Mapping of a Classical Temperature-Sensitive Replicase Mutant of Mouse Hepatitis Virus

Terry Hafer [1 2] And Dr. Paul Masters, PhD [1]
[1]Wadsworth Center, New York State Department of Health
[2]Pennsylvania State University

Abstract

Coronaviruses are positive-sense RNA viruses that can cause human Severe Acute Respiratory Syndrome (SARS) and Middle Eastern Respiratory Syndrome (MERS). Mouse Hepatitis Virus (MHV) is the prototype used here to analyze temperature-sensitive (ts) mutants of Alb2 that can grow at 33°C, but not 39°C. Previous results suggested that the mutation in Alb2 may occur within nonstructural proteins nsp1-11 of the 16-subunit replicase gene [1]. Analysis of the ts mutant Alb2 using RT-PCR and sequencing showed that Alb2 has a point mutation in the Y domain of nsp3 resulting in an amino acid change from Alanine to Valine. In addition, Alb2 was found to have a 47-nt deletion and a point mutation in the 3' Untranslated Region of nsp3. Six revertants were selected for further analysis. One revertant analyzed from nsp1- nsp11 was found to have a single primary site mutation in the Y domain of nsp3 causing it to revert to wild type MHV. Those remaining were sequenced only in the nsp3 region and all reverted back to WT. No second-site mutations were found in any of these six, and the 47-nt change and the point mutation in the 3' UTR were all maintained in them. The results of revertant analysis strongly suggest that the mutation responsible for temperature-sensitivity in Alb2 is the point mutation in the Y domain of nsp3. To complete the analysis of ts mutant Alb2, the remaining genome will be sequenced to determine if other mutations downstream of nsp11 might impact the Y domain of nsp3.

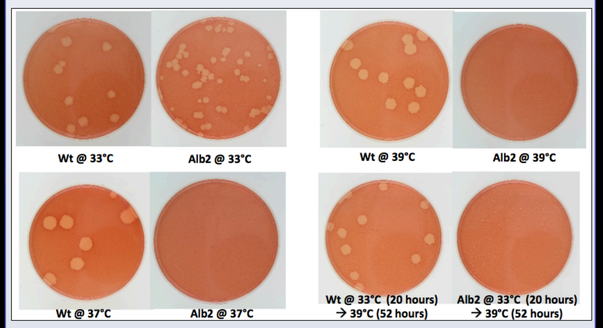

Figure 1: Phenotype of *ts*Alb2 Mutant. At 37ºC, *ts*Alb2 forms no plaques, meaning that it has become defective in some essential viral function at this temperature, making 37ºC the nonpermissive temperature. Typically, this would be the ideal temperature for a mammalian virus, as seen with the large plaques of the *wt* at this temperature. At 39ºC, the *wt* forms smaller plaques than at 37ºC, but *ts*Alb2 is once again unable to form plaques at this temperature. When growth is initiated at 33ºC and shifted to 39ºC, plaques begin to form in *ts*Alb2, however growth is arrested at the nonpermissive temperature. Tiny plaques form as a result.

Objectives

- Find the mutation responsible for the temperature-sensitivity phenotype in Alb2.
- Understand how revertant viruses can mutate to compensate for the temperature-sensitivity.

Materials and Methods

- The *ts* Alb2 mutant was sequenced from nsp1-11 (~12,000 bp) to find mutations.
  - Infect mouse cells with Alb2 mutant
  - Isolate RNA
  - Generate cDNA
  - Amplify through PCR
  - Send for sequencing; Compare sequences of Alb2 mutant with *wt*.
- Generate revertants for revertant analysis
- Sequence viral RNA of each revertant using the same process as in the *ts* Alb2 mutant, and search for primary or secondary site mutations

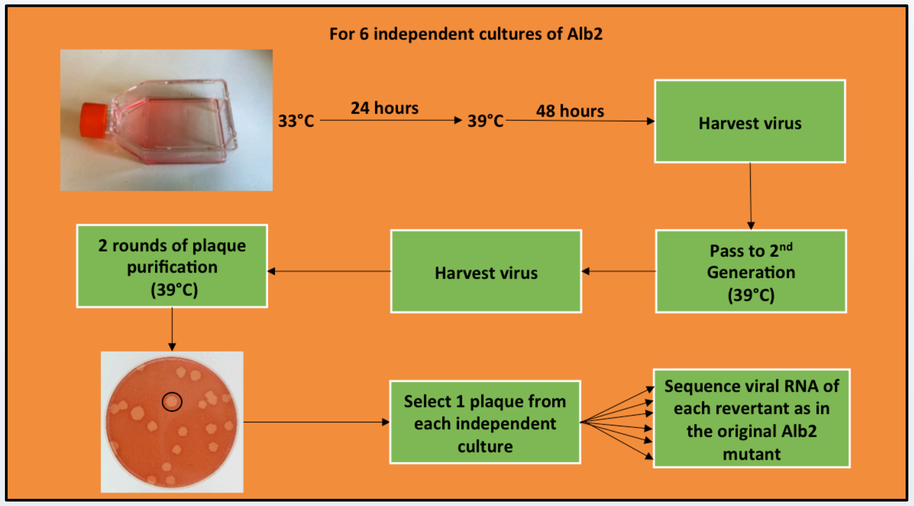

Figure 2: Generation of Revertants. Generation of revertants was carried out using the above methods. The infected flasks were initiated at the permissive temperature (33ºC), but then shifted to the nonpermissive temperature (39ºC) in order to select for revertants. Plaque purification was done by infecting cells with the virus on a cell-culture dish. The infection was overlayed with agarose and growth media.

Results

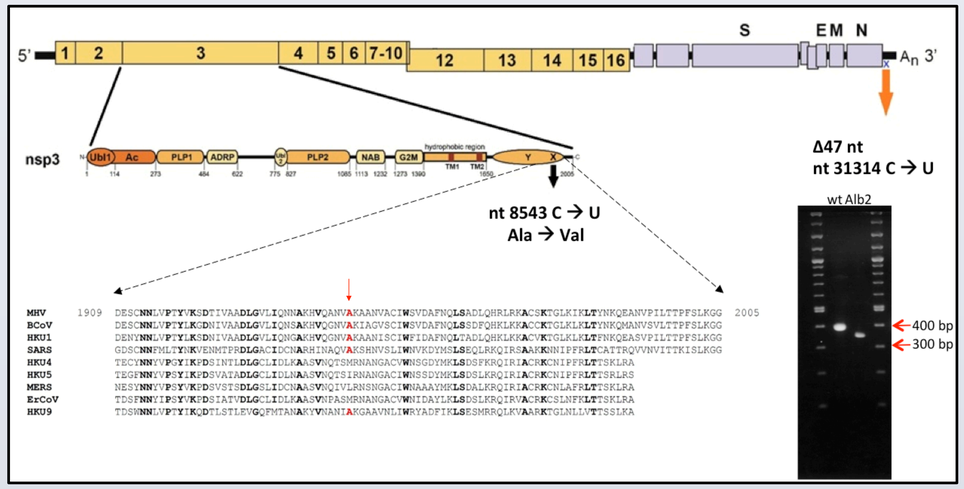

Figure 3: *Ts*Alb2 Mutation Search. Sequencing of *ts*Alb2 revealed a change in amino acid 1946 in nsp3, Alanine (shown in red) to Valine (nt 8543 C → U) in the Y-domain of nsp3. The Y-domain is highly conserved among all coronaviruses. In addition, a PCR using different primers revealed what appeared to be a 50-nt deletion in the 3' UTR. Sequencing results revealed a 47-nt deletion in the 3' UTR, in addition to a point mutation (nt 31314 C → U).

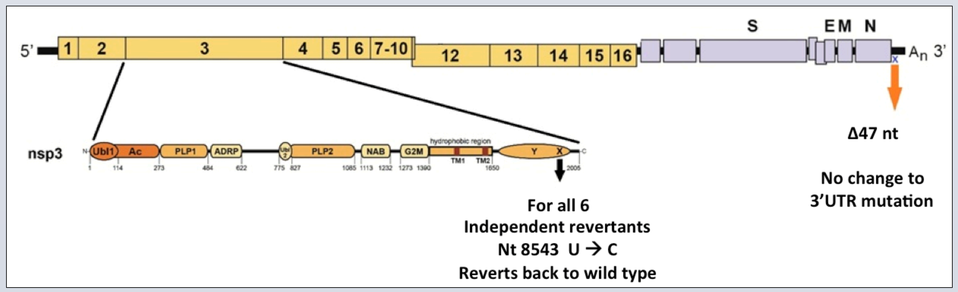

Figure 4: Revertant Analysis. All six independent revertants showed a reversion back to the *wt* at the location of the *ts*Alb2 mutation (nt 8543 U → C, aa Val → Ala). In addition, all six revertants maintained the 47-nt deletion and point mutation of *ts*Alb2.

Conclusions

- Alb2 has the following mutations:
  - Point mutation in the Y domain of nsp3
  - Point mutation in the 3'UTR
  - 47 nucleotide deletion
- The results of the revertant analysis strongly suggest that the mutation responsible for temperature sensitivity lies in the Y domain of nsp3.
- The deletion and point mutation in the 3'UTR are not responsible for temperature sensitivity in Alb2.
- The N-terminus of nsp3 interacts with the N protein, and has been shown to be indispensable to the virus, but little is known about the Y-domain and its function [2].
- Further work must be done to understand the role of the Y-domain of nsp3.

Future Work

- Sequence the rest of the genome – are there any more mutations?
- By reverse genetics, change amino acid 1946 of nsp3 to a different amino acid that is similar to valine.
- In order to obtain 2nd site mutations:
  - Try to obtain smaller plaque
  - Isolate revertants at 37 degrees rather than 39 degrees
  - This may show how different proteins or different parts of nsp3 are interacting with the Y-domain.

References

1. Sawicki SG, Sawicki DL, Younker D, Meyer Y, Thiel V, et al. (2005) Functional and genetic analysis of coronavirus replicase-transcriptase proteins. PLoS Pathog 39: 310-322.
2. Hurst KR, Koetzner CA, Masters PS (2013) Characterization of a Critical Interaction between the Coronavirus Nucleocapsid Protein and Nonstructural Protein 3 of the Viral Replicase-Transcriptase Complex. J Virol 87: 9159-9172.

Acknowledgements

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