Identification of the Mutations Responsible for the Phenotype of Three MHV RNA-negative ts Mutants

[1]S. SIDDELL, [2]D. SAWICKI, [1]Y. MEYER, [1]V.THIEL, AND [2]S. SAWICKI
[1]Institute of Virology and Immunology, University of Würzburg, 97078, Germany: [2]Department of Microbiology and Immunology, Medical College of Ohio, Toledo

1. INTRODUCTION

A number of laboratories have isolated and characterized conditionally lethal, temperature-sensitive (*ts*) mutants of the A59 strain of murine hepatitis virus (MHV) (Koolen *et al* 1983; Sturman *et al* 1987; Schaad *et al* 1990). By and large, these studies have focused on *ts* mutants with an RNA-negative phenotype, i.e. mutants that are unable to synthesize viral RNA at the restrictive temperature. RNA positive mutants that are able to synthesize viral RNA but fail to produce plaques at the restrictive temperature have also been isolated and analyzed (Masters *et al* 1994; Ricard *et al* 1995; Luytjes *et al* 1997).

We decided to initiate a systematic analysis of the phenotypes and genotypes of a collection of MHV-A59 *ts* mutants assembled from laboratories in Albany (Sturman *et al* 1987) Los Angeles and North Carolina (Schaad *et al* 1990), Utrecht (Koolen *et al* 1983) and Würzburg (Siddell, unpublished). Our intention is to characterize this set of mutants with respect to their phenotypes, i.e.

- growth characteristics at permissive and restrictive temperatures
- reversion or back mutation frequency
- ability to synthesize plus and minus strand RNA at the restrictive temperature and

The Nidoviruses (Coronaviruses and Arteriviruses).
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- relative replication and transcription rates (genomic RNA synthesis versus subgenomic mRNA synthesis) at the permissive temperature and after shifting to the restrictive temperature.
- and genotypes, i.e.complementation group of each mutant and causal mutation, identified by sequence analysis of *ts* mutant and revertant pairs.

In the long term, our goal is to provide further insights into the role of individual proteins encoded by the replicase gene in the assembly and function of the coronavirus replication-transcription complex

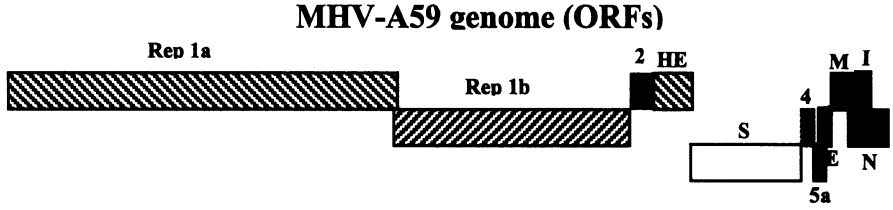
2. METHODS

Complementation analysis was done as described (Schaad *et al* 1990) and Sawicki *et al* (in preparation). The distinct phenotypes of Alb *ts*22, LA *ts*6 and Alb *ts*16 were determined as described (Younker *et al* 1998 and in preparation). The replicase gene sequence of wild-type MHV-A59, mutants, and revertants was determined by cycle sequencing performed on DNA fragments produced by RT-PCR of genomic RNA that was isolated from plaque-purified virus (Sawicki *et al,* in preparation).

3. RESULTS

Complementation analysis placed twenty-three mutants of MHV-59 into four complementation groups, numbered I-IV (Fig 1).

The mutations of three mutants, LA *ts*6 and Alb *ts*16 in cistron I and Alb *ts*22 in cistron II, were mapped by recombination and sequence analyses. Recombination analysis showed that mutations in LA *ts*6 and Alb *ts*16 were close to one another and distant from Albany *ts*22. Using Alb *ts*18 that has a mutation in the S gene (Ricard *et al* 1995) as a recombination marker, Alb *ts*16 was predicted to have a mutation close to and 5' of LA *ts*6, and Alb *ts*22 to have a mutation distant from and 3' of both LA *ts*6 and Alb *ts*16. We sequenced the entire replicase gene of Alb *ts*16 and *ts*22 and of LA *ts*6, revertants of each of the mutants and the wild-type parental MHV-A59 virus. The results of this analysis are summarized in Table 1.



ts, RNA-negative mutants with mutant ORF 1ab Rep genes

Cistron I	Cistron II	Cistron III	Cistron IV
Alb *ts*2	Alb *ts*22	Wü *ts*18	Alb *ts*17
Alb *ts*6	Ut *ts*261*	Wü*ts* 38	Wü *ts*36
Alb *ts*8	LA *ts*18 ?	Ut *ts*145	LA *ts*18 ?
Alb *ts*9		LA *ts*18 ?	
Alb *ts*16			
Alb *ts*19*			
LA *ts*3			
LA *ts*6			
LA*ts*10			
NC *ts*2			
NC *ts*3			
NC *ts*11*			
Ut *ts*88			
Ut *ts*261*			
Ut *ts*329			
Wü ts21*			

Figure 1. MHV-A59 RNA-negative mutants with replicase gene mutations. (*) mutants not producing revertants

Table 1. Identification of the mutations responsible for the phenotype of three RNA-negative-MHV ts mutants.

Mutant	Mutated Protein	Wild-type	ts	Revertant
Alb ts16	$p33^{3Clpro}$	Phe_{219}	Leu_{219}	Phe_{219}
LA ts6	p15	Gln_{65}	Glu_{65}	Gln_{65}
Alb ts22	$p102^{POL}$	His_{868}	Arg_{868}	His_{868}

In addition to being RNA-negative *ts* mutants, i.e., mutants unable to cause the synthesis of viral RNA when the infection is initiated and maintained at 37°C or higher, LA *ts*6, Alb *ts*16, and Alb *ts*22 have distinct phenotypes. These distinct phenotypes are observed when the infection is initiated at the permissive temperature (34°C or lower) and the infected cultures shifted to the restrictive temperature (40°C) after viral RNA synthesis has commenced. With Alb *ts*22, both plus and minus strand synthesis ceased immediately. With LA *ts*6, minus strand synthesis stopped immediately but plus strand synthesis continued at the same level as was occurring at the time of temperature shift; and, 30-60 min after shift to 40°C, plus strand synthesis began to diminish and by 3-4 hours was undetectable. With Alb *ts*16, both minus and plus strand synthesis was not greatly affected by a shift to 40°C. However, the rate of plus strand synthesis did not increase when Alb *ts*16 infected cultures were shifted to 40°C early, when the rate of viral RNA synthesis was increasing, i.e., between 5 and 8 hours at 30°C. Adding cycloheximide at the time of shift to 40°C, which inhibits minus strand synthesis within 15 min, caused plus strand synthesis to decline after 30-60 min (Younker *et al* 1998).

The phenotype of Alb *ts*22 is consistent with a mutation in the polymerase gene that makes elongation of nascent viral RNA temperature sensitive. Interestingly, we found that even at 30-34°C, Alb *ts*22 caused 4-5 fold less viral RNA synthesis compared to revertants of Alb *ts*22, the parental MHV-A59 virus, Alb *ts*16 or LA *ts*6. Moreover, Alb *ts*22 produced relatively more genomic than subgenomic mRNA (Younker *et al*, in preparation). It will be necessary to explain how a single amino acid change in the polymerase gene product, p102, that changed His_{868} to Arg_{868} results in both a *ts* phentype and an altered phenotype at permissive temperature.

The phenotype of LA *ts*6 would result from the failure to synthesize minus strand templates at 40°C. Eventually, the minus strands synthesized at permissive temperature are turned over (see Tao, Sawicki and Sawicki, these Proceedings) and plus strand synthesis declines coincidentally. The single amino acid change in the p15 protein that changes Gln_{65} to Glu_{65} apparently prevents the formation of minus strand polymerase activity at the restrictive temperature.

The phenotype of Alb *ts*16 results from a single amino acid change in the $p33^{3CLpro}$ protein that changes Phe_{219} to Leu_{219}. The mutated 3C-like protease activity would affect the rate of proteolytic cleavage of the replicase polyproteins, pp1a and pp1ab, at the restrictive temperature. Only enough minus strand polymerase activity would be formed at restrictive temperature to allow for the replenishment of minus strand templates being lost due to turnover. However, not enough minus strand templates would be produced at restrictive temperature to raise the rate of plus strand synthesis.

Alternatively, the mutation in the 3C-protease might specifically prevent the formation of plus strand polymerase activity, or the conversion of the minus strand polymerase activity to plus strand polymerase activity. As with alphaviruses, a precursor polyprotein in MHV might function in minus strand activity and proteolytic processing would unveil the plus strand polymerase activity (Wang *et al* 1994).

4. CONCLUSIONS

Our analysis of three MHV-A59 RNA-negative *ts* mutants allows us to make a number of interesting conclusions. First, the mutants Alb *ts*16 and LA *ts*6 both fall into the same complementation group (cistron I) and yet they have very distinct phenotypes. Indeed, the mutations responsible for these phenotypes are located in two different replicase polyprotein processed products, $p33^{3CLpro}$ and p15, present in pp1a and in pp1ab. One possible interpretation of this data is that the complementation group defining cistron I is in fact equivalent to the pp1a or pp1ab polyprotein precursor and $p33^{3CLpro}$ and p15 are cis-acting, i.e. the cleavage events that define these proteins occur after they have associated into a functional complex. This situation would be analogous to the relationships found for the Sindbis virus nsP2/nsP3 proteins (Wang *et al* 1994).

Second, our results demonstrate that the replicase gene products encoded in ORF 1a are involved directly in coronavirus RNA synthesis.

Third, LA *ts*6 was put into an "A" complementation group (Schaad *et al* 1990) and mapped to the 5' end of the genome by recombination analysis (Baric *et al* 1990). Based on our sequencing of LA *ts*6, the causal mutation is at the C-end of pp1a, close to where Fu and Baric (1994) suggested mutants belonging to their "C" complementation group would map. At this time we have no explanation for this discrepancy, which calls at least for a re-examination of past "cistron" assignments as causal mutations are identified.

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