polymerases have different enzymatic requirements and properties (1). Furthermore, the RNA products of the early polymerase are of negative-strand polarity, compared with the genomic RNA, whereas those of the late polymerase are of positive-strand polarity (P. R. Brayton, M. M. C. Lai, and S. A. Stohlman, unpublished data). Therefore, MHV RNA replicates through a negative-stranded RNA intermediate which serves as the template for the synthesis of mRNAs. Since multiple subgenomic mRNAs have been detected, it is of interest to determine whether the negative-stranded RNA intermediate is a single genomic species or includes multiple subgenomic and genomic species corresponding to the positive-stranded RNA. In this study, we examined this issue by directly examining the negative-stranded RNA species and the double-stranded replicative-form (RF) RNA in MHV-infected cells. We found that only a negative-stranded genomic RNA intermediate could be detected.

MATERIALS AND METHODS

Viruses and cells. MHV strain A59 (MHV-A59) was used throughout. In some experiments, strain JHM was also used. These two strains have been described previously (8). Virus was grown in DBT cells in Dulbecco minimal essential medium (DMEM) supplemented with 1% fetal calf serum.

Preparation of intracellular virus-specific RNA. Preparation of intracellular [^{32}P]RNA from MHV-infected cells has been described (5, 6). Briefly, L-2 cells were

preincubated with DMEM containing 1/10 the normal concentration of phosphate and 0.5% fetal calf serum for 10 to 12 h before infection. After virus adsorption, the cells were incubated in phosphate-free DMEM containing twice the normal concentration of vitamins and amino acids, 2 μg of actinomycin D per ml, 1% dialyzed fetal calf serum, and 250 μCi of $^{32}P_i$ (ICN Pharmaceuticals) per ml. At 7 to 9 h p.i., the cells were chilled on ice and lysed with a buffer containing 10 mM Tris (pH 8.5), 60 mM NaCl, 1 mM EDTA, and 0.5% Triton N-101. Nuclei were removed by centrifugation at 1,800 × *g* for 5 min. Sodium dodecyl sulfate (SDS) was added to the supernatant fluid to a final volume of 1% SDS, and the supernatant fluid was then extracted with chloroform-phenol (1:1). The RNA was precipitated with 2 volumes of ethanol.

For preparation of unlabeled intracellular RNA, the same protocol was followed except that the infected cells were grown in unmodified DMEM supplemented with 1% fetal calf serum throughout infection.

Preparation of double-stranded RNA. The intracellular [^{32}P]RNA extracted as described above was precipitated by sedimentation at 15,000 × *g* for 15 min. The RNA was resuspended in NTE buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 0.001 M EDTA). NaCl was added to obtain a final concentration of 2 M, and the solution was left at 0 to 4°C for 48 h. The RNA was then sedimented at 13,000 rpm for 30 min. The supernatant was diluted with an equal volume of water and precipitated with 2 volumes of ethanol. For digestion with RNase, the RNA was pelleted at 12,000 rpm for 15 min and suspended in low-salt buffer (0.01 M Tris-hydrochloride [pH 7.4], 0.001 M EDTA), and SSC buffer was added to the solution to a final concentration of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium acetate). The RNA was digested with RNase A (20 μg/ml) at 37°C for 60 min. The RNA was then extracted with phenol-chloroform (1:1) and precipitated with 2 volumes of ethanol.

Agarose gel electrophoresis of RNA. Electrophoretic analysis of RNA was generally performed in 1% agarose gels made in RE buffer, pH 8.1 (50 mM boric acid, 5 mM sodium borate, 1 mM EDTA, and 10 mM sodium sulfate (5, 6), except when RNA was to be transferred to nitrocellulose filters (see below). Electrophoresis was at 90 V for 5.5 h. After electrophoresis, the wet gel was wrapped with cellophane and autoradiographed.

For extraction of RNA from the agarose gel, the agarose fraction containing RNA was homogenized in a buffer containing 0.3 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 0.001 M EDTA, and 0.5% SDS and continuously agitated at room temperature for 12 h. After the gel pieces were removed, the RNA was precipitated with 2 volumes of ethanol.

RNA blotting and filter hybridization. Intracellular RNA was analyzed by a modification of the filter hybridization method of Thomas (15). Briefly, intracellular virus-specific RNA was denatured in 1 M glyoxal–50% dimethylsulfoxide (DMSO)–10 mM sodium phosphate buffer (pH 6.8) at 50°C for 1 h (10). The RNA was then electrophoresed in a 1% agarose gel made in 10 mM phosphate buffer (pH 6.8) at 90 V for 5.5 h. After electrophoresis, the RNA was transferred to nitrocellulose membrane filters by the procedure described by Thomas (15). Hybridization was performed in 10 ml of buffer containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 6.8), 250 μg of sonicated, denatured salmon sperm DNA per ml, 0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone, 250 μg of rRNA per ml, 10% dextran sulfate, and 5 × 10^6 cpm of 60S MHV [^{32}P]RNA (specific activity, 5 × 10^6 to 10 × 10^6 cpm/μg). Hybridization was performed at 42°C for 3 days. The RNA blots were washed with four changes of room-temperature 2× SSC containing 0.1% SDS and then with two changes of 50°C 0.1× SSC containing 0.1% SDS. They were then exposed to Kodak XR film at −70°C with an intensifying screen.

RESULTS

Characterization of negative-stranded RNA in MHV-infected cells. We first characterized the negative-stranded RNA in MHV-infected cells by a direct approach. The total cytoplasmic RNA of MHV-A59-infected L-2 cells was extracted at 7 to 9 h p.i., when the virus-specific RNA synthesis was at its maximum level (5). The total RNA was then denatured with DMSO-glyoxal by the procedure of McMaster and Carmichael (10) and separated by electrophoresis on a 1% agarose gel. A parallel control experiment with MHV-A59-specific intracellular [^{32}P]RNA analyzed under identical conditions revealed that there were at least six virus-specific RNA species (Fig. 1, lane A). These species have been extensively studied and represent positive-stranded genomic and subgenomic virus-specific mRNAs (5, 6). The unlabeled RNA was transferred from the agarose gel to a nitrocellulose membrane and hybridized to 60S [^{32}P]RNA extracted from the purified MHV-A59 virion (7). The viral genomic RNA contains only positive-stranded RNA (7, 8); therefore, this ^{32}P-labeled probe would detect only negative-stranded RNA species. Only one RNA band, corresponding in size to the virion genomic RNA, was detected (Fig. 1, lane B). There was occasionally some faint radioactivity in the lower part of the gel; however, it was not reproducible and did not represent discrete RNA bands.

This observation suggests that there is only a single negative-stranded RNA species in the MHV-infected cells and that it has approximately the same size as that of the genomic RNA. We could not rule out the possibility that there were very small amounts of negative-stranded subgenomic RNA; however, this possibility seems very unlikely, since the amount of smaller positive-stranded subgenomic mRNAs far exceeded that of genomic RNA (Fig. 1, lane A) (4, 9). Therefore, our data suggest that all the subgenomic and genomic mRNAs are transcribed from a negative-stranded genomic RNA template. Since the RNA band detected by this procedure was broad (Fig. 1, lane B), we could not rule out the presence of several species of negative-stranded RNA with very similar molecular weights. Alternatively, the heterogeneity of

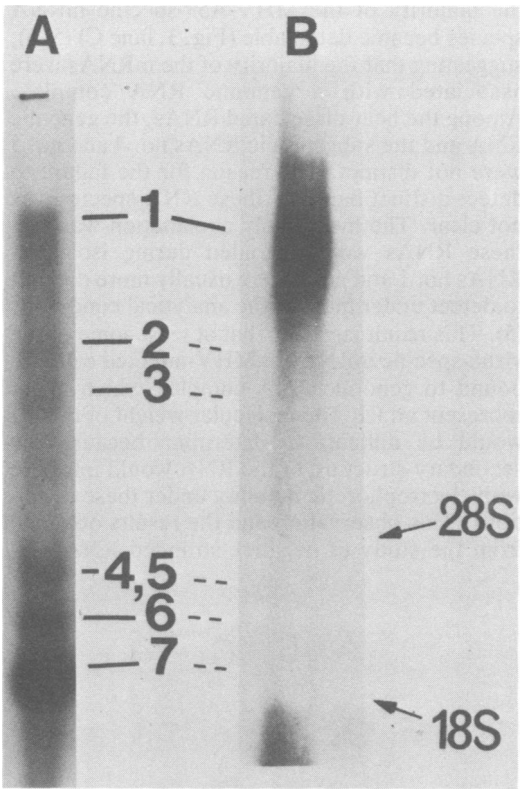

FIG. 1. Electrophoretic analysis of MHV-specific positive- and negative-stranded RNAs. Lane A, Intracellular [^{32}P]RNA from MHV-infected cells was heat denatured and separated by electrophoresis on a 1% agarose gel. After electrophoresis, the wet gel was exposed to Kodak film directly. Lane B, Unlabeled intracellular RNA was denatured with glyoxal-DMSO (10) and separated by electrophoresis as described for lane A. After electrophoresis, the RNA was transferred to a nitrocellulose filter and hybridized to viral genomic [^{32}P]RNA by published procedures (15). The filter was washed, dried, and exposed to Kodak film with an intensifying screen.

the RNAs could have been an artifact of extraction, as similar heterogeneity was noticed for the positive-stranded genomic RNA (Fig. 1, lane A).

Characterization of double-stranded RF RNA. To confirm the interpretation derived from the analysis of negative-stranded RNA, we studied the double-stranded RF RNA involved in the synthesis of MHV mRNA. Virus-specific intracellular [^{32}P]RNA was precipitated with 2 M NaCl at 0 to 4°C for 48 h. The double-stranded RNA in the soluble fraction was found to be enriched (Table 1). However, 24% of this fraction at most was RNase resistant. This value was consistently obtained in repeated experiments. The reason for the failure to obtain a higher percentage of double-stranded RNA was not clear. Presumably, the rest of the 2 M NaCl-soluble fraction was small single-stranded RNA which could not be precipitated with high salt concentrations. Similar observations have been made for the RF RNA of influenza virus (3). The soluble fraction was digested with RNase A (20 μg/ml) in 0.3 M NaCl to remove contaminating single-stranded RNA species so we could study the RF RNA. The resulting RNase-resistant RNA was then analyzed by electrophoresis on a 1% agarose gel. This RNA was a single species with an electrophoretic mobility only slightly slower than that of the genomic RNA (Fig. 2, lane A). This RNA was extracted from the gel, denatured with DMSO-glyoxal (10), and analyzed by agarose gel electrophoresis. A single genomic RNA species was detected (Fig. 2, lane B). The electrophoretic mobility of this RNA was slightly faster than or sometimes indistinguishable from that of the double-stranded RF RNA (data not shown). It was unclear which secondary structure rendered these RNAs electrophoretically similar. At the bottom of the gel, there was some faint dispersed radioactivity which might represent the remaining portions, after RNase A digestion, of the various RNA species bound to the genomic RNA (compare with Fig. 3, lane C; see below). This result showed that the double-stranded RF RNA in the MHV-infected cells consisted of a single genomic species. Although it was still possible that some double-stranded subgenomic RF RNA might have a configuration which rendered it inseparable from the genomic RF RNA under our electrophoretic condition, this possibility seems quite unlikely in view of the fact that the size of the smallest RNA is only $\frac{1}{10}$ that of the genomic RNA (5, 9, 13). This result, together with the result obtained from the filter hybridization analysis of negative-stranded RNA, strongly suggests that the template RNA for all of the MHV mRNAs is a single negative-stranded genomic RNA species.

Characterization of a partially double-stranded

TABLE 1. RNase resistance of various forms of MHV intracellular RNA[a]

Source of RNA	% RNase resistance
2 M NaCl (soluble)	24
2 M NaCl (precipitate)	2
RI RNA (Fig. 2, lane A)	100
RI RNA (Fig. 3, lane B)	61
RNA no. 6 (Fig. 3, lane C)	9
RNA no. 7 (Fig. 3, lane C)	9

[a] RNA was dissolved in 0.5 ml of a solution containing 0.3 M NaCl, 0.03 M sodium acetate, 0.01 M Tris-hydrochloride (pH 7.4), 1 mM EDTA, and 20 μg of RNase A per ml and incubated at 37°C for 60 min. The amount of RNase-resistant RNA was determined by precipitation with trichloroacetic acid.

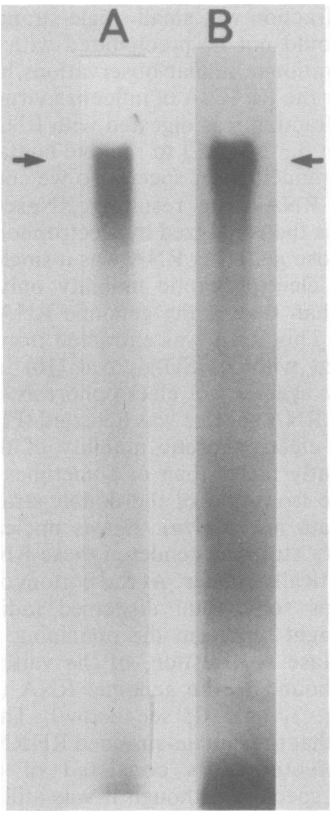

FIG. 2. Electrophoretic analysis of double-stranded RF RNA. Lane A, Double-stranded RF RNA (arrow) isolated from MHV-infected cells and then digested with RNase was analyzed by electrophoresis on 1% agarose gel as described in the legend to Fig. 1. Lane B, The RNA band in lane A was eluted from the gel, denatured with glyoxal-DMSO, and analyzed by agarose gel electrophoresis (arrow) as described for lane A.

RI. To better understand the RNA replicative intermediate (RI) involved in the synthesis of MHV RNA, we further analyzed the RNA in the 2 M NaCl-soluble fraction without RNase treatment. Any single-stranded RNA partially bound to the negative-stranded RNA could be identified electrophoretically. This RNA fraction was analyzed by agarose gel electrophoresis. There was only one RNA species detected under these conditions, and it was in the region of the gel corresponding to the genomic RNA (Fig. 3, lane B). This RNA had a migration rate similar to that of RNase-treated RF RNA (Fig. 3, lane A). Again, it was unclear which secondary structure rendered these RNA species electrophoretically indistinguishable. No discrete subgenomic RNA was detected even when no RNase digestion was performed. When this RNA species was heat denatured and analyzed by electrophoresis, the majority of the MHV-A59-specific mRNA species became detectable (Fig. 3, lane C) (5, 6), suggesting that the majority of the mRNAs were associated with a genomic RNA complex. Among the heat-dissociated RNAs, the genomic RNA and the subgenomic RNAs no. 4 and no. 5 were not distinct. The reason for the failure to detect distinct bands of these RNA species was not clear. The most likely explanation was that these RNAs were degraded during isolation. RNAs no. 1 and no. 4 were usually more difficult to detect under most of the analytical conditions (5). This result suggests that at least some of the virus-specific mRNAs in MHV-infected cells are bound to genomic RNA complex which might represent an RI. The molecular weight of this RI would be difficult to determine because the secondary structure of the RNA would interfere with electrophoretic mobility under these conditions. This observation and the results obtained from the study of negative-stranded RNA sug-

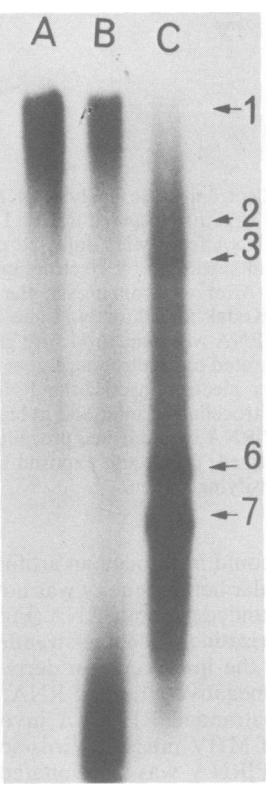

FIG. 3. Electrophoretic analysis of partially double-stranded RI RNA. RNA obtained from the 2 M NaCl-soluble fraction without RNase digestion was analyzed by electrophoresis on a 1% agarose gel. Double-stranded RF RNA (as described for Fig. 2, lane A) was used as a control (lane A). Lane B, Native RI RNA. Lane C, RI RNA heated at 100°C for 1 min before electrophoresis.

gest that this RI consists of various mRNA species associated with a negative-stranded genomic RNA.

To further understand the structure of the various RNA species detected in the MHV-infected cells, we determined the RNase resistance of each RNA species after the species was eluted from the agarose gel. The double-stranded RF RNA was completely resistant to RNase digestion (Table 1). In contrast, RNA isolated directly from the 2 M NaCl-soluble fraction was only 61% resistant to RNase. This result confirmed the hypothesis that the latter form represented an RNA RI which contained single-stranded RNA tails. In contrast, the RNA species released from the RI by heat treatment were sensitive to RNase digestion, consistent with the idea that they represented single-stranded RNA species.

DISCUSSION

From the study of the virus-specific negative-stranded RNA and double-stranded RF RNA in MHV-infected cells, we concluded that there was only one negative-stranded genomic RNA species and that this species served as the template for the synthesis of multiple positive-stranded mRNAs. Therefore, the replication of MHV RNA can be summarized as in Fig. 4. In this scheme, the positive-stranded genomic RNA from incoming virus is first transcribed by the early RNA polymerase (1) into a negative-stranded genomic RNA. This process presumably involves faithful copying of all of the genomic sequences, except for the 3'-polyadenylic acid sequences. However, no details of the structure of the negative-stranded RNA, except for its size, are currently known. The next step in replication is the synthesis of multiple genomic and subgenomic mRNAs. This step apparently involves transcription by the late RNA polymerase (1) of the full-length negative-stranded RNA. This is a very complex step, and very few details about it are currently known. Transcriptional mapping data have shown that the UV target size of each mRNA is the same as its physical size (4), suggesting that each mRNA is transcribed individually at its own initiation point rather that being derived from a genomic RNA. Furthermore, as we have shown previously, the synthesis of subgenomic mRNAs may involve some forms of RNA processing (6). One of these processes could be involvement of RNA splicing (6; unpublished data). It is not clear how and when RNA processing occurs during mRNA synthesis.

The second unanswered question is whether all of the mRNAs are synthesized from the same negative-stranded RNA molecule. The finding in this study that no double-stranded subgenomic RF RNA was detected after RNase digestion suggests that the entire negative-stranded RNA template is protected by binding to mRNAs. Thus, any single molecule of negative-stranded template RNA is probably used for the synthesis of more than one mRNA species and also of genomic RNA (Fig. 4, model a). It is not known, however, whether all of the mRNAs are synthesized on the same RNA template simultaneously. An alternative possibility is that the MHV-specific negative-stranded RNA is always present as a double-stranded RNA. In that case, mRNAS would be synthesized by a conservative mechanism (Fig. 4, model b) as opposed to a semiconservative one (model a). At the present time, our data did not enable us to determine whether the negative-stranded MHV RNA was ever present as free molecules. Our data also did not enable us to rule out the possibility that there were very small amounts of negative-stranded

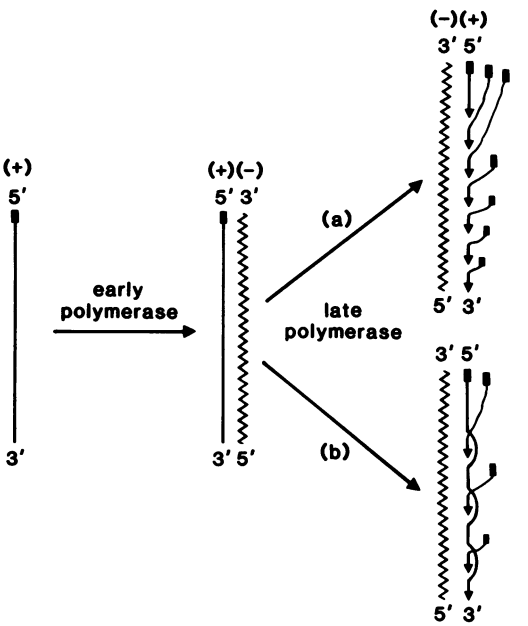

FIG. 4. Proposed scheme for MHV RNA replication. This scheme assumes that nascent RNA strands are immediately displaced by other newly synthesized strands, so that there are multiple single-stranded RNA tails in the RI. The shorter positive-stranded RNA tails complementary to the 5'-end of the negative-stranded RNA template in the RI represent newly synthesized subgenomic mRNAs. The solid rectangles at the 5'-ends of the positive-stranded RNAs represent the presumptive leader sequences (6; unpublished data). The number of RNA strands in the RI is arbitrarily assigned. No evidence has been obtained that the nascent positive strands contain the leader sequences. (a) Semiconservative mechanism for mRNA synthesis; (b) conservative mechanism.

subgenomic RNA. However, if this was the case, the rates of synthesis of the smaller mRNAs must have been very fast, since the amount of smaller subgenomic RNAs far exceeded that of genomic RNA.

This replication scheme raises an interesting question: how is the synthesis of various mRNAs regulated? It has been shown that the smaller mRNAs (no. 7 and no. 6) are far more abundant than the rest of the mRNAs (4, 5, 9). Furthermore, the relative rate of synthesis of most of mRNAs is constant throughout viral replication (9). Therefore, there must be a mechanism which regulates the frequency of transcription of each mRNA species on the negative-stranded RNA template. This remains one of the outstanding unanswered questions regarding the replication scheme of MHV RNA.

It should be noted that our finding of a single negative-stranded genomic RNA is compatible with previously reported UV transcriptional mapping data (4). In that study, UV light was administered to MHV-infected cells at 6 h p.i., at which time synthesis of negative-stranded RNA should have already been completed. Therefore, the data obtained in that study pertain only to the synthesis of positive-stranded RNA. Our present findings also suggest that if RNA splicing or another modification was involved in MHV RNA synthesis, it most likely took place during the synthesis of mRNA, rather than during the synthesis of negative-stranded RNA. Therefore, late RNA polymerase, rather than early RNA polymerase (1), may possess properties which are responsible for such RNA processing.

Our present findings thus distinguish the mechanism of RNA synthesis for coronavirus from that for other positive-stranded-RNA viruses, e.g., togavirus and picornavirus. In the case of togaviruses, several genomic and subgenomic RF RNAs have been detected (12, 14). There is a subgenomic RF RNA corresponding to the subgenomic mRNA of alphaviruses. Furthermore, the RI involved in the synthesis of alphavirus subgenomic mRNA contains a negative-stranded genomic template which is sensitive to RNase at the site of initiation of subgenomic mRNA (12). This appeared not to be the case with coronaviruses, which use a unique mechanism of RNA synthesis. Further understanding of coronavirus RNA synthesis will require the study of the sequences of the mRNAs and the detailed structure of the RI. Such experiments are in progress.

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