ALTERED PROTEOLYTIC PROCESSING OF THE POLYMERASE POLYPROTEIN IN RNA(-) TEMPERATURE SENSITIVE MUTANTS OF MURINE CORONAVIRUS

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ABSTRACT

We examined the synthesis and processing of the polymerase polyprotein in RNA(-) temperature sensitive mutant of murine coronavirus strain A59. These temperature sensitive mutants of MHV-A59 synthesize viral RNA at the permissive temperature (33.0°C), but are unable to synthesize viral RNA at the nonpermissive temperature (39.5°C). The ts mutants have been mapped to five different complementation groups in the polymerase gene. The 5'-most complementation groups, Groups A and B, map to a region encoding an autoprotease responsible for the cleavage of p28, the amino-terminal product of the polymerase polyprotein. We screened six temperature sensitive mutants to determine if there was an alteration in the proteolytic processing of the polymerase polyprotein, particularly in the cleavage of the p28 protein. Two mutants, tsNC9 and tsLA16, had altered proteolytic products at both the permissive and nonpermissive temperatures. One Group B temperature sensitive mutant, designated tsNC11, was defective in the production of p28 protein at the nonpermissive temperature. To further localize the site of the mutation in tsNC11, RNA representing the 5'-most 5.3 kb region of the polymerase gene was transfected into tsNC11-infected cells and virus production monitored. The transfected RNA was able to complement the defect in tsNC11, resulting in viral RNA synthesis and production of viral particles at the nonpermissive temperature. These results indicate that a gene product from the 5.3 kb region of gene 1 is required for coronavirus RNA synthesis.

INTRODUCTION

The unique transcription and recombination mechanisms of coronaviruses implicate a polymerase with interesting properties. The polymerase may be able to disassociate and re-associate with template RNA to generate recombinant RNA molecules and seems to be able to identify relatively small intergenic sequences interspersed in the 31 kb RNA genome and initiate leader primed transcription (1). In addition, functional domains such as a helicase, polymerase, zinc-finger motif and proteinases have been predicted from the primary sequence of the polymerase gene (2). Our understanding of the complex functions of the MHV polymerase gene have been hampered because of the large size of the gene (22 kb) and its gene product (800 kDa) (2, 3). It has been previously shown that the gene 1 protein product is a polyprotein which is cleaved to yield subunits (4-7), but little is known about the

functional domains of either the polyprotein or the subunits. We have initiated a genetic approach to investigate the functional domains of the polymerase gene. Temperature sensitive mutants of MHV-A59 which are defective for RNA synthesis at the nonpermissive temperature and have been mapped to the 5'-end of the polymerase gene (8, 9) were analyzed for alteration in expression of the 5'-most proteolytic product, p28. To further localize the lesion in the ts mutant, RNA encoding the 5'-most open reading frame was transfected into ts virus infected cells and analyzed its ability to complement the ts defect. This approach allowed us to more precisely localize a mutation in one temperature sensitive mutant of coronavirus, tsNC11, and provides evidence that p28 may function in coronavirus RNA synthesis.

MATERIALS AND METHODS

Viruses and Cells. Temperature sensitive mutants of MHV-A59 designated tsNC8, tsNC9, tsNC11, tsNC13, tsLA6 and tsLA16 were derived by chemical mutagenesis and mapped to gene 1 by complementation and recombination analysis as previously described (8, 9). Virus isolates were plaque purified three times and stocks were prepared in DBT cells. Plaque assays were performed on monolayers of DBT cells in MEM containing 3% FCS and 0.4% Noble agar. Plates were incubated at 33.0°C or 39.5°C for 48 hr, and plaques visualized with neutral red.

Labeling of infected cells and immunoprecipitation. Duplicate monolayers of DBT cells were infected at a m.o.i. of 5 PFU/cell for 1 hr at 32.0°C. At 1.0 hr postinfection the inoculum was replaced with MEM containing 3% FCS and Actinomycin D (2 µg/ml). At 5.5 hr postinfection, medium was replaced with methionine-free MEM and half the cultures were shifted to 39.5°C. At 6.25 hr postinfection monolayers were labeled for 45 min with medium containing 200 µCi/ml (³⁵S)-trans-label (ICN), after which the cells were lysed and prepared for immunoprecipitation. Immunoprecipitations were carried out using an antipeptide antiserum specific for p28 (5). Products were analyzed on 10% SDS-polyacrylamide gels followed by autoradiography.

in vitro transcription and transfection of RNA. Plasmid pT7-NBgl consists of MHV-JHM gene 1 sequence from nucleotide 187 to nucleotide 5273 inserted downstream from the T7 polymerase promoter (5). Plasmid DNA was linearized by digestion with BamHI (nucleotide 467), Xho I (nucleotide 1129) or EcoRI (which cuts in the polylinker following the MHV-JHM sequences) and capped RNA was synthesized in vitro with T7 RNA polymerase as previously described (5). The resulting RNA was transfected into tsNC11 infected DBT cells using lipofectin (Gibco/BRL) following the procedure of Makino (10).

RESULTS AND DISCUSSION

As an initial approach to investigate the functions of the coronavirus polymerase gene products, we screened temperature sensitive mutants of MHV-A59 for alterations in expression or processing of the 5'-most polyprotein cleavage product, p28. These temperature sensitive mutants of MHV-A59 are unable to synthesize viral RNA at the nonpermissive temperature (39.5°C). A large panel of ts mutants were mapped by complementation and recombination studies into 5 groups (8, 9). Group A and B mutants map to the 5'-most 10 kb of gene, suggesting that the ts defect may reside in the p28 protein (encoded in the first 1 kb of the RNA genome), or in the proteinase domain (encoded in the 3.5 to 4.5 kb region of the RNA genome). The temperature sensitive mutants were screened by labeling duplicate dishes of infected cells with (³⁵S)-methionine at the permissive and nonpermissive temperatures, followed by immunoprecipitation of the cell lysate with antisera specific for p28 (5). Two ts mutants, tsNC9 and tsLA16, displayed an additional, higher molecular weight band that was co-precipitated with p28 at both the permissive (32.0°C) and nonpermissive (39.5°C) temperature (Figure 1A). A third ts mutant, tsNC11, produced p28 at levels comparable to wild type MHV-A59 at the permissive temperature. However, little or no p28 was detected in tsNC11 infected cell lysates labeled at the nonpermissive temperature (Fig. 1B). This reduction in the level of p28 in the tsNC11 infected cells at the

Figure 1. Immunoprecipitation of p28 from MHV-A59 and temperature sensitive mutant infected DBT cells labeled with (35 S)-methionine at the permissive and nonpermissive temperature.

nonpermissive temperature suggested that tsNC11 may have a defect in either the autoprotease domain responsible for cleavage of p28, the p28 cleavage site or in the p28 coding region.

To further test whether the loss of the p28 protein product was responsible for the ts phenotype of tsNC11, we established a protocol to provide translatable RNAs in trans to determine if the gene products produced from these RNAs would act in trans to complement the ts phenotype. The RNAs used for these experiments were synthesized from the pT7-NBgl plasmid DNA (5). As shown in Figure 2, this plasmid DNA contains the 5' open reading frame of MHV strain JHM downstream of a T7 RNA polymerase promoter. Plasmid DNA was linearized by digestion with Eco RI and capped RNA synthesized via T7 RNA polymerase. The capped RNA was then transfected in tsNC11 infected DBT cells using lipofectin (10). At four hours after infection and transfection, the cells were shifted to the nonpermissive temperature and incubated for an additional 16 hours. Supernatants were harvested from the infected/transfected cultures and plaque assays were performed at both the permissive and nonpermissive temperatures.

Figure 2. Schematic diagram of pT7-NBgl plasmid DNA. The open reading frame encodes the p28 protein and the proteinase domain responsible for autoproteolytic cleavage of p28. Restriction enzyme sites used to linearize plasmid DNA before in vitro transcription with T7 RNA polymerase are indicated.

Figure 3. Fusion of tsNC11 infected cells incubated at 39.5°C following mock transfection (A) or transfection with 10 ug RNA transcribed from pT7-NBgl/EcoRI DNA (B).

The first suggestion that transfected RNA may be able to complement the defect in tsNC11 was from a simple observation of the amount of virus induced fusion detected in tsNC11-infected/pT7-NBgl RNA transfected cultures compared to mock transfected virus- infected cells (Fig. 3). The number and extent of fusion seen in mock transfected, tsNC11-infected cells that had been shifted to the nonpermissive temperature at 4 hours after infection, was very low (Fig. 3A). In contrast, cells which had been infected with tsNC11 and transfected with 10 ug of capped RNA synthesized from pT7-NBgl/Eco RI DNA showed multiple, large syncytia characteristic a productive MHV infection (Fig. 3B).

To determine if the amount of syncytia formation correlated with the amount of virus produced from these cultures, plaque assays were performed on the supernatants collected from the cultures. As shown in Figure 4, there was greater than 3-fold increase in the amount of virus produced following transfection with 10 ug of pT7-NBgl/Eco RI RNA. This data indicates that the RNA or the gene product translated from the transfected RNA may act in trans to complement the defect in tsNC11 and thereby increase the production of infectious viral particles at the nonpermissive temperature. Interestingly, although there was a corresponding 3-fold increase in the amount of virus that produced plaques at the nonpermissive temperature, we did not detect a significantly higher reversion frequency (the number of infectious virus particles detected at 39.5°C divided by the number detected at 33.0°C). This data indicates that the increase in virus titer is probably due to complementation rather than a recombination event which would repair the temperature sensitive defect in the original tsNC11 genome., and therefore produce large amounts of virus which are no longer temperature sensitive.

	<u>33.0°C</u>	<u>39.5°C</u>	<u>39.5°C/33.0°C</u>
lipofectin alone	8.3 x 10 ³	4.8 x 10 ¹	5.8 x 10 ⁻³
1.0 ug RNA	7.0 x 10 ³	9.5 x 10 ¹	14.0 x 10 ⁻³
5.0 ug RNA	15.5 x 10 ³	13.8 x 10 ¹	8.9 x 10 ⁻³
10.0 ug RNA	28.8 x 10 ³	12.0 x 10 ¹	4.2 x 10 ⁻³

Figure 4. Determination of infectious particles released into the supernatant from tsNC11-infected/RNA transfected DBT cells. Virus titer is expressed in PFU/ml.

In summary, we have developed a transfection assay system which allows us to compliment the defect in a MHV RNA(-) temperature sensitive mutant, tsNC11. The data indicate that a gene product from the first 5.3 kb of gene 1 is sufficient to compliment the defect in RNA synthesis. We plan to use this assay to map the location of the ts lesion. In addition, we plan to exploit the high rate of recombination of coronaviruses to recover "repaired" recombinant viruses (11, 12), and thereby definitively show that the putative ts lesion is responsible for the particular ts phenotype of the virus. These experiments will provide information on the role of individual protein subunits in coronavirus RNA synthesis and for the mechanism of recombination of coronaviruses.

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