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Characterization of Two RNA Polymerase Activities Induced by Mouse Hepatitis Virus

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RNA-dependent RNA polymerase activity was found in mouse hepatitis virus strain A59 (MHV-A59)-infected cells. The enzyme was induced in the infected cells and could not be detected in the MHV-A59 virion. Two peaks of RNA polymerase activity, one early and the other late in infection, were detected. These polymerase activities were in temporal sequence with early and late virus-specific RNA synthesis. Both of them were found to be associated with membrane fractions. There were significant differences in the enzymatic properties of the two polymerases. The early polymerase, but not the late polymerase, could be activated by potassium ions in the absence of magnesium ions and also had a lower optimum pH than the late polymerase. It was therefore probable that the enzymes represent two different species of RNA polymerase and perform different roles in virus-specific RNA synthesis. The effects of cycloheximide on MHV-specific RNA synthesis were determined. Continuous protein synthesis was required for both early and late RNA synthesis and might also be required for shutoff of early RNA synthesis.

Mouse hepatitis virus (MHV), a member of *Coronaviridae* (19), contains a single-stranded RNA of positive polarity (9, 21). The 60S RNA genome of MHV has a molecular weight of 5.4×10^6 and contains polyadenylic acid sequences of about 100 nucleotides at the 3′ end and a cap structure at the 5′ end (9, 10). The RNA contains possibly seven genes (8) which are known to code for three structural proteins, including gp 90/180, pp60, and gp 23 (18). Therefore, the genome has the potential to code for at least four nonstructural proteins, some of which have been identified (1, 3, 14), although their functions are still unclear.

The mechanism of MHV RNA replication is largely unknown. Recently, it was shown that seven mRNA species, including one equivalent to the genomic RNA, can be detected in association with polysomes in MHV-infected cells late in infection (8, 15, 20). These RNAs overlap in their sequences and account for all of the coding regions of the viral genome (8). It was also shown that coronaviruses do not contain reverse transcriptase activity (M. M. C. Lai, unpublished observation) and that neither the host cell nucleus nor continued host cell RNA synthesis is required for MHV replication (4, 22). Therefore, coronaviruses might have a multiplication strategy very similar to that of the other positive-stranded class IV RNA viruses (2, 5, 6, 11, 13, 16). If this is the case, MHV would presumably code for an RNA polymerase which synthesizes negative-stranded RNA early in the infection. The negative-stranded RNA would then serve as template for the synthesis of the seven species of RNA detected late in MHV-infected cells (8, 15, 20).

Conceivably, the synthesis of positive-stranded RNA might require a new or different form of RNA polymerase from the one responsible for the synthesis of negative-stranded RNA. During coronavirus replication, neither RNA polymerase nor negative-stranded RNA has yet been detected. Since the MHV genomic RNA is infectious (21), the virion is not likely to package any RNA-dependent RNA polymerase. In this paper, we report the detection of two types of RNA polymerase activity induced in MHV-infected cells. These polymerases are probably coded for by the MHV genome and are probably responsible for the replication and synthesis of MHV RNA.

MATERIALS AND METHODS

Viruses and cells. The A59 strain of MHV (MHV-A59), originally from J. Robb, University of California at San Diego, was used throughout this study. DBT cells, a continuous murine astrocytoma line (8), were grown on 150-mm plastic tissue culture plates. The virus was adsorbed onto DBT cells at a multiplicity of 1 at 0 to 4°C for 60 min and then incubated at 37°C in Dulbecco modified minimal essential medium containing 0.5% fetal bovine serum.

MHV-A59 and the Indiana strain of vesicular stomatitis virus were grown in DBT cells. Supernatant fluids

were clarified by centrifugation at 15,000 × *g* for 20 min to remove cellular debris. The virus was then pelleted through 20% sucrose in buffer containing 100 mM NaCl, 1 mM EDTA, and 50 mM Tris-maleate (pH 6.2) by centrifugation at 96,000 × *g* for 2.5 h as previously described (8). The pellet was suspended in 2× reticulocyte standard buffer and gently homogenized in a tight-fitting Dounce homogenizer.

Intracellular RNA synthesis. DBT cells, grown on 35-mm plastic tissue culture plates, were treated with 2 μg of actinomycin D per ml for 1 h before infection with MHV-A59. Virus was adsorbed onto the cells at a multiplicity of 1 for 1 h at 0 to 4°C. The cells were then refed with Dulbecco modified minimal essential medium containing 0.5% fetal bovine serum and 2 μg of actinomycin D per ml. For determination of total RNA synthesis, 100 μCi of [^{3}H]uridine per ml (ICN Pharmaceuticals) was added immediately after virus adsorption, and the total RNA synthesis was determined at 9 h postinfection (p.i.). For determination of rate of RNA synthesis at different time points, the [^{3}H]uridine was added on the half-hour, and the RNA synthesis was determined 30 min later. Cycloheximide (Sigma) was added hourly after infection to different plates to achieve a final concentration of 100 μg/ml. Cells were solubilized with 10% sodium dodecyl sulfate and precipitated with 5% trichloroacetic acid, and the amount of labeled product was determined as previously described (8).

Subcellular fractionation. At designated times after infection, the medium was removed, and the cells were washed five times with sterile buffer containing 0.01 M Tris-hydrochloride (pH 7.4) and 0.14 M NaCl at 0 to 4°C. All further manipulations were done at 0 to 4°C. Cells were scraped into buffer containing 0.01 M Tris-hydrochloride (pH 7.4) and 0.14 M NaCl and pelleted by centrifugation at 500 × *g* for 5 min. Cells were suspended in 5 ml of a 0.3 M sucrose solution, allowed to swell for 10 min, and then disrupted using 20 strokes in a Dounce homogenizer. Cellular disruption was monitored by microscopic examination. The preparation was centrifuged at 800 × *g* for 7 min to pellet the nuclei and other large debris. This fraction was designated the nuclear fraction. The supernatant was then centrifuged at 13,000 × *g* for 20 min to pellet the membranous structures. This pellet, suspended in 2× reticulocyte standard buffer (0.01 M Tris hydrochloride [pH 7.4]–0.01 M NaCl–0.0015 M $MgCl_2$) and gently homogenized in a Dounce homogenizer, was designated the membrane fraction. This fraction contained mostly membranous structures, and no nuclei or intact cells were seen by light microscopy. The supernatant was designated the soluble fraction.

Assay of cell-associated polymerase. The assay for cell-associated RNA-dependent RNA polymerase was a modification of the protocol reported by Polatnick and Arlinghaus (12). In the reaction, a 50-μl sample of cellular protein, suspended to a concentration of 1 to 2 mg/ml in 2× reticulocyte standard buffer was assayed in a 200-μl (total volume) solution containing a final concentration of 50 mM Tris hydrochloride (pH 8.0), 4.25 mM magnesium acetate, 10 mM phosphoenol pyruvic acid, 40 μg of phosphoenol pyruvate kinase per ml, 17.5 mM 2-mercaptoethanol, 20 μg of actinomycin D per ml (Merck Sharp & Dohme), 10 μCi of [5,6-^{3}H]UTP (specific activity 50 Ci/mmol) (ICN Pharmaceuticals), and 0.5 mM each of ATP, CTP, and GTP (Sigma). In later experiments, phosphoenol pyruvic acid and phosphoenol pyruvate kinase were omitted. The amount of labeled product synthesized after 1 h at 37°C was determined by precipitation with 5% trichloroacetic acid containing 50 mM sodium pyrophosphate as previously described (8).

For determination of polymerase ionic requirements, the protein sample was suspended in 50 mM Tris-hydrochloride (pH 7.4), and the polymerase reaction was performed in the reaction mixture without magnesium acetate for which various salts were substituted. For pH requirements, the protein sample was suspended as in the standard reaction, but the final solution contained either 50 mM Tris-maleate (pH 6.0 and 6.5) or 50 mM Tris-hydrochloride (pH 7.0, 7.5, 8.0, and 8.5).

Assay for virion-associated polymerase. Virion-associated RNA-dependent RNA polymerase activity was determined by the method of Huang et al. (7). Briefly, 50 μl of purified virus suspended at a concentration of 0.5 mg/ml in 2× reticulocyte standard buffer was assayed in a total volume of 200 μl of solution. The reaction mixture contained 50 mM Tris hydrochloride (pH 7.3), 5.3 mM magnesium acetate, 100 mM sodium chloride, 3.3 mM 2-mercaptoethanol, 830 μg of Triton N-101 per ml, 10 μCi of [5,6-^{3}H]UTP, and 0.5 mM each of ATP, GTP, and CTP. The amount of labeled product produced after 60 min at 35°C was determined by following trichloroacetic acid precipitation as described above.

RESULTS

Effects of cycloheximide on MHV RNA synthesis. To determine whether de novo protein synthesis is required for the replication of MHV RNA, we first examined the effects of cycloheximide on the rate of MHV RNA synthesis. As we previously reported, actinomycin D (1 to 3 μg/ml) does not affect the viral RNA synthesis whereas it inhibits host DBT cell RNA synthesis at least 95% (8). It was shown (8) that the rate of viral RNA synthesis in the presence of actinomycin D reached a peak at 6 to 7 h p.i. and that no early viral RNA synthesis was detectable under the conditions previously reported (8), perhaps due to residual host cell RNA synthesis. To study early virus-specific RNA synthesis, the cells were treated with actinomycin D for 1 h before infection, and the infection was synchronized by adsorbing the virus at 0 to 4°C. Using this approach, early virus-specific RNA synthesis became detectable. As shown in Fig. 1a, the kinetics of virus-specific RNA synthesis exhibited two peaks: early RNA synthesis peaked at 2 h p.i., whereas the major RNA synthesis started at 5 h p.i. and continued until 9 h p.i. The second peak of RNA synthesis under these conditions is slightly later than that reported previously (8). To determine whether RNA synthesis was dependent on de novo protein synthesis, cycloheximide (100 μg/ml) was added at various time points, and the rate of RNA synthesis was determined 30 min later. RNA synthesis was

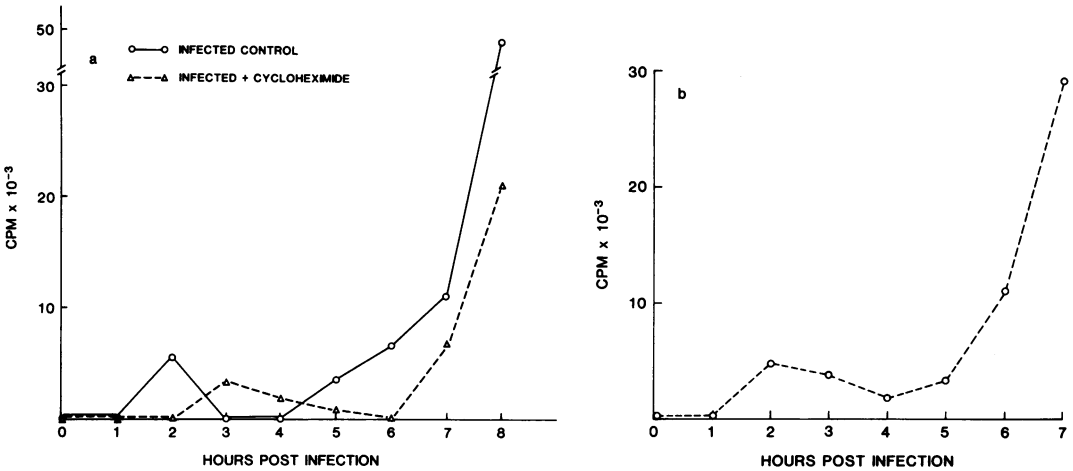

FIG. 1. Rate of RNA synthesis in the presence of cycloheximide. Cycloheximide (100 μg/ml) was added to the infected cells at hourly intervals. At 30 min after the addition of cycloheximide, [^{3}H]uridine was added and the trichloroacetic acid-precipitable counts were determined after a 30-min incubation. For instance, the sample to which cycloheximide was added at 0 h p.i., [^{3}H]uridine was added at 0.5 h p.i., and which was harvested at 1 h p.i., is represented by 1 h p.i. on the abscissa. (b) Accumulated RNA synthesized in the presence of cycloheximide. [^{3}H]uridine was added to the cultures at 0 h p.i., and cycloheximide was added at hourly intervals p.i. Total uridine incorporation was determined at 9 h p.i. For instance, the sample to which cycloheximide was added at 1 h p.i. and which was harvested at 9 h p.i. is represented by 1 h p.i. on the abscissa.

inhibited by cycloheximide throughout infection (Fig. 1a), suggesting that continuous de novo protein synthesis was required for MHV RNA synthesis. Notably, the rate of RNA synthesis when cycloheximide was added 2 to 3 h p.i. was higher than infected controls. This difference was minor but was reproducible. This unexpected result suggested that a protein responsible for shutting off early viral RNA synthesis might be synthesized in the infected cells.

The effects of the continuous presence of cycloheximide on cumulative MHV RNA synthesis were also assessed. [^{3}H]uridine was added at the time of infection, and the cycloheximide was added at different time points after infection. Total RNA synthesized was determined at 9 h p.i. Figure 1b shows that, if cycloheximide was added before 1 h p.i., virus-specific RNA synthesis was not detectable. As expected, the later the cycloheximide was added, the more total RNA was synthesized, suggesting that protein synthesis was required throughout the infection. Surprisingly, the total RNA synthesized when cycloheximide was added 3 to 4 h p.i. was less than that when cycloheximide was added at 2 h p.i. This result is consistent with the higher rate of RNA synthesis in the presence of cycloheximide than in infected control cells within the same period (Fig. 1a). This further suggested the possibility that, at 2 to 3 h p.i., some protein was synthesized which was required for turning off early RNA synthesis. Therefore, the data obtained from the cycloheximide studies suggest that de novo protein synthesis was required for early RNA synthesis, late RNA synthesis, and possibly also for switching-off of early RNA synthesis.

Detection of RNA polymerase activity. The requirement of MHV RNA synthesis for de novo protein synthesis (Fig. 1) suggested that an RNA polymerase might be synthesized at both early and late times during infection. To detect such an RNA polymerase activity, infected cells were disrupted at 1 h and 6 h p.i. and were separated into nuclear, membrane, and soluble fractions. Each subcellular fraction was assayed for RNA polymerase activity without adding any exogenous RNA. RNA polymerase activity could be detected in the three subcellular fractions from infected cells at both 1 h at 6 h p.i. (Table 1). The highest activity was associated with the membrane fraction at both time points. In contrast, uninfected cells contained very little RNA polymerase activity. We therefore concluded that an RNA polymerase activity was induced in the MHV-infected cells.

We further characterized the properties of these RNA polymerase activities. We found that deletion of any nucleoside triphosphate or Mg^{2+} almost completely abolished polymerase activity (Table 2). In contrast, deletion of phosphoenol pyruvic acid and phosphoenol pyruvate kinase had little effect. Therefore, the ATP-generating system was deleted from subsequent

TABLE 1. Association of RNA polymerase activity with subcellular fractions of DBT cells

Fraction	Incorporation of [5,6-^{3}H]UTP (fmol mg of protein^{-1} h^{-1})			
	MHV-A59 infected		Uninfected	
	1 h p.i.	6 h p.i.	1 h p.i.	6 h p.i.
Nuclear	78.0	58.0	6.7	6.7
Membrane	215.0	314.0	1.5	1.5
Soluble	63.0	50.0	1.5	1.5

experiments. Incubation of either template or products with RNase rendered the products completely acid soluble. In contrast, DNase had no effect on the polymerase products. These results established that the polymerase activities induced in the MHV-infected cells are RNA-dependent RNA polymerase.

Detection and characterization of two RNA polymerase activities. As shown above, the RNA polymerase activity was detected both at 1 h and 6 h p.i. It was not clear whether both polymerases represented the same enzyme. We first studied the kinetics of RNA polymerase activity in the MHV-infected cells. The polymerase activity reached a peak at 1 h p.i. and then gradually declined until it was almost undetectable at 3 h p.i. (Fig. 2). The second peak of polymerase activity appeared at 6 h p.i. The kinetics of appearance of RNA polymerase activities paralleled closely the kinetics of RNA synthesis (Fig. 1), although the peaks of RNA synthesis appeared slightly behind those of the polymerases in temporal sequence. Similar results showing two temporal peaks of polymerase activity were obtained in L-2 cells (data not shown). This result suggested that the polymerases detected at 1 h and 6 h p.i. represent separately synthesized RNA polymerases.

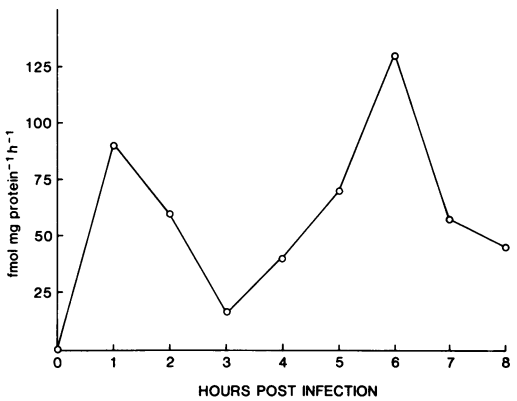

FIG. 2. Kinetics of RNA polymerase activities in MHV-A59-infected DBT cells. The membrane fractions from infected cells harvested at hourly intervals after infection were used to assay for polymerase activity.

TABLE 2. Requirements of RNA-directed RNA polymerase activity

Reaction system	Incorporation of [5,6-^{3}H]UTP			
	1 h p.i.		6 h p.i.	
	fmol mg of protein^{-1} h^{-1}	% Complete assay	fmol mg of protein^{-1} h^{-1}	% Complete assay
Complete assay	367	100	412	100
−Mg^{2+}[a]	14	4	20	5
−PEP & PEP kinase[b]	377	103	413	100
−ATP	12	3	4	1
−CTP	20	5	11	4
−GTP	19	5	4	1
−ATP,CTP,GTP	0	0	0	0
Template + DNase[c]	364	99	409	99
Template + RNase[d]	0	0	0	0
Heated product + DNase[e]	419	114	417	101
Heated product + RNase[f]	0	0	0	0

[a] Membrane pellet was suspended in 2 × reticulocyte standard buffer without $MgCl_2$.

[b] PEP, Phosphoenol pyruvate.

[c] Suspended membrane pellet was incubated at 37°C for 30 min with 50 μg of DNase I/ml before assay.

[d] Suspended membrane pellet was incubated at 37°C for 30 min with 20 μg of RNase A/ml before assay.

[e] At the end of the 1-h assay, the mixture was heated to 100°C for 2 min, cooled to 37°C, and incubated with 50 μg of DNase I per ml for 15 min. The reaction was then terminated.

[f] At the end of the 1-h assay, the mixture was heated to 100°C for 2 min, cooled to 37°C, and incubated with 20 μg of RNase A per ml for 15 min. The reaction was then terminated.

We studied the enzymatic requirements of the two RNA polymerase activities to determine whether they represented two different polymerases with different functions. Both the early and the late RNA polymerases had an optimum enzyme activity at 5 mM Mg^{2+} and 5 mM Na^+, and both activities were stimulated only slightly by 2 mM Mn^{2+} (Fig. 3). A significant difference in the ability of K^+ to activate the early and late polymerases was noted. The early enzyme had an optimum activity at 20 mM K^+, whereas the late enzyme was only slightly stimulated by a wide range of K^+ concentrations. Furthermore, the optimum pH for the early enzyme was pH 7.5, whereas that for the late enzyme was pH 8.0 (Fig. 4). This difference was minor but reproducible. In the presence of the optimum Mg^{2+} concentration, neither monovalent cation had

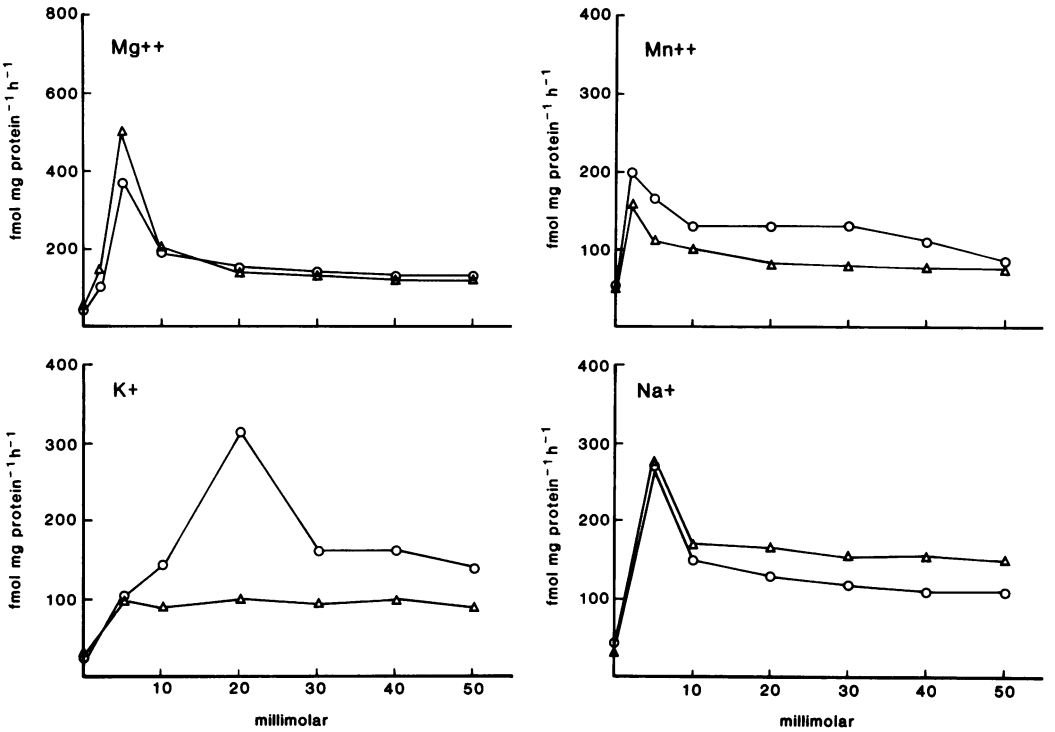

FIG. 3. Effects of ionic concentration on the early (1 h p.i.) and late (6 h p.i.) polymerases. The reactions were performed in standard reaction buffer (Materials and Methods) except that the magnesium acetate was replaced with different ions at the concentrations indicated. Symbols: 0, 1 h p.i.; △, 6 h p.i.

any stimulatory effect (data not shown). These results suggested that the polymerase activities detected at 1 h and 6 h p.i. represent two different RNA polymerase species.

Lack of virion-associated RNA polymerase. To test whether the RNA polymerase was packaged into the MHV virion, we examined purified preparations of MHV for RNA polymerase activity using the conditions optimized for the RNA polymerase activity for vesicular stomatitis virus (7). Table 3 illustrates that no RNA polymerase activity was found associated with the purified MHV virion. In addition, no detectable products were synthesized when we assayed purified virions using the cell-associated RNA polymerase assay system (data not shown).

DISCUSSION

We report in this communication the presence of two RNA polymerase activities in MHV-infected cells. These two polymerases are very similar in their enzyme requirements, but differ in several properties: the polymerase detected at 1 h p.i. (referred to as early polymerase) can be activated by potassium ions in the absence of magnesium ions, whereas the polymerase detected at 6 h p.i. (referred to as late polymerase) cannot. Also, the early polymerase has an optimum pH of 7.5, whereas that of the late polymerase is pH 8.0. Furthermore, the experiments

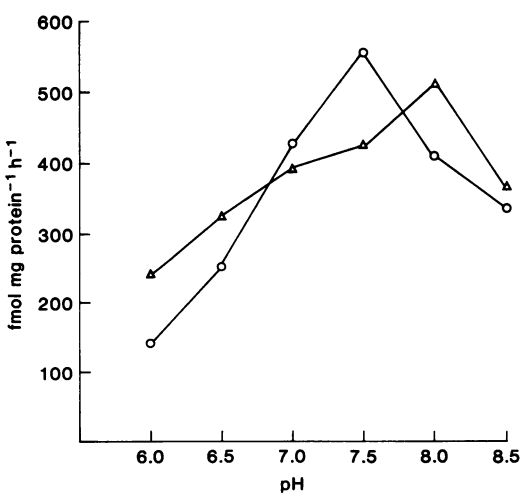

FIG. 4. Effect of pH on the 1 h (0) and 6-h (△) p.i. polymerase.

TABLE 3. Virion-associated RNA polymerase assay

Virus	Incorporation of [5,6-^{3}H]UTP (cpm mg of protein^{-1} h^{-1})	Incorporation of [5,6-^{3}H]UTP (fmol mg of protein^{-1} h^{-1})
VSV[a]	12,510	75.8
MHV-A59	255	1.5

[a] VSV, Vesicular stomatitis virus.

using cycloheximide suggest that the appearance of both the early and the late polymerase activities requires new protein synthesis. Preliminary data in our laboratory further showed that the RNA products of the early polymerase were primarily negative-stranded, whereas those of the late polymerase were positive-stranded RNA (unpublished observation). These results suggest that the early and late polymerases in the MHV-infected cells represent two different types of polymerase. It is not known, however, whether they represent two different enzymes or two different forms of the same enzyme. In the latter case, either a viral protein or a cellular protein would be required for the modification of the early polymerase. The presence of RNA polymerase activities in transmissible gastroenteritis virus (a porcine coronavirus)-infected cells has also been reported recently (D. Dennis and D. Brian, personal communication). Therefore, coronaviruses might share the same replication mechanism.

By analogy to alphaviruses (17), the MHV-induced RNA polymerase might be encoded by the largest gene in the viral genome, i.e., gene *A* in MHV (8). This gene is localized at the extreme 5′ end of the genome and can potentially code for proteins of up to 150,000 to 200,000 molecular weight (8). So far, no viral proteins have been linked to this genetic region. It is also unknown whether this region codes for a single or for multiple proteins.

It is noteworthy that the RNA polymerase activities in our system were detected without addition of exogenous RNA template. Therefore, the polymerase activities detected probably reside in a replication or transcription complex which contains viral RNA, RNA polymerase, and any other essential proteins in tightly bound form. Consistent with this interpretation, the highest amounts of polymerase activities were detected in the membrane fractions. These membrane fractions might contain replication and/or transcription complexes. Very recently, we have indeed succeeded in separating these membranes into two different fractions containing polymerase activity. Presumably, the polymerases contained within these two fractions would correspond to the early and late polymerases. As a consequence of such assay conditions, the polymerase activities detected reflect not only the polymerase itself but also the quantity and nature of the RNA templates in the membrane complexes. The attempt is being made to solubilize these RNA polymerases, the assay of which should reflect more accurately the actual activity of the polymerases.

The cycloheximide experiments also revealed that a dependence on de novo protein synthesis for shutting off early RNA synthesis might exist. Presumably, early RNA species represent negative-stranded RNAs which will serve as templates for positive-stranded RNA synthesis later in the infection. The negative-stranded RNA may continue to be synthesized when the synthesis of the hypothetical switching-off protein is inhibited by cycloheximide at 2 to 3 h p.i. If this interpretation is correct, this system might allow the accumulation of negative-stranded RNA, thus facilitating study of the MHV-RNA synthesis mechanism. The nature of such a hypothetical shut-off protein is unclear; however, the MHV genome has enough coding capacity for three to four nonstructural proteins, some of which might perform such regulatory functions.

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