STUDIES INTO THE MECHANISM OF MHV TRANSCRIPTION

Ralph S. Baric[1], Chien Kou Shieh[2], Stephen A. Stohlman[2], and Michael M.C. Lai[2]

[1]University of North Carolina
School of Public Health
Department of Parasitology and Laboratory Practice
348 Rosenau Hall, 201H
Chapel Hill, North Carolina 27514

[2]University of Southern California
School of Medicine
Department of Microbiology and Neurology
2025 Zonal Avenue
Los Angeles, California 90033

INTRODUCTION

Coronaviruses are enveloped, plus-polarity RNA viruses which replicate by a unique mode of RNA transcription.[1,2,3] Previous studies in our laboratory have indicated that RNA recombination occurs at very high frequency during mixed infection with two heterologous strains of MHV[4,5]. These data, coupled with the presence of discrete larger leader-containing RNAs which range from 47 to 1000 nucleotides in length in MHV-infected cells,[6] suggest that discrete RNA intermediates are synthesized during transcription which may dissociate and reassort between viral RNA templates to generate recombinant viruses by a copy-choice mechanism[4]. Therefore, the larger leader-containing RNAs in MHV-infected cells may represent functional intermediates of RNA transcription and recombination. In this paper, we have analyzed the origin, structure, and probable mechanism of synthesis of these RNAs. These data provide evidence that MHV RNA transcription pauses at sites corresponding to hairpin loops in the RNA template or product strands and that these RNA intermediates may dissociate and reassociate with the RNA template intermittently during the course of transcription.

We shall also present a new approach to identify the sequences encoded at the intergenic start sites for transcription which function in subgenomic mRNA synthesis. This approach utilizes a murine retrovirus

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expression vector to synthesize virus-like negative-stranded RNAs that can be recognized and transcribed by the viral polymerase.

METHODS

Preparation of Intracellular RNA and Replicative Intermediate RNA

RNA was extracted by the phenol/chloroform method between 5-7 hours postinfection when DBT or L2 cells exhibited 70% CPE[6]. To purify replicative intermediate RNA from 4S RNA fractions, intracellular RNA was separated on 15-30% sucrose gradients in NTE[6,7]. The 18-40S RNA fractions were collected as RI-containing intermediates, while the small RNAs were collected in the 4-10S RNA fractions.

Nick Translations of MHV cDNA Clones and Filter Hybridization

The cDNA clones of MHV genomic RNA used in this study are summarized in fig 1. Clones F82 and C96 represent overlapping regions at the 5'-end of the genomic RNA[8]. F82 is a 3.8 kb clone encoding the leader RNA sequences, while C96 encodes internal sequences in gene A. Two other clones, representing internal sequences of the MHV genome, were also used: clone D63 contains all of gene D and E and portions of gene C and F (Shieh, unpublished), and clone PHN42 spans the entire gene F[9] (kindly provided by Dr. Heiner Neiman, Geissan, Germany). An internal XbaI fragment of clone D63 (this probe encodes nucleotides 47-375 in gene D[10]), designated D63X, was used as a gene D probe. Several subclones were constructed from clone PHN42. E1-500, located between nucleotides 90-609 in gene F[9], was used to detect small RNAs synthesized during an RNA6 transcription. Subclone E1-300 (534-780 in the E1 RNA)[9] was used as control, while subclone E1-L contains the first 84 nucleotides of the E1 RNA, including the 72-nucleotide leader RNA sequence. This fragment was used to detect small leader RNAs.

Computing Stability of MHV RNA Secondary Structure

Predictions for regions of secondary structure were made using the Zucker RNA folding program through Bionet[12]. Stability of individual hairpin loops, within the MHV sequence, was calculated from the thermodynamics by adding a base pair to a double-stranded helix at 25°C as reported by Tinoco et al.[13] and modified by Salser[14].

Transfections and Selection for G-418 Resistant Cell Populations

The murine retrovirus expression vector, pNeoG, encodes G-418 antibiotic resistance in eukaryotic cells. Into this vector we inserted a 1.5 kb fragment containing the full-length gene F cDNA, PHN42 (fig 1b), and the chloramphenicol transferase (CAT) gene in the antisense orientation. The new vector, pNeoG-63, has the 5'-end of the CAT gene linked to

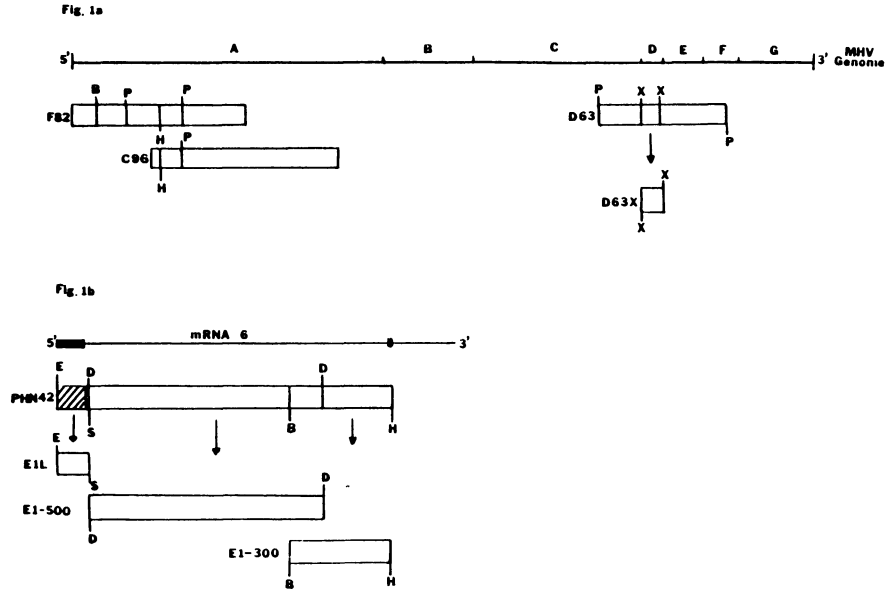

Figure 1: The structure of the MHV cDNA probes. Clone F82 contains the complete 5'-end of the genome and overlaps with clone C96. Clones D63X, E1-500, and E1-300 were used to detect small RNAs originating during transcription of mRNA4 (gene D) and mRNA6 (gene F). Probe E1-L was used to detect small leader RNAs containing the leader sequence.

the 3'-end of PHN42 which contains the intergenic region for mRNA7 transcription (see fig 7). Upon infection with MHV, the intergenic region for mRNA7 transcription in pNeoB-63 could be used to initiate $CAT^{(+)}$ RNA synthesis. Plasmids were transfected into L2 cells using the Calcium-phosphate method[15]. G-418 resistant colonies were selected in 850 ug/ml G-418 antibiotic over a 2 to 3 week period.

Preparation of Strand-Specific RNA CAT Probes

The CAT gene was inserted into PT7-1 and PT7-2 at the SMA 1 site in both orientations. The plasmid DNA was linearized and ^{32}P-UTP labeled RNA transcribed in vitro with the T7 polymerase. The RNA was purified by G-50 column chromatography. Dot blots of infected and uninfected NeoG63 cell lines were performed as described in the literature[16]. The blots were probed with ^{32}P-UTP labeled $CAT^{(+)}$ or $CAT^{(-)}$ RNA at 65°C for 36 hours and immediately washed in 2xSSC containing 0.01% SDS at 75°C. Following several additional washes at 75°C in 0.2xSSC containing 0.1% SDS, the blots were treated with lug/ml RNaseA in 2xSSC for 15-20 minutes, dried and exposed to XAR-5 film.

RESULTS

Construction of Specific cDNA Probes

To determine the structure and origin of the small RNAs present in

MHV-infected cells, we constructed several cDNA probes specific for different regions in the MHV genome. Three genes were chosen for the study, namely A, D, and F. Gene A probably encodes an RNA polymerase(s), while genes D and F encode a nonstructural protein p14, and the E1 structural protein[9,10], respectively. Since gene A represents the 5'-most region of the genome, small RNAs originating from this gene represent products of RNA replication. The other two genes represent two internal genes which have been well characterized[9,10]. The map locations of the probes used in this study are depicted in fig 1 and are characterized in more detail in the Methods section.

Analysis of Small RNAs Originating Within the Leader RNA Sequence

To determine the structure and origin of the small RNAs present in MHV-infected cells, we first used a leader-specific cDNA probe, E1-L, to identify small RNAs terminated within or around the leader sequence. Intracellular RNA was separated on 8% polyacrylamide gels, transferred to Z-probe paper, then probed with the leader specific probe[6]. As shown in fig 2, several distinct small RNAs, ranging from 47-84 nucleotides in length, were detected. The small RNAs correspond in lengths between the

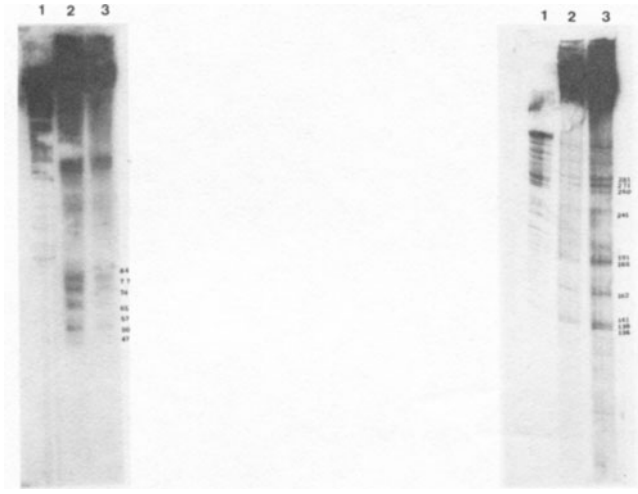

Figure 2: Analysis of the MHV leader-containing RNAs. Intracellular RNA extracted from MHV-infected cells was separated by electrophoresis on 8% polyacrylamide gels and transferred to Z-probe paper. The nick-translated leader-specific probe (E1-L) was hybridized to the blot. Lane 1: M13 sequencing T ladder. Lane 2: MHV-infected L2 cells. Lane 3: MHV-infected DBT cells. The sizes of T bands were determined from the accompanying A, G, C, and T ladders of M13 sequencing (data not shown).

Figure 3: Analysis of MHV small RNAs originating at the 5'-end of gene A. Intracellular RNA was separated by electrophoresis under conditions which separate RNAs between 100-300 nucleotides in length (Methods). Following transfer to Z-probe paper, RNAs were probed with the nick-trans-lated clone F82 (Panel A) Lane 1, M13 sequencing T ladder; Lane 2, A59-infected L2 cells; Lane 3, A59-infected DBT cells.

5′-end of the genome and hairpin loop structures around nucleotides 42-56, 52-72, 80-143 and a postulated transcriptional termination signal (UUUAUAAA) for MHV leader RNA synthesis[8] (table 1).

Larger leader-containing RNA species were present in MHV-infected cells, consistent with previously published results[6]. To determine if any of these larger leader-containing RNAs represent intermediates of RNA replication, the RNA blots were probed with cDNA clone F82 (fig 1a), representing the 5′-end of the genome. Several distinct RNA species ranging from 136 to larger than 281 nucleotides in length were resolved (fig 3). It is noteworthy that the internal probe, C96, detected no small RNAs (data not shown). These data suggest that these RNAs represent transcriptional intermediates derived from the 5′-end of the genomic RNA during RNA replication. The sizes of these small RNAs correspond to the lengths between the 5′-end and the sites of potential hairpins (table 1).

Analsyis of Small RNAs Originating from Internal Genes

The leader-containing RNAs larger than 110 nucleotides in length, detected by the leader-specific cDNA probe (E1-L), were heterogenous. However, the 5′-gene A probe (F82) detected discrete species, implying that the larger leader-containing RNAs may contain RNA species derived from other portions of the genome during transcription. Probes encoding the 5′-end of gene F (E1-500) (fig 1b) detected RNA species of 118, 123, 127, 143/145, 150, 158, 164, 169, and several poorly resolved RNAs ranging between 241-260 and 270-281 nucleotides in length (fig 4a). These

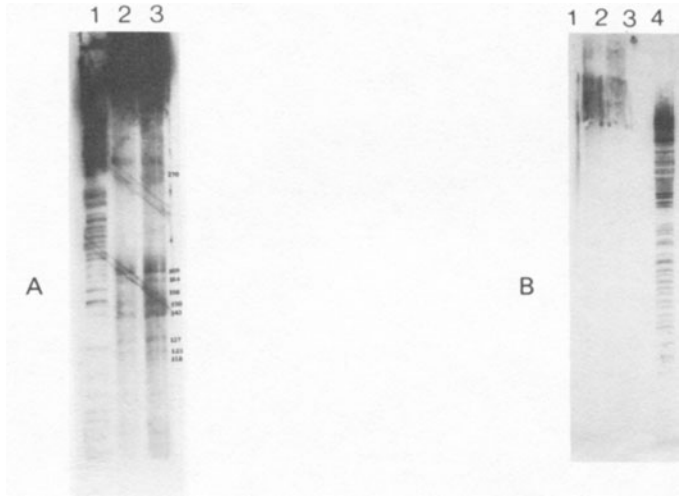

Figure 4: Analysis of small RNAs originating within genes D and F. Intracellular RNA was separated and transferred as described in figure 4. Nick-translated probes specific for gene F (Panel A, C) or gene D (Panel B) were used. Similar amounts of RNA were used for each blot. Panel A: RNAs detected with E1-500. Lane 1: M13 T ladder; Lane 2: A59-infected L2 cells; Lane 3: A59-infected DBT cells. Panel B: RNAs detected with E1-300. Lane 1: M13 T ladder; Lane 2: uninfected DBT cells; Lane 3: A59-infected DBT cells; Lane 4: A59-infected L2 cells.

RNA species differ from those detected with the gene A probe (fig 3) or a probe (E1-300) representing the 3'-end of gene F between nucleotides 534-780 (fig 4b). The gene D specific cDNA probe, D63x, also detected a different set of small RNA species of 117, 135, 160, 168, 205, 218 nucleotides in length (data not shown). Smaller amounts of RNA of 173 and 199 nucleotides in length were also detected. These data suggest that transcription of subgenomic mRNAs also terminate or pause at many sites.

Computer models of RNA folding suggest the presence of hairpin loops in regions of nucleotides 105-122, 114-141, 123-167, 193-226 and 242-285 within the sequence of mRNA6 (gene F) and in regions of nucleotides 106-131, 105-162, 168-204 and 211-235 within mRNA4 (gene D) (fig 5a,b). The predicted stabilities of these structures is shown in table 1. These data show that most of the small RNAs terminate within hairpin loop structures, suggesting a possible correlation between the generation of small RNAs and the presence of secondary structure in the template or product RNAs.

Presence of Small RNA Species on the Replicative Intermediate (RI) RNA

If the small RNA species detected in MHV-infected cells represent specific products released or bound to the template RNA during transcription, then discrete, small RNA species would be expected to be present in

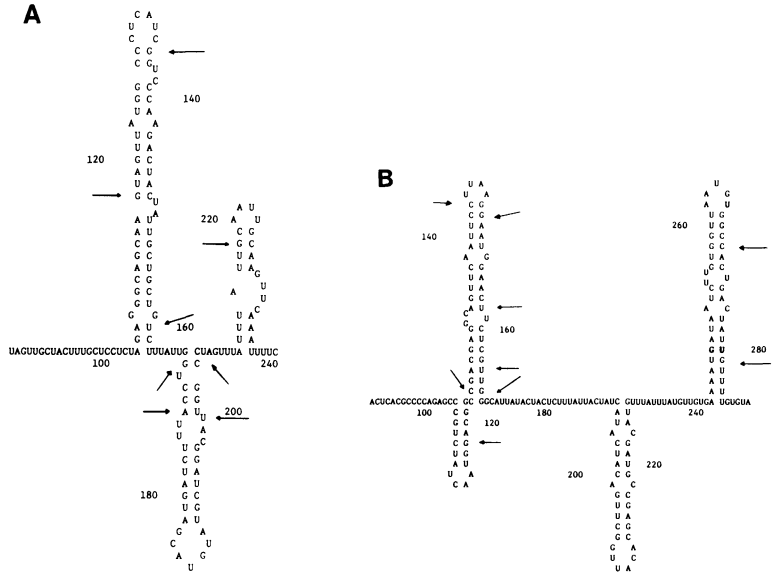

Figure 5: Predicted regions of secondary structure at the 5'-ends of mRNA4 (gene D) and mRNA6 (gene F). The sequences of mRNA4 and mRNA6 were derived from Skinner and Siddell[10] and Armstrong et al[9] respectively. Arrows mark the possible termination points of the small RNAs detected in MHV-infected cells. The free energies of the hairpin loops are shown in table 1. In cases of overlapping secondary structures, only the more stable structure is shown. Panel A: mRNA4; Panel B: mRNA6.

Table 1. Stability of MHV Hairpin Loop Structures Present within the MHV RNA.

Origin	[a]Hairpin loop locations	Free energy (Kcal/mole)	[b]Size of RNA Intermediates detected
Leader	42-56	-0.8	47,50,57
Leader	52-72	-1.6	47,50,57,65,74,77,84
mRNA1	80-143	-18.7	136,138,141
mRNA1	165-188	-6.9	188,191
mRNA1	148-320	-77.1	162,188,191,241,260,271,281
mRNA4	106-131	-13.0	117,135
mRNA4	105-162	-34.8	117,135,160,168,173
mRNA4	168-204	-12.3	168,173,199,205
mRNA4	211-235	-6.6	218
mRNA6	105-122	-8.2	118,123
mRNA6	114-141	-16.1	127,143/145
mRNA6	123-167	-26.4	127,143/145,150,158,164,169
mRNA6	193-226	-6.2	none
mRNA6	242-285	-12.0	241-260,270-281

a) Locations are expressed in numbers of nucleotides from the 5'-end of respective mRNAs.

b) Expressed in number of nucleotides. The data were derived from fig 2, 3 and 4. In the case of overlapping secondary structures, small RNAs of appropriate size were placed in each region of hairpin loop structure, since it could not be determined which loop structure induced pausing at this site.

the RI structure which is involved in RNA transcription. Conversely, if RNA transcription proceeds in a continuous manner, the RNA species on the RI RNA should be heterogeneous, without predominant RNA species. To distinguish these possibilities, 18-40S RI RNA was isolated and analyzed for the presence of small RNAs using two probes, F82 and E1-500 (fig 1), which encode the 5'-end of gene A and F, respectively. As shown in fig 6, the small RNA species present on the RI RNA are the same as those present in whole lysates (fig 3, 4a). Furthermore, the 4S RNA fraction also contains an identical set of small RNAs, indicating that some of the small RNAs were dissociated from the template RNA. These results strongly suggest that the small RNA species are true transcriptional intermediates.

Model System for Analyzing the Sequences Which Function in Subgenomic Transcription

We have recently devised a new approach to analyze which sequences, encoded at the intergenetic "start" sites for subgenomic transcription, function in viral mRNA synthesis. This approach is depicted in fig 7 and utilizes murine retrovirus expression vectors to synthesize virus-like negative stranded RNAs that can be recognized and transcribed by the MHV polymerase. We have constructed the vector pNeoG-63 which encodes a

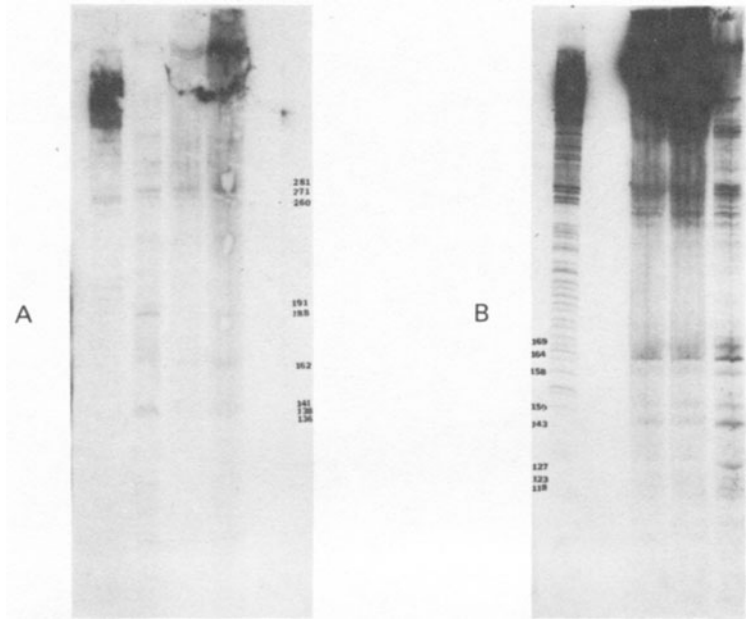

Figure 6: Analysis of small RNAs present on RI or as free RNAs. Intracellular RNA was extracted from MHV-infected cells and separated on 10-30% sucrose gradients. The 18-40S RNA, representing the RI, and 4S RNA fractions were collected separately and analyzed with F82 (Panel A) and E1-500 (Panel B) separately. Panel A: Lane 1, M13 T ladder; Lane 2, 4S RNA fractions; Lane 3, 18-40S RNA; Lane 4, total intracellular RNA from A59-infected cells; Lane 5, uninfected DBT cells. Panel B: Lane 1, M13 T ladder; Lane 2, uninfected DBT cells; Lane 3, total intracellular RNA from A59-infected cells; Lane 4, 18-40S RNA; Lane 5, 4S RNA fractions.

negative-sensed CAT gene and a negative-sensed MHV cDNA (PHN42) (fig 7). The orientation of these cDNA clones is such that the intergenic region for mRNA7 transcription is in the appropriate orientation to mimic the (-)-sensed intergenic region on the viral template and act as a "start" signal for $CAT^{(+)}$ RNA expression upon infection of cells with MHV. Cultures of L2 cells were transfected with pNeoG-63 and individual colonies selected with 850 ug/ml G-418 antibiotic. Several colonies were selected and intracellular RNA was extracted. By dot blot analysis using strand specific CAT RNA probes, these clones were shown to synthesize $CAT^{(-)}$ RNA, but not $CAT^{(+)}$ RNA (data not shown). More importantly, the cloned cell lines pNeoG-63 #2 and a mixed population of pNeoG-63 cells were shown to express $CAT^{(+)}$ RNA upon infection with MHV (fig 8). Since $CAT^{(+)}$ RNA was not detected in any controls, except those cells transiently transfected with pSV2 CAT, these data indicate that cellular RNAs containing viral regulatory sequences can be recognized and transcribed by the viral polymerase. We are currently modifying this system so that the role of individual nucleotides in subgenomic mRNA synthesis can be evaluated.

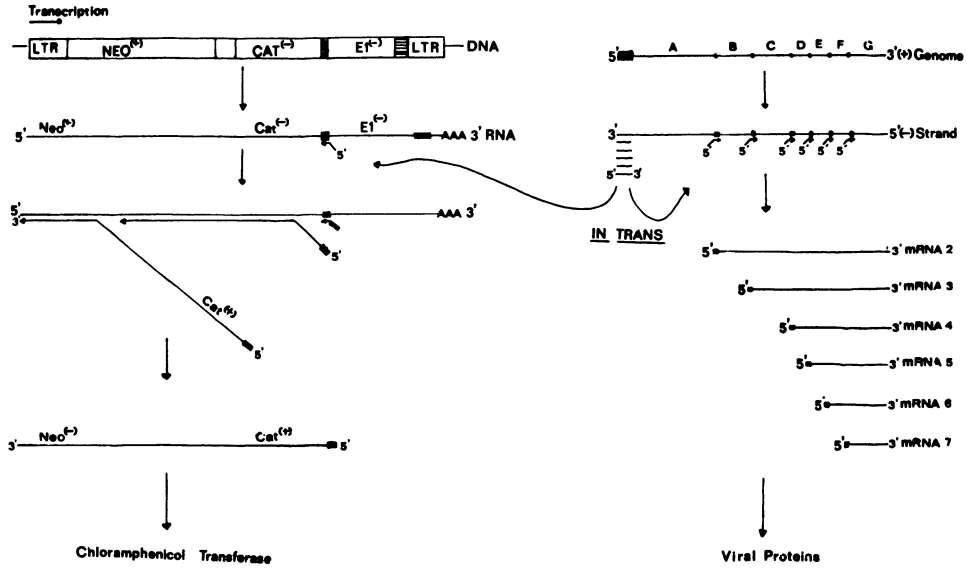

Figure 7: Strategy for mapping the intergenic sequences which function in MHV subgenomic RNA synthesis. Utilizing a murine retrovirus expression vector, we have constructed pNeoG-63 containing an antisense CAT and E1 gene. Upon infection of stably transfected cells, the MHV leader RNA will act in trans to initiate $CAT^{(+)}$ mRNA synthesis.

DISCUSSION

Previous findings in our laboratory indicate that RNA recombination occurs at a high frequency during a mixed infection with two strains of MHV[4,5]. This high frequency is reminiscent of RNA reassortment described for viruses with segmented RNAs and suggests that MHV replication might involve the generation of free segmented RNA intermediates. These intermediates could participate in RNA recombination by a copy-choice mechanism. The detection of multiple discrete species of leader-containing RNAs, which range from 47 to more than 1,000 nucleotides in length, in MHV-infected cells further supports this model[6]. In this report, we examined the origin, structure and mechanism of synthesis of RNAs. The data suggest that these RNA species are likely to represent transcriptional pausing products of MHV RNA synthesis. In general, the pausing sites correspond to the potential hairpin loops present in the template or product RNAs. This mechanism is reminiscent of transcriptional pausing by QB phage in which the QB RNA polymerase pauses in regions of hairpin loop structures[17]. Pausing also occurs during DNA-directed DNA synthesis[18], DNA-directed RNA synthesis[19] and in reverse transcriptase reactions[20]. Similar to the mechanism of QB RNA transcription, the majority of MHV small RNAs terminate at the 3' side of hairpin loop structures in the product or template strands, which have free energies ranging from

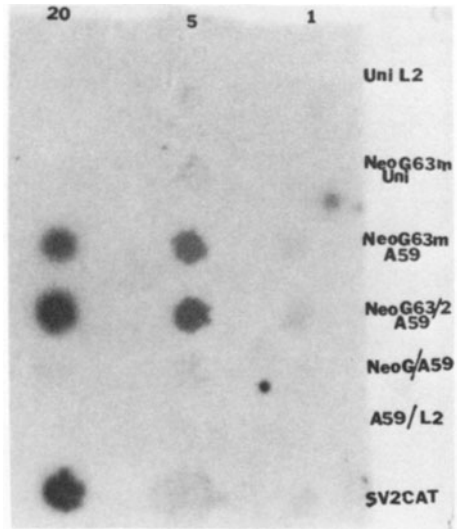

Figure 8: Induction of CAT$^{(+)}$ RNA Synthesis by MHV. Mixed populations or cloned pNeoG-63 cell lines were infected with MHV and intracellular RNA extracted 7-9 hours post-infection. The RNA was blotted onto nitrocellulose paper and probed for the presence of CAT$^{(+)}$ RNA with CAT$^{(-)}$ RNA probes transcribed in vitro. Lanes 1-7: L2 cells uninfected, pNeoG-63 mixed population-uninfected; pNeoG-63 mixed population-infected; pNeoG-63 clone 2, infected; pNeoG (vector devoid of CAT sequences)-infected; L2 cells-infected; pSV2 CAT transfected L cells.

-1.6 cals/mole to -77.1 Kcals/mole (figure 6 and table 1). The stabilities of these hairpin loops are comparable to those of QB (-9.8 to -23.8 K cals) and tRNA (-4.5 to -15.0 Kcals) hairpin loops, whose existence in the RNA has been demonstrated by various physical and biochemical methods[21,22]. Whether the pausing RNA intermediates of QB or other transcriptional pausing model systems are dissociated from the RNA template is not known; however, the data presented in this paper clearly show that at least some of the MHV RNA intermediates are separated from the template RNA strand.

Currently, there is no direct evidence to prove that these free small RNAs actually rebind to the RNA template and participate in continuing transcription; however, the high frequency of RNA recombination[4] suggests that such rejoining does occur. The recent isolation of several MHV recombinants with multiple crossovers further strengthened this probability[23]. More importantly, the crossover sites in two recently isolated recombinants, A-1 and A-5, have been mapped within a region of the leader sequences (nucleotides 35-60 from the 5'-end)[23], where three small RNA species were detected (fig 2). The isolation of these recombinants strongly suggests the functional roles of the pausing RNA intermediates in transcription and recombination. Interestingly, poliovirus recombination also occurs predominantly in regions of secondary structure[24], and that during the transduction of the proto-oncogene c-fps by a retrovirus,

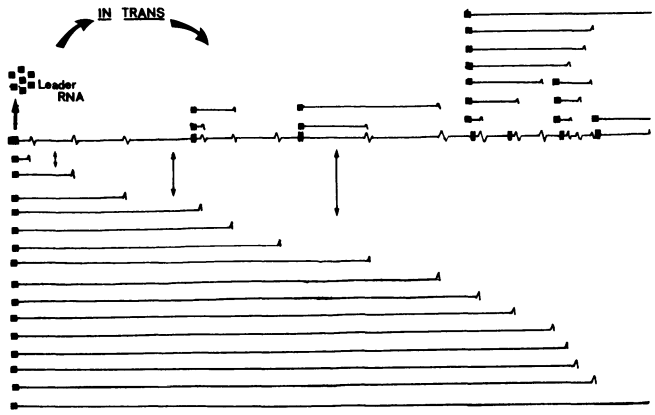

Figure 9: Proposed model for MHV discontinuous transcription. In this model, MHV positive-sensed RNA transcription initiates at the 3'-end of the negative strand and "pauses" in regions of secondary structure in either the product or template strands. These intermediate RNAs can dissociate from the (-) strand template and reassociate to function as primers for the synthesis of full-length parental and recombinant RNAs.

right-handed recombination occurred near or in stable hairpin loop structures[25]. These data, coupled with the fact that reverse transcriptase "pauses" in regions of secondary structure, suggest a common mechanism of recombination for picornaviruses, retroviruses and coronaviruses, involving paused transcriptional intermediates[20]. In addition, it has previously been shown that short oligonucleotides can act as primers for RNA synthesis in vitro[26], and that the nascent (+) strands on RI RNA are held by very short regions of heteroduplex[27]. Thus, it seems likely that the free RNAs in MHV-infected cells could dissociate and reassociate freely in RNA elongation. Recently our laboratory has shown that the MHV leader RNA can act in trans to participate in RNA transcription[28].

The biochemical and biological data presented here strongly supports a mechanism of MHV transcription which is discontinuous and nonprocessive (fig 9). In this model, the viral polymerase pauses in regions of secondary structure, producing discrete nascent RNA intermediates on replicative intermediate RNA. Either a portion, or all, of these small RNAs dissociate from the template strand and then reassociate to act as primers for continuing RNA elongation of parental or recombinant RNAs. Further proof of this model will require studies using an in vitro transcription system.

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