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SHORT COMMUNICATIONS

Further Characterization of Mouse Hepatitis Virus RNA-Dependent RNA Polymerases

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Two temporally and enzymatically distinct RNA-dependent RNA polymerase activities associated with membranes of the mouse hepatitis virus (MHV)-infected cells have been identified previously [Brayton *et al.*, *J. Virol.* **42**, 847-853 (1982)]. In this paper, the subcellular distribution and functions of these two polymerases were examined. Fractionation of the postnuclear membranes by sucrose gradient sedimentation showed that the early polymerase activity (detected at 1 hr p.i.) was homogeneous, while the late polymerase (6 hr p.i.) was associated with two distinct membrane fractions. The early polymerase synthesized a single RNA species of viral genomic size and negative sense. In contrast, the light peak of the late polymerase synthesized genomic-sized RNA of positive sense, while the heavy peak of the activity synthesized positive-sensed genomic and subgenomic mRNAs. These findings suggest that the light peak of the late polymerase represents a replication complex while the heavy peak represents a transcription complex. They also establish the essential features of the mode of replication of MHV.

Mouse hepatitis virus (MHV), a murine coronavirus, contains a single-stranded, positive-sensed RNA genome with a molecular weight of 5.4×10^6 (10, 19). The genome contains at least seven genes encoding for the three structural proteins associated with the nucleocapsid (pp 60) and the envelope (gp 25 and gp 90/180), and possibly four or more nonstructural proteins (11, 16, 17). MHV-infected cells contain seven virus-specific mRNAs, which are transcribed from a genomic-length, negative-stranded template (8) and have a "nested set" structure (7, 12). Each mRNA contains polyadenylic acid sequences at the 3'-end as well as a cap structure at the 5'-end (10, 11).

The synthesis of both negative-stranded and positive-stranded RNA probably requires virion-encoded, RNA-dependent RNA polymerases, since it depends upon

de novo protein synthesis (3). Such RNA polymerase activities have recently been demonstrated in MHV strain A59-infected cells (3). Furthermore, the polymerase detected at 1 hr postinfection (p.i.) (early polymerase) and that detected at 6 hr p.i. (late polymerase) had different cationic requirements and pH optima (3). Since these enzymatic activities appeared just prior to the start of early and late virus-specific RNA synthesis, respectively, they are probably responsible for the virus-specific RNA synthesis in the infected cells. In this report, we provide evidence that the early polymerase synthesizes a single genome-sized, negative-stranded RNA, while the late polymerase synthesizes positive-stranded, genomic and subgenomic mRNAs. This result establishes that these polymerases are responsible for MHV RNA synthesis in infected cells. Furthermore, late in the infection the RNA polymerase activities were separated into two membrane-associated complexes, which could represent separate replication and transcription complexes.

We utilized DBT cells (10) infected with the A59 strain of MHV (MHV-A59) throughout this study. We have previously

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shown that both the early and late RNA polymerase activities in this cell line are associated with the postnuclear membrane fractions (3). In order to further characterize the nature and functions of the early and late RNA polymerase activities, we first separated the postnuclear membrane fractions by sucrose gradient sedimentation. These membranes were isolated from the MHV-A59-infected cells at 1 and 6 hr p.i. according to the published procedures (3), pelleted by sedimentation, resuspended in 9% sucrose in $2 \times$ RSB (RSB: 0.01 M Tris-HCl (pH 7.4), 0.01 M NaCl, and 0.0015 M MgCl₂) containing 40 μ g dextran sulfate/ml, and centrifuged in a 9 to 50% sucrose gradient made in the same buffer in an SW27.1 rotor at 25,000 rpm for 1.5 hr. The gradients were fractionated and each fraction was then assayed for the RNA polymerase activity (3).

As shown in Fig. 1a, the membrane preparations from the A59-infected cells at 1 hr p.i. contained a single peak of polymerase activity. The enzymatic properties, including the requirements for Mg²⁺, Mn²⁺, K⁺, and Na⁺, and pH optimum, were identical to those of the unfractionated early polymerase (data not shown) (3). In contrast, the membrane preparation from the infected cells 6 hr p.i. was separated into two polymerase-active populations. The peak of activity which has approximately the same sedimentation characteristics as the early polymerase was designated the light (L) fraction. The second peak which sedimented more rapidly was designated the heavy (H) fraction (Fig. 1b). The polymerase activities contained in these two fractions were examined separately for cationic requirements and pH optima. They were essentially indistinguishable from each other and from the unfractionated late polymerase (3) (data not shown).

To determine the biological functions of these separate polymerase activities, we first examined the polarity of the RNA products made by these membranes. The radiolabeled RNA products were extracted from the membrane fractions and hybridized with at least 100-fold excess of either MHV-A59 genomic RNA or cDNA. As

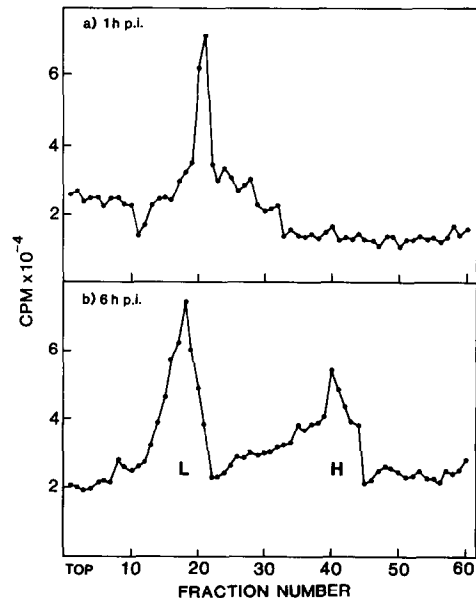


FIG. 1. Sucrose gradient sedimentation of the polymerase-containing membrane components. The postnuclear membrane fractions were prepared from infected cells at 1 and 6 hr p.i. according to the published procedures (3). They were suspended in 9% sucrose in $2 \times$ RSB containing 40 μ g dextran sulfate/ml and centrifuged in a 9 to 50% sucrose gradient made in the same buffer in an SW27.1 rotor at 25,000 rpm for 1.5 hr. Fractions of 0.2 ml each were collected and each fraction was assayed for RNA polymerase activity (3). (a) 1 hr p.i., (b) 6 hr p.i., (L) light activity peak, (H) heavy activity peak. Sedimentation was from left to right.

shown in Table 1, the products of the early polymerase hybridized completely with MHV-A59 genomic RNA, but did not hybridize appreciably with the cDNA complementary to the genomic RNA. Since the genomic RNA is positive stranded, this result suggests that the RNA synthesized by the early polymerase is negative stranded. In contrast, the products of both the L and H fractions of the late polymerase were hybridized completely to the cDNA but not to the genomic RNA, consistent with previous observations that the *in vivo* RNA products late in the infection are mRNAs of positive polarity (7, 12, 14). As a control, the [³H]uridine-labeled genomic RNA hybridized completely to cDNA but not to the genomic RNA, indicating that the cDNA

TABLE 1

HYBRIDIZATION OF THE *IN VITRO* RNA PRODUCTS OF THE EARLY AND LATE POLYMERASE PEAKS WITH MHV-A59 RNA OR cDNA

| Source of RNA products ^a | Percentage hybridization to probe ^c | |
|--|--|-------------------|
| | Genomic RNA | cDNA ^b |
| Early polymerase (1 h.p.i.) | 102 | 9 |
| Late polymerase (L) (6 h.p.i.) | 8 | 125 |
| Late polymerase (H) (6 h.p.i.) | 12 | 115 |
| [³ H]Uridine-labeled genomic RNA | 4 | 99 |

^a The postnuclear membrane fractions (3) were separated by sucrose gradient sedimentation as shown in Fig. 1. The fractions corresponding to the various peaks of polymerase activities were pooled separately and used for the *in vitro* synthesis of RNA according to the procedures published previously (3), except that the reactions were performed for only 30 min to minimize the degradation of RNA products. The [³H]UMP-labeled RNA products were extracted twice with phenol-chloroform (1:1) in the presence of 0.1% sodium dodecyl sulfate (SDS), and separated from the free nucleotides by chromatography on a Sephadex G-50 column. The RNA in the void volume was collected and precipitated with ethanol. The [³H]Uridine-labeled virion genomic RNA was prepared as previously described (10).

^b DNA complementary to the total virion genomic RNA was prepared by a modification of the procedure described by Taylor *et al.* (18). Briefly, the reaction was performed in a buffer containing 5–10 µg viral RNA; 50 mM Tris-hydrochloride (pH 8.0); 8 mM MgCl₂; 40 mM KCl; 1 mM each of dATP, dCTP, dGTP, and TTP; 10 units of reverse transcriptase, and 60 µg of DNase I-digested calf thymus DNA, and incubated at 42° for 2 hr. The reaction was stopped by addition of 10 mM EDTA and 0.5% SDS, and the DNA products were then isolated by chromatography on a Sephadex G-50 column (10 × 1 cm). The RNA templates were removed by incubating in 0.2 M NaOH at room temperature for 18 hr.

^c Nucleic acid hybridization was performed in a 40-µl volume containing 1000 to 2000 cpm of [³H]UMP-labeled RNA products, 1–2 µg cDNA or genomic RNA, 25 µg tRNA, 0.05% SDS, 0.01 M Tris-hydrochloride (pH 7.4), and 0.75 M NaCl, incubated at 68° for 4 hr, and then gradually cooled over a period of 12–18 hr. Following digestion with RNase A (10 µg/ml) in the presence of 0.3 M NaCl at 32° for 30 min, the amount of [³H]UMP-labeled RNA products hybridized was determined by precipitation with 5% trichloroacetic acid.

used is representative of the entire genomic sequences.

The species and size of the RNA products synthesized by the RNA polymerases in the different membrane fractions were also examined by agarose gel electrophoresis. As shown in Fig. 2, the product of the early polymerase is the genome-sized RNA, which is identical to the single species of negative-stranded RNA detected in the MHV-A59-infected cells (8). Surprisingly, the RNA made by the late polymerases (6 hr p.i.) associated with the L fraction of the late polymerase was also exclusively genome-sized RNA (Fig. 2B). In contrast, products made by the H fraction consist of genomic-sized RNA plus RNA species which comigrated with viral mRNAs 5, 6, and 7 (Fig. 2C). No clear-cut RNA bands

corresponding to the viral mRNAs 2 and 3 were observed. However, when the RNA products made by the total unfractionated polymerases from the lysates late in the infection (6 h p.i.) were examined, all seven mRNA species, which correspond to the genomic and subgenomic mRNAs detected in the infected cells, were noticeable (data not shown). These results indicate that the *in vitro* RNA products of the early polymerase are genome-length RNA of negative sense while those of the late polymerase are genomic and subgenomic RNAs of positive sense. This conclusion is consistent with the replication scheme of mouse hepatitis virus proposed previously (8). Furthermore, since the L fraction synthesizes only the positive-stranded genomic RNA, it could represent a replication

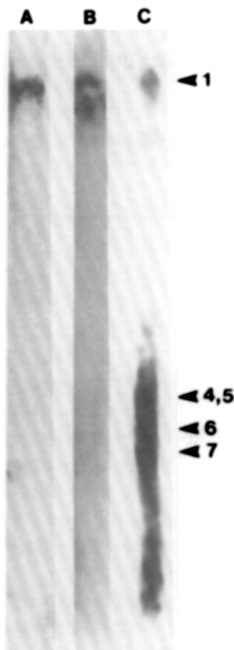


FIG. 2. Agarose gel electrophoresis of the RNA synthesized *in vitro* by various membrane fractions. The polymerase-active membrane fractions separated as in Fig. 1 were used for *in vitro* synthesis of RNA. The RNA products were analyzed by electrophoresis on 1% agarose gels made in RE buffer at 90 V for 5 hr (7). (A) 1 hr p.i., (B) 6 hr p.i. light, (C) 6 hr p.i. heavy. Markers indicate 1, genomic-sized RNA; and 4-7, subgenomic mRNAs. ^{32}P -Labeled intracellular RNA from the infected cells were run in parallel as size markers.

complex, while the H fraction may represent a transcription complex which is responsible for the synthesis of all of the MHV mRNAs. Currently, we do not have evidence to indicate whether the genomic RNAs synthesized by the L and H fractions have different fates, i.e., whether they are encapsidated into virus particles or used as mRNAs. Nevertheless, the possibility that the MHV replication and transcription functions are structurally separated is interesting. This subdivision could explain the finding that, late in the infection, the genomic RNA replicates at a faster rate than the subgenomic mRNAs (Lai, unpublished observation). It might be expected that the structure of the replicative-intermediate (RI) RNA will be different

for these two membrane structures. Such issues are presently being examined.

The separation of RNA polymerase activities into two membrane-associated complexes has also been noted with a porcine coronavirus, transmissible gastroenteritis virus (4), although it is not clear whether these two activities represent the late polymerases corresponding to those in MHV-infected cells. Similar observations have also been made with picornavirus and togaviruses (1, 15), despite the fact that they have different modes of replication from that of coronavirus.

The data presented in this report clearly indicate that the membrane-associated polymerase activities detected by *in vitro* polymerase assays represent the authentic polymerase activities in the MHV-infected cells. They also provide support for the previously proposed replication scheme of MHV (8). Since the mRNA synthesis of MHV involves a very unique mechanism of fusing a leader RNA with the body sequences of mRNAs (9), the MHV polymerase activity, particularly that of the late polymerase, should have a very unique property. The studies of these properties will require purification of the RNA polymerases. Such studies are in progress.

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