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NEGATIVE STRAND RNA SYNTHESIS BY TEMPERATURE-SENSITIVE MUTANTS OF MOUSE HEPATITIS VIRUS

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1. ABSTRACT

Mutants of the A59 strain of mouse hepatitis virus (MHV) were studied to determine the effects of temperature shift on negative and positive strand RNA synthesis. Mutant LA 9 was originally reported to have a selective temperature sensitive defect in negative strand synthesis. We found that this mutant continued to synthesize negative strands for at least one hour after temperature shift. LA 6 was found to possess a temperature sensitive defect in negative strand synthesis. Following temperature shift, negative strand synthesis rapidly declined. The effect of temperature shift on negative strand synthesis by LA 6 was similar to the effect of cycloheximide treatment of the parental A59 virus. Temperature shift of Alb 16 infected cells did not stop negative strand synthesis but did prevent a corresponding rise in the rate of positive strand synthesis. Therefore, we suggest that Alb 16 is a conversion mutant because it cannot convert newly synthesized negative strands into templates for positive strand synthesis at the nonpermissive temperature.

2. INTRODUCTION

Coronaviruses are positive strand RNA viruses and their genome must be translated after entry of the virus into the cytoplasm. The translation products of the genome are nonstructural proteins, which have been characterized only partially, and must include the viral RNA-dependent RNA polymerase. The coronavirus RNA polymerase would be responsible for producing both negative and positive strand templates. Previously we had shown that the rate of positive strand RNA synthesis in MHV infected cells was coupled to negative strand synthesis and the number of negative stands controlled the rate of posi-

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tive strand synthesis (Sawicki and Sawicki, 1986). During the early phase of infection, negative strand synthesis occurred coincidentally with positive strand synthesis. After the rate of both positive and negative strand synthesis reached a maximum at 5–6 hours p.i. at 37°C the rate of negative strand synthesis declined and the rate of positive strand synthesis became relatively constant. If translation was inhibited by cycloheximide before the maximum rate of viral RNA synthesis was attained, negative strand synthesis ceased abruptly and the rate of positive strand synthesis leveled and then declined beginning about 30 min. after translation had halted. We interpreted these data to mean that new polymerase was required for the production of negative strands. Once negative strands were produced they functioned as templates for positive strand synthesis. However, positive strand synthesis continued unabated for only a limited time after inhibition of translation. Therefore, positive strand synthesis required the production of either new polymerase or a protein factor that is necessary for polymerase activity. We have been studying RNA negative, temperature sensitive mutants of MHV/A59. These mutants fail to produce viral RNA when cells are infected and maintained at the nonpermissive temperature. Schaad et al. (1990) suggested that the rate of positive strand synthesis could be increased in the absence of negative strand synthesis in the temperature sensitive mutant LA 9. After shifting to the nonpermissive temperature after viral RNA synthesis had started at the permissive temperature, it appeared that positive strands, but not negative strands accumulated. In fact, they did not measure the rate of negative strand synthesis, e.g., the incorporation of ^{3}H-uridine into positive or negative strands, but looked for the accumulation of negative strands after temperature shift. We obtained LA 9 and LA 6 from Dr. Ralph Baric and Alb 16 from Dr. Larry Sturman (Sturman et al. 1987) and determined whether or not they had a specific defects in negative strand RNA synthesis.

3. RESULTS

All of the mutants possessed a ts RNA negative phenotype, i.e., they did not make viral RNA if the infection was initiated and maintained at 39.5°C the nonpermissive temperature. To study the effect of temperature on negative strand synthesis by these mutants, we allowed viral RNA synthesis to begin and reach about 20% of the maximum rate at 30°C, the permissive temperature. Then we shifted the infected cells to 40°C and labeled the viral RNA with ^{3}H-uridine in the presence of dactinomycin. To determine negative strand synthesis we followed our published procedure (Sawicki and Sawicki, 1986) of first isolating RF RNA by cellulose CF-11 chromatography and subjecting denatured RF RNA to hybridization with an excess of unlabeled virion RNA.

When the parental A59 virus was shifted from 30°C to 40°C at 9 hours post infection and pulse labeled with ^{3}H-uridine for two half hour periods, the rate of positive strand synthesis increased about two fold during the second half hour pulse period compared to the first half hour pulse (Fig. 1A). Similarly, the rate of negative strand synthesis increased about two-fold in the second half-hour relative to the first half hour after shift (Fig. 1B). The radioactivity in RF RNA also increased about two fold but the percent labeled RF RNA as negative strands remained constant. We interpret this to mean that the negative strands produced after shift were utilized as templates for positive strand synthesis to increase the rate of positive strand synthesis. With the addition of cycloheximide at the time of shift, positive strand synthesis remained the same during the second half hour pulse period compared to the first half hour pulse period (Fig. 1A). However, cycloheximide caused a dramatic reduction in negative strand synthesis (Fig. 1B). We reported previously

(Sawicki and Sawicki, 1986) that cycloheximide immediately inhibited negative strand synthesis whereas positive strand synthesis began to decline only after 30–60 min. after its addition. Therefore, the inhibition of negative strand synthesis after the addition of cycloheximide prevented the increase in the rate of positive strand synthesis which was observed after shift in the absence of cycloheximide.

Schaad et al. (1990) reported that the ts mutant LA 9 of the A59 strain MHV possessed a specific temperature sensitive defect in negative strand synthesis: Positive strands and infectious virus, but not negative strands, accumulated after temperature shift. Since they had not measured negative strand synthesis directly, we determined if negative strand synthesis by LA 9 was inhibited immediately by temperature shift. When LA 9 infected cells were shifted from 30°C to 40°C at 9 hours post infection and pulse labeled with ^{3}H-uridine for two half hour periods, the rate of positive strand synthesis increased about two fold during the second half hour pulse period compared to the first half hour pulse (Fig. 1C). The rate of negative strand synthesis also increased about two-fold in the second half-hour relative to the first half hour after shift (Fig. 1D). When cycloheximide was added at the time of shift, LA 9 positive strands synthesis declined slightly during the second half hour pulse period compared to the first half hour pulse period (Fig. 1C); and, cycloheximide caused a dramatic reduction in negative strand synthesis (Fig. 1D). If LA 9 had a specific defect in the synthesis of negative strands, we would have observed a similar reduction in the incorporation of ^{3}H-uridine into negative strands in the presence as in the absence of cycloheximide. Clearly, this was not the case. Rather, we observed that the increase in positive strand synthesis that occurred immediately after temperature shift was coupled to an increase in negative strand synthesis. Therefore, we could not confirm that LA 9 had a specific temperature sensitive defect in negative strand synthesis.

When shifted between 2 and 10 hours post infection to the nonpermissive temperature, LA 9 infected cells increased their rate of positive strand synthesis approximately five-fold (data not shown). With LA 9 we found the time of temperature shift to be critical. Shifting early when RNA synthesis is below detectable levels had little or no effect on viral RNA synthesis or virus yield. Shifting too late, when viral synthesis had reached its maximum rate, produced no immediate reduction in viral RNA synthesis or viral yield. We confirmed the original observation (Schaad et al., 1990) that LA 9 increased the rate of viral RNA synthesis and virus yield if the time of temperature shift was after six hours post infection.

We also screened two other ts mutants (LA 6 and Alb 16) for defects in negative or positive strand synthesis. LA 6 infected cells were shifted from 30°C to 40°C at 9 hours post infection and pulse labeled with ^{3}H-uridine for three twenty minutes periods. The rate of positive strand synthesis remained the same for the three pulse periods (Fig. 1E). Notice that the rate of ^{3}H-uridine incorporation into viral RNA was less with LA 6 than with the parental A59 or LA 9. LA 6 at the permissive temperature showed three to six fold less viral RNA synthesis compared to the parental A59 virus or revertants of LA 6. With LA 6 negative strand synthesis decreased dramatically after shift (Fig. 1F). With the addition of cycloheximide at the time of shift, positive strands synthesis remained the same during the three twenty minutes pulse (Fig. 1E) and cycloheximide caused a dramatic reduction in negative strand synthesis (Fig. 1F). We concluded that with LA 6 synthesis of negative strands, but not positive strands, was temperature sensitive. The failure to produce more negative strands after shift resulted in the failure to increase positive strand synthesis. Therefore, temperature shift in the presence and absence of cycloheximide produced nearly the same results.

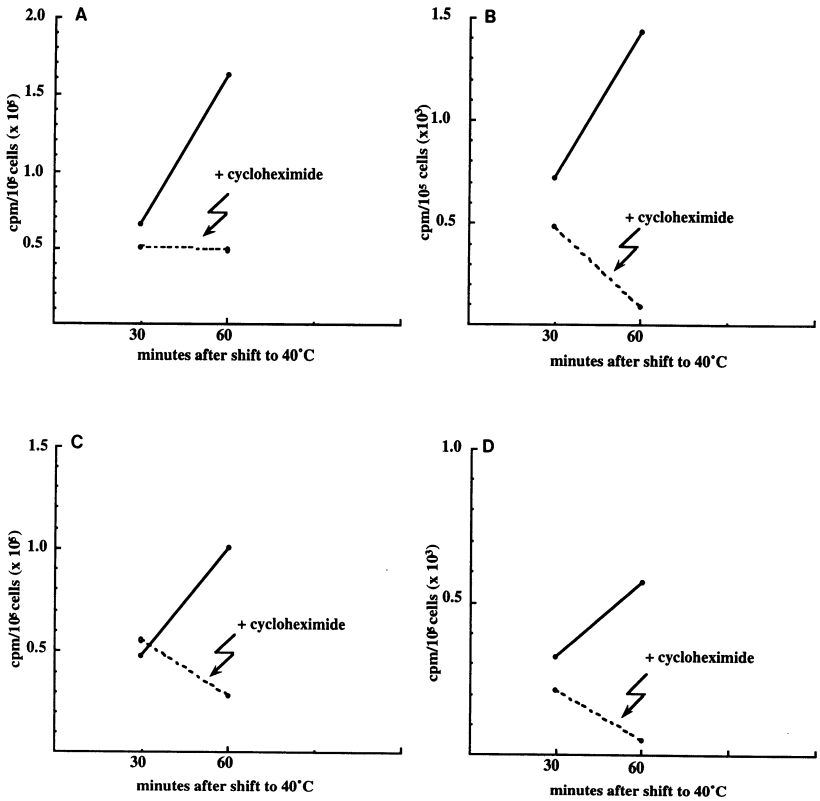

Figure 1. Viral and negative strand synthesis by parental A59 and temperature sensitive mutants after temperature shift. 17Cl-1 cells were infected and maintained at 30°C and shifted to 40°C at 8 hours for Alb 16 or 9 hours for A59, LA 9, and LA 6 viruses and labeled for 30 or 20 minute pulses with 200 μCi/ml of [^{3}H]uridine and 20 μg/ml dactinomycin in the presence or absence of 100 μg/ml cycloheximide. Viral RNA was determined by acid precipitation from cell lysates. RF RNA was purified by column chromatography on CF-11 after RNase T1 digestion. Labeled negative strands were obtained from the RF RNA after they were denatured, annealed to a 100-fold excess unlabeled virion RNA, and treated with RNase A and T1. (A) A59, viral RNA synthesis. (B) A59 negative strand synthesis. (C) LA 9, viral RNA synthesis. (D) LA 9, negative strand synthesis. (E) LA 6, viral RNA synthesis. (F) LA 6, negative strand synthesis. (G) Alb 16, viral RNA synthesis. (H) Alb 16, negative strand synthesis.

When Alb 16 (Sturman et al., 1987) was shifted at 8 hours post infection and pulse labeled with ^{3}H-uridine for three twenty minute periods, the rate of positive strand synthesis increased slightly or remained constant (Fig. 1G). The rate of negative strand synthesis remained relative constant for one hour after temperature shift (Fig. 1H). With the addition of cycloheximide at the time of shift, positive strand synthesis remained the about the same (Fig. 1G), whereas negative strand synthesis declined (Fig. 1H) as it did for the parental A59 virus. Therefore, Alb 16 has an interesting phenotype with regard to negative strand synthesis. Negative strand synthesis is not as sensitive to temperature shift as it is to cycloheximide. Nevertheless, positive strand synthesis did not increase significantly during the first hour after temperature shift, which mimics the effect of cycloheximide.

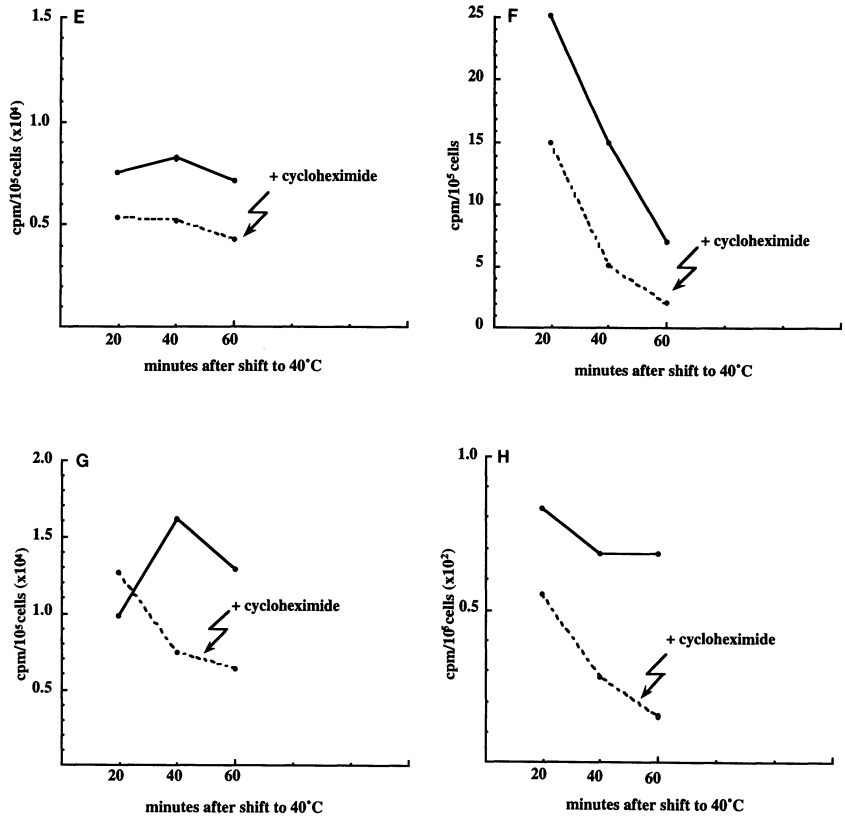

Figure 1E-H.

4. DISCUSSION

The use of RNA-negative temperature sensitive mutants should give some insight into mechanisms coronaviruses use to synthesize RNA. Previously, Schaad et al. (1990) had concluded that LA 9 had a temperature sensitive defect in negative strand synthesis. We could not confirm their results for negative strand synthesis but could confirm their results for positive strand synthesis and virus yield. Temperature shift with this mutant allowed negative strands to be synthesized for about one hour before the synthesis of negative strands decreased. This was in turn followed by a five fold increase in the rate of positive strand synthesis. The timing of the shift was critical. If the rate of viral RNA synthesis was 20% of the maximum or higher at the time of the shift, then a full burst of viral RNA synthesis and virus yield occurred. If the shift was earlier, then less viral RNA and virus were made.

The mutant LA 6 had a temperature sensitive defect in producing polymerase that would synthesize negative strands, but the polymerase producing positive strands was unaffected by shift to the nonpermissive temperature. Since negative strand synthesis was

not more sensitive to temperature shift than was the parental A59 virus to cycloheximide, we concluded the mutation in LA 6 affected the initiation of negative strand synthesis or the formation of the negative strand replicase. Without the continued production of negative strands the rate of positive strand synthesis remained constant after the shift to the nonpermissive temperature.

The mutant Alb 16 had an interesting phenotype. Negative strand synthesis was not inhibited immediately by temperature shift; however, the rate of positive strand synthesis did not increase. We (see Sawicki, D.L., and Sawicki, S.G., this volume) called alphavirus mutants with this phenotype conversion mutants because the newly formed polymerase which has negative strand activity cannot be converted to a polymerase with positive strand activity.

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CELLULAR PROTEIN hnRNP-A1 INTERACTS WITH THE 3'-END AND THE INTERGENIC SEQUENCE OF MOUSE HEPATITIS VIRUS NEGATIVE-STRAND RNA TO FORM A RIBONUCLEOPROTEIN COMPLEX

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1. ABSTRACT

We previously showed that several cellular proteins specifically bind to the 3'-end and the intergenic sequences of the negative-strand RNA of mouse hepatitis virus (MHV), and proposed that these distant RNA sequences can be brought together by cellular and viral proteins (Furuya and Lai, 1993; Zhang et al., 1994; Zhang and Lai, 1995). The cellular protein p35 has been identified as a heterogeneous nuclear ribonucleoprotein (hnRNP) A1. We have now expressed hnRNP-A1 as a glutathione-*S*-transferase (GST) fusion protein and demonstrated that the amino terminal two-thirds of hnRNP-A1 interacted with the two MHV regulatory RNA sequences (3'-end and intergenic sequences) through protein-RNA interaction while its carboxy-terminal glycine-rich domain mediated homomeric (protein-protein) interactions. In a partially reconstituted reaction, in which the two MHV RNA fragments and the purified GST-hnRNP-A1 fusion protein were mixed, an RNP complex was formed. Depletion of either hnRNP-A1 or one of the RNA components abolished the complex formation. These results indicate that hnRNP-A1 can mediate the formation of an MHV RNP complex, which includes both the negative-strand leader and intergenic sequences. Site-directed mutagenesis revealed that mutations in the MHV intergenic se-

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