Mouse Hepatitis Virus Minus-strand Templates are Unstable and Turnover During Viral Replication

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1. INTRODUCTION

During the first 6 hours after infection at 37°C, MHV-A59 infected cells accumulate seven species of minus strands, one equal in length to the viral genome RNA and six that are subgenomic and with sizes equal to those of the six subgenomic viral mRNA (Sawicki and Sawicki 1990). These templates are found in double-stranded replicative intermediates (RIs and RFs) that together with viral polymerases are active in transcription of the viral plus strand species. Thus, replication of MHV-A59 conforms to the general rules discovered for plus strand RNA viruses. It is unique in having a genome of ~32 kb and in utilizing a novel discontinuous transcription mechanism to form the templates for its 3' nest set of viral mRNA (Sawicki and Sawicki 1990, 1995, 1998;). Our studies have been verified by Schaad and Baric (1994) and Baric and Yount (2000).

Our studies of the kinetics of MHV-A59 RNA synthesis found both plus and minus strand syntheses occurred throughout the infectious cycle (Sawicki and Sawicki 1986). Minus strand synthesis (as determined by the incorporation of ^{3}H-uridine) slowed after reaching a maximum rate, which was at about the same time plus strand synthesis reached its maximum rate. Plus strand synthesis then remained at a constant or slowly decreasing rate for several hours. Minus strand synthesis was inhibited almost immediately after stopping translation, while plus strand synthesis continued for about 30-60 min and then declined. Because stopping minus strand synthesis early by

The Nidoviruses (Coronaviruses and Arteriviruses).
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blocking protein synthesis stopped plus strand synthesis from increasing, the number of minus strands appears to determine the rate of plus strand synthesis. These studies form the bases for our perspective on coronavirus transcription. Replication complexes (RCs) are formed when newly translated viral polymerase copies the genome into minus strands: The formation of RCs requires de novo protein synthesis. The minus strands are then converted into transcription complexes (TCs) producing plus strands. MHV TCs contain both genome and subgenome-length templates (Sawicki and Sawicki 1990). TCs producing genomes have similar, if not identical, properties as TCs making subgenomic mRNA because both show the same sensitivity to translation inhibition (Sawicki and Sawicki 1986). We had performed these analyses at times after plus strand synthesis had reached its maximum and minus strand synthesis was at a low rate. Therefore, the ^{3}H-uridine we detected in RF's was almost exclusively (>90%) in plus strands. From those experiments it was apparent that subgenomic mRNA was produced from anti-subgenomes, whereas TCs with anti-genomes were producing exclusively genomes. We began a study to investigate why overall rates of transcription decreased late in infection and yet minus strand synthesis continued.

2. RESULTS

17Cl-1 cells infected with MHV-A59 at an MOI of 100 and continuously labeled in the presence of 20 μg of actinomycin D/ml with ^{3}H-uridine beginning at 1 h p.i. accumulated radiolabeled RNA that was viral mRNA and viral RI/RF RNA. Surprisingly, late in infection there was less accumulated RI/RF RNA as detected by either ethidium bromide staining (Fig 1) or autoradiography than had been in these infected cells earlier. Thus, levels of viral RI/RF RNA previously made by infected cells up to 6 h p.i. was found to decline significantly after 6 h p.i. This suggested the viral RI/RF RNA was not stable but rather turned over during replication. Contrary to the RI/RF RNA, viral plus strands were stable and accumulated over time (data not shown).

2.1 MHV RI/RF RNA and their minus strand templates are unstable

To further investigate this phenomenon, infected cells were treated with cycloheximide (CHI) at different times after infection. We included the early periods, when plus and minus strand syntheses were exponentially

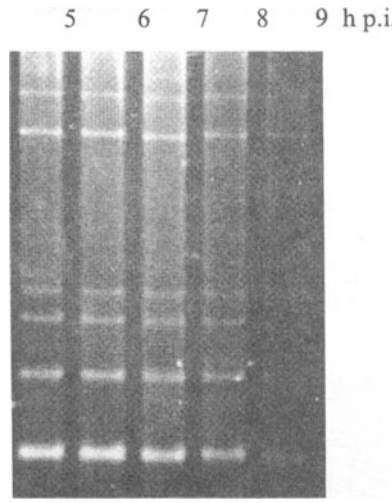

Figure 1. MHV-infected 17Cl-1 cells were harvested at the times indicated, the RNA was treated with RNase T1(1U/μg RNA), run on 0.8% agarose-TBE gels and stained with ethidium bromide.

increasing, and later periods, when plus strand synthesis was in its linear phase and minus strand synthesis was occurring at low rates. Treatment with CHI led to an abrupt cessation in minus strand synthesis and to a steady and linear decline in the rate of plus strand synthesis after a lag period of about 60 minutes. This pattern was the same whether cells were treated at 4 h p.i., an early period in infection, or at 5 h p.i or at 6 h p.i., confirming and extending our earlier results (Sawicki and Sawicki 1986). Thus, the ability of infected cells to continue linear RNA transcription late in infection before overall rates began to decline requires new protein synthesis. We investigated whether the synthesis of new minus strands were also needed during the late period because coronaviruses do not shut off minus strand transcription late but continue to synthesize new templates. We added ^{3}H-uridine to infected cells beginning at 6 h p.i. Radiolabel accumulated in RI/RF RNA and in new minus strands since the percentage of labeled RI/RF RNA that was in minus strands increased with time from a low of 5-10% at the start of the labeling period (6.5 h p.i.) to over 35% at 8 h p.i. This meant that at least 70% of minus strands in RI/RF RNA made during the first 6 hours were replaced by minus strands made after 6 h p.i. If verified, this indicated coronavirus minus strands are unstable templates and only function for a limited time and they must be continuously replaced by newly made ones for plus strand transcription rates to continue. It also suggested the loss of plus strand synthesis after CHI-treatment might result from the loss of the minus strand templates and not necessarily from the turnover of the plus

strand replicases or transcriptases. Such template turnover may also be under the control of a unique viral function.

2.2 Loss of minus strands is reversible but requires new protein synthesis

Removal of the CHI after a 4 h treatment period resulted in a burst of new minus strand synthesis and a return of plus strand synthesis to normal levels ongoing in untreated cultures (Fig 2). The newly synthesized minus strands accounted for 70% or more of the total minus strand templates in viral RI and RF RNA. This result indicates that essentially all the old templates have been replaced by newly synthesized ones.

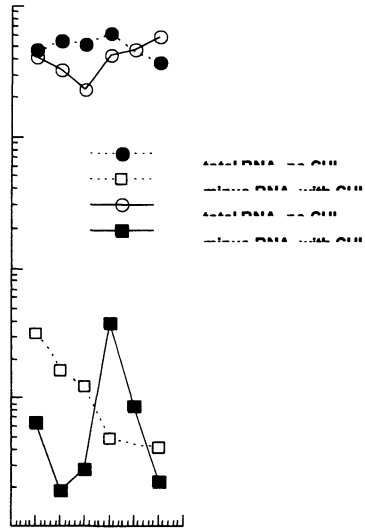

Figure 2. MHV-infected cells were pulse-labeled for 1 hour periods with ^{3}H-uridine in the presence of actinomycin D and harvested at the times indicated. One set of cultures, treated with CHI from 5-9 hr p.i., was washed free of CHI and labeled in the absence of CHI. Incorporation/50,000 cells is shown.

2.3 Turnover is specific for coronavirus RI/RF RNA

Was the turnover of minus strands specific to MHV or did MHV infected cells produce or activate a nuclease that would degrade any viral RI/RF RNA? To investigate this issue, cells were infected with both MHV-A59 and the alphavirus Semliki Forest virus (SFV). Unlike MHV, alphavirus

RI/RF RNA is stable and does not turnover normally either late in infection or after treatment of cells with protein synthesis inhibitors. In cells infected with MHV and superinfected with SFV 1-2 hours later, which allowed the more slowly growing MHV to start transcription and accumulate TCs, only the MHV-A59 RI/RF RNA was lost over time. Therefore, turnover was specific to MHV RI/RF and their minus strands.

2.4 RI/RF minus strands are released in single-stranded form before they are degraded

We determined whether turnover of minus strands in MHV infected cells involved their release from double-stranded into a single-stranded form. If so, infected cells labeled continuously from 1 h p.i. would contain radiolabel in single-stranded minus strands. RNA from infected cells was either digested directly with RNase T1 to obtain the nuclease-resistant RF cores of the viral RIs or first allowed to hybridize by incubation at 68°C for 30 minutes and then at 25°C for another 30 minutes before RNase T1 treatment. Both samples were then passed through CF-11 columns to obtain the nuclease-resistant cores and the amount of labeled RF RNA was determined. The results (Table 1) indicated that after 6 h p.i. MHV-infected cells had less and less total RI/RF RNA with time but they also had a percentage of their total minus strands in single-stranded form. Hybridization before RNase digestion allowed single-stranded minus strands to form hybrids with the excess of viral plus strands (genomes and mRNA). Hybridization led to the recoverey of an additional, equal amount of RF RNA compared to lysates that were digested with RNase directly (Table 1). With time, however, the absolute amount of both RI/RF RNA and recoverable single-stranded minus strands decreased, explaining the steady decline of overall plus strand synthesis observed late in infection.

Table 1. Pre-hybridization recovered free minus strands late in infection. 17CL-1 cells were infected with MHV-A59 at an MOI of 100 and maintained at 37°C. Cultures were labeled continuously with ^{3}H-uridine (200 μCi/ml) in the presence of actinomycin D from 1 hr p.i. and harvested at the times indicated. Half of each infected cell total RNA sample was pre-hybridized by heat-fast cooling, followed by incubating at 68°C for 30 minutes and then at 25°C for 30 minutes. Viral RNA untreated or pre-hybridized was digested with RNase T1 and chromatographed on CF-11 cellulose to purify the RFs. The maximum RF cpm was at 6 h p.i.

	RI/RF RNA cpm/50,000 cells		% of maximal	
h pi	No hybridization	hybridization	No hybridization	hybridization
4	7,883	7,475	57	53
6	13,801	14,122	100	100
7.5	3,246	6,617	24	47
9	1,236	2,357	9	17

3. CONCLUSIONS AND MODEL

Coronavirus infected cells synthesize minus strand templates that function for only a limited time as components of transcription complexes active in viral genome and subgenomic mRNA production. Thereafter, the minus strand are released from the transcription or the entire transcription complex dissociated; in either case, the minus strands were found in single-stranded form before they were degraded. This provides an explanation for why coronavirus infected cells continue to synthesize minus strand RNA after the maximum rate of plus strand transcription has been achieved. Thus, coronaviruses, and possibly other nidoviruses, employ a strategy for replication that requires constant production of both polyprotein forms of the viral polymerase components and minus strand templates. In poliovirus infected cells, templates for viral plus strand synthesis are also replaced at a slow, constant rate (Baltimore 1969). Paralleling but different from coronaviruses, the poliovirus minus strand components of RIs either were converted to inactive RFs after about 10-20 initiation cycles or were degraded. They were not found in single-stranded form in infected cells (reviewed in Koch and Koch 1985). The mechanism that specifically targets the MHV templates for degradation is of interest.

ACKNOWLEDGMENTS

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