MHV Subgenomic Negative Strand Function

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1. INTRODUCTION

Mouse hepatitis virus (MHV), a member of *Nidovirales*, contains a ~32 KB linear, single-stranded, positive polarity RNA genome. Upon entry into the cell, the viral genome is transcribed into 7-8 subgenomic mRNAs ranging in size from ~1.0-32.0 KB. The positive strand mRNAs are arranged in a 3' co-terminal nested set, and each contains a 5' end ~72 nucleotide (nt) leader RNA sequence which is derived from the 5' end of the genome. Leader RNA sequences are joined to body sequences of each subgenomic length mRNA at highly conserved transcriptional start (TSE) sites located just upstream from the coding sequences of each viral gene (Baric et al., 1983; Sethna et al., 1989). In addition to the viral mRNAs, both full-length as well as subgenomic length negative strand RNAs and replicative form (RF) RNAs have been detected in porcine transmissible gastro-enteritis virus (TGEV), bovine coronavirus (BCV) and MHV-infected cells (Baric et al., 1983, Sethna et al., 1989; 1991; Sawicki and Sawicki, 1990). The subgenomic length negative strand RNA contains antileader RNA sequences (Sethna et al., 1991). It has been suggested that the subgenomic length negative strands function as dead end products of transcription (Yokomori et al., 1992; Jeong and Makino, 1992). In this manuscript, we provide direct evidence that the subgenomic negative strands function as the principle templates for the synthesis of each corresponding mRNA.

2. MATERIALS AND METHODS

2.1 Virus, Cells and Analysis of RNA

MHV-A59 was grown on DBT and 17Cl1 cells. Radiolabeling of viral RNAs was performed in 17Cl-1 cells. After infections with MHV-A59, the virus inoculums were removed and the cultures overlaid with Δ MEM medium (pH 6.8) containing 2% fetal clone II, 0.05 μ g/ml gentamicin and 0.25 μ g/ml kanamycin. Cultures were radiolabeled with 300 μ Ci/ml 32 P-orthophosphate for various times to radiolabel virus-specific mRNA and RI RNAs. The RNA was isolated as described previously (Schaad and Baric, 1994).

Cultures of 17Cl-1 cells were infected with MHV-A59 at a MOI of 10. At 6.0 hrs postinfection, cultures were radiolabeled with 1000 μ Ci/ml of 32 P-orthophosphate for 5, 15, 30, 45 and 60 min. Total RNA was isolated and purified. Viral mRNA and RF RNA were analysed according to the protocol described by Sawicki and Sawicki, 1990. To calculate the % molar ratios of mRNA and RF RNA, the % CPM in each viral RNA was divided by the molecular weight of the respective mRNA/RF RNA and normalised to 100% (Schaad and Baric, 1994).

Figure 1. Saturation Kinetics of Full Length and Subgenomic Length Replicative Intermediate RNAs and mRNAs.

3. RESULTS

For positive strand RNA viruses, it is understood that viral mRNA is transcribed from a complementary negative strand RNA. Nascent viral RNAs are rapidly radiolabeled and located in a partially single-stranded, partially double-stranded RI RNA (Simons and Strauss, 1972). Following RNase digestion, much of the recently transcribed RNA remains affiliated with double-stranded RF RNA cores (Figure 1). Previous studies by Sethna *et al.*, 1989, 1991 used probe hybridization to identify the presence of full length and subgenomic length negative strand RNAs during transmissible gastroenteritis virus infection. These experiments could not address whether these negative strands were templates for mRNA synthesis. These experiments, however, were criticised because the subgenomic length negative strand RNAs could be dead-end products of transcription engaged in the synthesis of a single positive strand RNA (Jeong and Makino, 1992, Yokomori *et al.*, 1992). If negative strands were actively engaged in continual nascent plus strand synthesis, both full length and subgenomic length RI RNAs should rapidly incorporate radiolabel and then saturate as the nascent positive strands become maximally radiolabeled and then dissociate from the RI complex (Simons and Strauss, 1972). In contrast, mRNA levels should increase steadily until degradation and transcription rates become equal.

To study the saturation kinetics of full length and subgenomic length RI RNAs, cultures of 17Cl-1 cells were infected with MHV-A59 at a MOI of 10 and radiolabeled with 1000 $\mu\text{Ci/ml}$ ^{32}P -orthophosphate for 5, 15, 30, 45 and 60 min. Under these conditions, nascent positive strands are preferentially labelled as rates of negative strand synthesis are reduced at later times postinfection. The intracellular RNAs were isolated and analysed for mRNA and RF RNA. Similar to findings reported by Sawicki and Sawicki, 1990, that increasing amounts of radiolabel were incorporated into all viral mRNAs following longer labelling periods (data not shown). In agreement with previous reports (Sawicki and Sawicki, 1990), full length and subgenomic length RF RNAs were rapidly labelled after a 5-min pulse. Increasing amounts of radiolabel were evident in the RF RNAs with longer labelling periods (15-30 min); after which time the amount of radiolabel slowly increased in the RF RNAs (data not shown). For example, rapid increases in the levels of RF RNA 4 and RF RNA 5 were noted during the first 30 min pulse labelling period, after which total counts in these RF RNAs became relatively constant. In contrast total counts in mRNA 4 and 5 increased steadily over the same labelling period (Figure 2). Such findings were consistent with the hypothesis that the nascent plus strands on full length and subgenomic length RI RNAs rapidly saturated with label and that

all negative strands remained actively engaged in the synthesis of many new mRNAs.

Figure 2. Radiolabeling of mRNA and RF RNA 4 and 5.

Figure 3. Saturation Kinetics of mRNA and RF RNA 4 and 5.

3.1 Saturation Kinetics of Genomic and Subgenomic Length RI RNAs

Labelling kinetics of individual MHV full length and subgenomic length RF RNAs reflected the kinetics of total RF RNA labelling. To provide additional evidence that the full length and subgenomic length negative strand RNAs were in transcriptionally active RI structures, we compared the saturation kinetics of mRNAs and RF RNAs 4 and 5 over the 1 hr time period (Figure 3). Importantly, while steady increases in mRNA 4 and 5 were noted throughout the labelling period, their respective RF RNAs rapidly saturated with label. By comparing the percent label incorporated as a function of total mRNA or RF RNA, saturation kinetics revealed that over 70% of the radiolabel was incorporated into full length and subgenomic length RF RNAs within 30 min of the addition of label (Figure 3). Only

slight increases in the amount of RF RNA 4 or 5 were detected after this time. In contrast under identical conditions, <10% of the total label were incorporated into mRNA 4 or 5 within the first 30 minutes. These data were consistent with the hypothesis that both full length and subgenomic length negative strands remained in transcription active RI complexes engaged in the continual synthesis of numerous positive strand RNAs.

While the slight increase in total levels of the RF RNAs noted after 30 min was most likely due to new negative strand synthesis and hotter pools of nucleotide precursors, this could also represent RF RNA structures which had "burned out" and were accumulating during infection. If this were the case, then the relative percent molar ratio's of the transcriptionally-active full length RF RNA should decrease over time in proportion to the slow rate of increase in the molar ratio's of the slowly accumulating "burned out" subgenomic RF RNAs. To address this possibility, we calculated the percent molar ratio of each RF RNA and mRNA during the labelling period. Relative percent molar ratios of the viral mRNAs reflected the relative abundance of each RF RNA and remained relatively constant throughout the labelling period as well (data not shown).

4. DISCUSSION

Several discontinuous transcription models have been proposed to explain the presence of leader RNA sequences on positive strand RNAs and antileader RNA sequences on full-length and subgenomic length negative strand RNAs (Baric *et al.*, 1983; Sethna *et al.*, 1989; Sawick and Sawicki, 1990). It is generally agreed that these smaller RNAs do not originate from larger precursors. The leader-primed transcription model proposed that a free leader RNA was synthesised from the 3' end of the full length minus strand (figure 4). Free leader RNA then acts in trans and binds with the different intergenic start elements (TSE) in a full length minus strand template to prime transcription of each of the subgenomic mRNAs (Baric *et al.*, 1983). This model was heavily based on the finding of a single full length negative strand RNA and RF RNA in infected cells and the presence of mRNA 7 nascent plus strands on purified full length RI RNA (Lai *et al.*, 1982; Baric *et al.*, 1983). The former observation is clearly incorrect. The latter observation can no longer be viewed as definitive evidence for "leader-primed transcription" as such RNAs may represent template switching between full length and subgenomic length RI RNAs associated with high frequency RNA recombination (Baric *et al.*, 1990).

Figure 4. Models of Discontinuous Transcription. Panel A. Leader Primed Transcription. Panel B: transcription attenuation model.

All other data referenced in support of leader primed transcription will also support the transcription attenuation model for Nidovirales transcription (Makino et al., 1986; Li et al., 1997). In this model, the body TSE elements in the genome length template act as discontinuous elongation sites that allow for the polymerase/nascent minus strand to detach and reinitiate transcription (in cis or in trans) at the 5' end of the genome. This model predicts that all subgenomic negative strands originate from the genomic RNA directly, rather than from mRNA. In support of this hypothesis, tandem placement of a coronavirus promoter resulted in enhanced mRNA synthesis from the downstream most initiation sites (Krishman et al., 1996). During EAV transcription, the intergenic TSE element in subgenomic mRNA is derived from the TSE sequences encoded in the genomic RNA during negative strand synthesis rather than from leader sequences encoded at the 5' end of the genome. These studies support a mechanism of discontinuous negative strand synthesis reminiscent of the process of copy choice RNA recombination (Van Marle et al., 1999). The finding of a EAV replicase mutant deficient in subgenomic negative strand synthesis further supports

the role of these RNA templates in mRNA synthesis (Van Marle et al., 1999). In light of these findings, clearly, transcription attenuation is the most attractive mechanism of Nidovirus discontinuous transcription of the subgenomic RNAs.

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