TEMPERATURE-SENSITIVE MUTANTS OF MHV-A59

L. S. Sturman[1], C. Eastwood[1], M. F. Frana[2], C. Duchala[2], F. Baker[1], C. S. Ricard[1], S. G. Sawicki[3] and K. V. Holmes[2]

[1]Wadsworth Center for Laboratories and Research
New York State Department of Health
Albany, NY 12201

[2]Uniformed Services University of the Health Sciences
Bethesda, MD 20854

[3]Medical College of Ohio
Toledo, OH 43614

Sequencing of complete coronavirus genomes will soon be accomplished, and the size and locations of all of the viral genes and the amino acid sequence of the gene products will then be apparent. These important advances will be a major aid to understanding the fascinating biology and pathogenesis of this group of viruses. The next goal will be to understand the interactions of viral macromolecules during coronavirus replication, and to identify domains on the viral proteins which are responsible for these molecular interactions.

A genetic approach to understanding coronavirus replication has begun in several laboratories. Complementation analysis of conditional lethal mutants of MHV-JHM and MHV-A59 was used to identify 6 RNA^- complementation groups (Leibowitz et al., 1982; Van der Zeijst, personal communication). There should be 3 additional complementation groups to account for the viral structural proteins. This number of complementation groups (9) correlates rather well with the known number of viral proteins (Siddell, 1983), and the number of open reading frames identified to date (Skinner et al., 1985; Skinner and Siddell, 1985). Most of the mutants used for these studies were conditional lethal, temperature-sensitive mutants which had a difference of

M. M. C. Lai et al. (eds.), *Coronaviruses*

$> 10^4$ between the virus plaquing efficiencies at 39°C and 32°C (Haspel et al., 1978; Robb et al., 1979; Leibowitz et al., 1982; Koolen et al., 1983). As would be expected, these mutants were predominantly of the RNA^- phenotype at the non-permissive temperature.

We are particularly interested in identifying mutations which affect the viral structural proteins. Such mutations in other virus groups have been found to be more "leaky" and show less difference in yield or plaquing efficiency between the permissive and non-permissive temperatures than RNA^- mutants. The phenotypes of such mutants may include subtle alterations in plaque morphology, cytopathic effects (CPE), or host range among other phenotypes. We used nitrous acid to induce point mutations in MHV-A59 and then selected a series of 28 temperature-sensitive mutants of MHV-A59 by microscopic examination of virus-induced CPE in replicate cultures at 32° and 39°C. The mutants were plaque purified, and high titer stocks were prepared at 33°C. The phenotypes of several mutants are illustrated in Table 1.

Table 1

Mutant	Plaquing efficiency (39°/32°)	Plaque size (39°C)	CPE (39°C)
Alb 4	1.5×10^{-1}	small	yes
Alb 6	7.0×10^{-5}	normal	no
Alb 13	4.1×10^{-1}	small	yes
Alb 16	1.5×10^{-4}	normal	no
Alb 18	3.9×10^{-4}	normal	no

In this paper we describe some of the observations we have made in these ongoing studies. To identify which gene is altered, many properties of each mutant are being examined. We determined the yield of virus from mouse 17 Cl 1 cells, and compared the incorporation of ^{3}H-uridine into viral RNA in Actinomycin D treated 17 Cl 1 cells at permissive and non-permissive temperatures. Synthesis and processing of viral proteins were studied with Western blots and pulse chase experiments. The quantity and intracellular location of the viral structural proteins were analyzed by immunofluorescence microscopy. Structural proteins and thermolability of purified mutant virions were compared with those of wild type virus.

One of the mutants listed in Table 1, Alb 16, showed no CPE at 39°C, and no viral proteins could be detected in infected cells by immunofluorescence or Western blots at 39°C. The incorporation of ^{3}H uridine into viral RNA in Actinomycin D treated cells at 39°C was <2% of that of wild type virus. This mutant is like the mutants with RNA^- phenotype described previously by Leibowitz and Koolen. It showed a 10^4 fold difference in plaquing efficiency at 32° and 39°C. Additional RNA^- mutants of MHV-A59 in this collection of mutants fall into several complementation groups. It will be of considerable interest to determine whether these correspond to complementation groups previously identified for MHV-A59 (Van der Zeijst, personal communication), and whether RNA^- mutants of MHV-A59 will complement RNA^- MHV-JHM mutants (Leibowitz et al., 1982).

It is probable that RNA^- mutants have mutations in the viral polymerase gene or in one of the genes for the other non-structural proteins involved in RNA synthesis. Possibly mutations in leader RNA or certain non-coding regions could also lead to the RNA^- phenotype. To date, none of the RNA^- mutants has been assigned to a specific gene. Several of the complementation groups with RNA^- phenotype are represented so far by only one or two mutants. It is possible that some of these groups could represent intragenic complementation between different functional domains of the same gene product rather than intergenic complementation (Pringle, 1982). It therefore is important to obtain larger numbers of RNA^- mutants, and to map the locations of the mutations on the viral genome.

Although Alb 6 and 18 resemble Alb 16 in their efficiency of plaquing, CPE and plaque characteristics (Table 1), they both make some RNA at 39°C. Preliminary data from pulse chase experiments suggest that the synthesis of E2 and N of Alb 18 is similar to wild type virus at 32° and 39°C. However, Alb 18 makes less of the E1 glycoprotein at 39°C than does wild type virus. Nevertheless, the E1 protein in the mutant can be glycosylated at both 32° and 39°C. Since E1 is required for virus assembly (Holmes et al., 1981), at the non-permissive temperature virion formation may be reduced. It is not yet clear whether this mutation is due to a change in transcription or translation of the E1 mRNA, or to a change in the E1 glycoprotein.

Alb 6 has a complex phenotype. Although at 32°C this mutant makes RNA and structural proteins comparable to the wild type virus, at 39°C it produces low levels of viral RNA. This mRNA can be translated *in vivo* to

make normal amounts of N protein, but reduced amounts of both the E1 and E2 glycoproteins (data not shown). The reduced amount of fusion produced by this mutant during replication at 39°C may be due to the reduced amount of E2 on the plasma membrane. In virions purified from the medium of 17 Cl 1 cells infected with Alb 6 at 33°, the ratio of 180K (uncleaved) to 90K (cleaved) E2 is like that of wild type virus (Figure 1). Therefore it appears unlikely that the reduced amount of fusion in Alb 6 at 39°C is due to a mutation affecting the ability of the E2 glycoprotein to be cleaved by cellular proteases which is required to activate cell fusion (Frana et al., 1985; Sturman et al., 1985). It will be of considerable interest to determine whether the ratio of Alb 6 virus-specific mRNAs in the cell is different at 32° and 39°C.

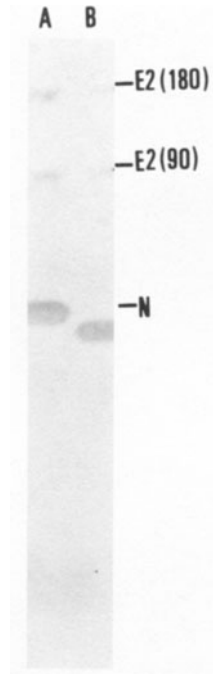

Figure 1. Structural proteins in purified virions of MHV-A59 mutants. Virions were purified from the supernatant medium over 17Cl 1 cells infected at 33°C with mutants Alb 6 and Alb 4. Western blot analysis of viral proteins from SDS-PAGE gels using antibody directed against E2 and N proteins shows that approximately 50% of the E2 in each of the mutant virions is cleaved from 180 to 90K, comparable to wild type MHV-A59 in these cells (Frana et al., 1985). The N protein in virions of Alb 4 (Lane B) migrates faster than that of Alb 6 (Lane A). Arrows on the right indicate the position of proteins from wild type MHV-A59 virions.

A large number of mutants with small or minute plaques at 39°C but wild type plaques at 32°C were identified. Different plaque mutants have different sizes. The plaque morphologies at 39° of two of these, Alb 4 and Alb 25, are compared with wild type virus (parent 5A2) in Figure 2. These mutants showed differences in cell fusion at 39°C when compared with wild type virus. Previous collections of chemically induced mutants of MHV were selected for absence of syncytia at 39°C. Since the mutants in this study were selected differently, they may include some involving alterations in a structural protein or its regulation, processing or expression.

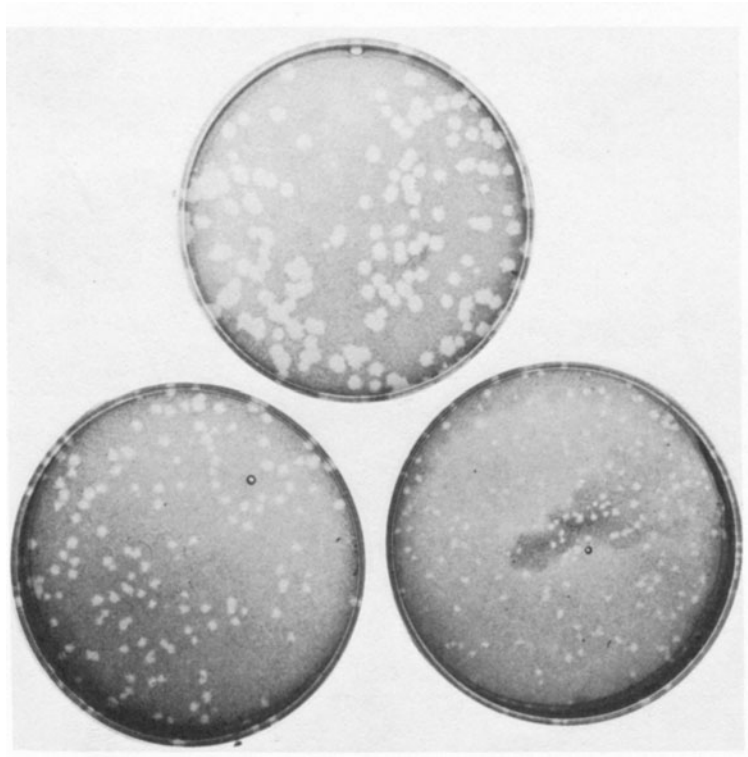

Figure 2. Plaque morphology of wild type and mutant MHV-A59 at 39°C. Some mutants, such as Alb 4 and Alb 13 (Table 1), have comparable numbers of plaques at 39° and 32°C. However, at 39°C the mutant plaques are small or minute. Plaques of different size produced by two mutants, Alb 4 (left) and Alb 25 (right), at 39°C are compared with plaques of parent MHV-A59 (designated 5A2) (top) in monolayers of 17Cl 1 cells.

Mutations in several different genes may result in the temperature-dependent small plaque phenotype. For Alb 13 (Table 1), the small plaque size and reduced cell fusion at 39°C appears to be associated with some defect in the processing of the E1 glycoprotein. Western blotting of intracellular viral proteins from wild type virus and Alb 13 grown at 32° and 39°C showed comparable amounts of N and E2, but a reduced ratio of glycosylated to non-glycosylated E1 in Alb 13 (Figure 3). This defect was more pronounced at 39°C. It will be interesting to determine whether the virions formed at 39°C contain an altered ratio of glycosylated to non-glycosylated E1.

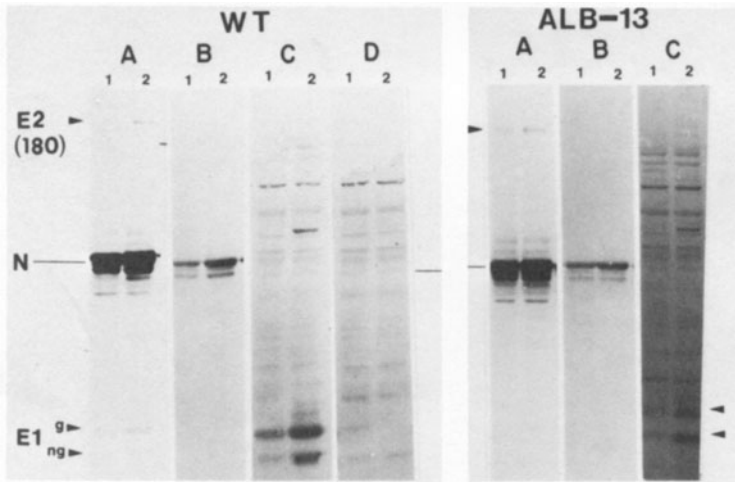

Figure 3. Intracellular virus-specific proteins of wild-type MHV-A59 and mutant Alb 13 at 32° and 39°C. Proteins from cytoplasmic extracts of 17Cl 1 cells infected for 14 hours with wild type MHV-A59 (left) or Alb 13 (right) were analyzed by SDS-PAGE and Western blotting with antisera directed against E2 and N (A), N (B) and E1 (C). (D) shows uninfected cell extract incubated with antibody (C). For each serum, the lane on the left shown proteins from cells grown at 32° and the lane on the right, at 39°C. Arrows indicate the position of E2 (180K), and glycosylated (g) and nonglycosylated (ng) E1.

Although Alb 13 and Alb 4 had the same temperature-dependent small plaque phenotype, they were apparently very different mutations. SDS-PAGE of the intracellular proteins of Alb 4 and wild type virus at 32° and 39°C, and immunoblotting with antisera directed against each of the viral structural proteins revealed that the nucleocapsid protein, N, of Alb 4

migrated faster (47K) than that of wild type virus (50K). This faster migrating N was seen in cells infected with Alb 4 at both 32° and 39°C, so the faster migration of N was not a temperature-sensitive defect. In cells infected with wild type virus, there are several bands of protein which react with antibody directed against N (Sturman and Holmes, 1983), but only the slowest migrating (50K) form is incorporated into virions (Holmes, 1984). In contrast, in purified virions of Alb 4 only the 47K form of N was observed (Figure 1). In addition to this striking difference in the electrophoretic mobility of N, Alb 4 also showed greatly enhanced thermolability in comparison with wild type virus. At 45°C, pH 6 for 2 hours, Alb 4 was inactivated 100 X more effectively than wild type virus. One intriguing hypothesis to explain all of these observations about Alb 4 is that the 47K N of this mutant is thermolabile and does not effectively perform some essential function, such as protecting the viral RNA, at the elevated temperature. This would result in greater inactivation of released virus in cultures held at 39°C. Further study of this interesting mutant may help to identify one of the functional domains of the nucleocapsid protein.

The examples described above show the complexity of determining the gene assignments for viral mutants. Many parameters of the expression of each virus gene must be evaluated, and the phenotype must be correlated with any observed change. Sequencing the altered gene will be required to elucidate where in the genome the mutation occurs, what kind of mutation it is, and what alterations in the protein may result. Multiple revertants of each mutation must be studied to ascertain whether all elements of the altered phenotype can be attributed to a change at a single genetic locus. These studies may elucidate complex temporal relationships between various virus functions during the replicative cycle. The genetic studies are made more complex by the high frequency of spontaneous mutation during replication of viral RNA by the RNA-dependent RNA polymerase.

While these studies have focussed upon characterization of chemically induced mutations selected to show temperature-sensitive changes in CPE, the same considerations pertain to mutants isolated in other ways, such as selection with neutralizing monoclonal (MC) antibodies. Many investigators are now attempting to understand the tissue tropism and virulence factors of coronaviruses using ts mutants, MCab-selected mutants or site directed mutagenesis. These studies must be cautiously interpreted, since any mutation which slows virus replication significantly may alter the temporal interactions between virus production *in vivo* and the immune response

migrated faster (47K) than that of wild type virus (50K). This faster migrating N was seen in cells infected with Alb 4 at both 32° and 39°C, so the faster migration of N was not a temperature-sensitive defect. In cells infected with wild type virus, there are several band of protein which react with antibody directed against N (Sturman and Holmes, 1983), but only the slowest migrating (50K) form is incorporated into virions (Holmes, 1984). In contrast, in purified virions of Alb 4 only the 47K form of N was observed (Figure 1). In addition to this striking difference in the electrophoretic mobility of N, Alb 4 also showed greatly enhanced thermolabiliy in comparison with wild type virus. At 45°C, pH 6 for 2 hours, Alb 4 was inactivated 100 X more effectively than wild type virus. One intriguing hypothesis to explain all of these observations about Alb 4 is that the 47K N of this mutant is thermolabile and does not effectively perform some essential function, such as protecting the viral RNA, at the elevated temperature. This would result in greater inactivation of released virus in cultures held at 39°C. Further study of this interesting mutant may help to identify one of the functional domains of the nucleocapsid protein.

The examples described above show the complexity of determining the gene assignments for viral mutants. Many parameters of the expression of each virus gene must be evaluated, and the phenotype must be correlated with any observed change. Sequencing the altered gene will be required to elucidate where in the genome the mutation occurs, what kind of mutation it is, and what alterations in the protein may result. Multiple revertants of each mutation must be studied to ascertain whether all elements of the altered phenotype can be attributed to a change at a single genetic locus. These studies may elucidate complex temporal relationships between various virus functions during the replicative cycle. The genetic studies are made more complex by the high frequency of spontaneous mutation during replication of viral RNA by the RNA-dependent RNA polymerase.

While these studies have focussed upon characterization of chemically induced mutations selected to show temperature-sensitive changes in CPE, the same considerations pertain to mutants isolated in other ways, such as selection with neutralizing monoclonal (MC) antibodies. Many investigators are now attempting to understand the tissue tropism and virulence factors of coronaviruses using ts mutants, MCab-selected mutants or site directed mutagenesis. These studies must be cautiously interpreted, since any mutation which slows virus replication significantly may alter the temporal interactions between virus production *in vivo* and the immune response

(Pringle, 1982) resulting in attenuation of virulence. Coronavirus replication involves a complex series of events determined by a small number of genes, many of which are multifunctional. Further studies of coronavirus mutants will yield valuable information about the functional domains and molecular interactions of these viral proteins, and should ultimately contribute to understanding how coronaviruses cause disease.

ACKNOWLEDGEMENTS

The authors thank E. C. Bauer for excellent technical assistance, and R. Williams and C. Stephensen for helpful discussions. This work was supported by grant GM 31698 and AI 18997 from the National Institutes of Health. The opinions expressed are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University School of Medicine of the Department of Defense.

References

Frana, M.F., Behnke, J.N., Sturman, L.S., and Holmes, K.V., 1985, Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Host-dependent differences in proteolytic cleavage and cell fusion, J. Virol., 56:912.

Haspel, M.V., Lampert, P.W., and Oldstone, M.B.A., 1978, Temperature-sensitive mutants of mouse hepatitis virus produce a high incidence of demyelination, Proc. Natl. Acad Sci., USA, 75:4033.

Holmes, K.V., Doller, E.W., and Sturman, L.S., 1981, Tunicamycin resistant glycosylation of a coronavirus glycoprotein: Demonstration of a novel type of viral glycoprotein, Virology, 115:334.

Holmes, K.V., 1984, Assembly of Coronaviridae, Chapter 7, pp. 100-119 in "Assembly of Enveloped RNA Viruses", M. DuBois-Dalcq, K.V. Holmes, and B. Rentier, eds., Springer-Verlag, Vienna.

Koolen, M.J.M., Osterhaus, A.D.M.E., van Steenis, G., Horzinek, M.C., and van der Zeijst, B.A.M., 1983, Temperature-sensitive mutants of mouse hepatitis virus strain A59: Isolation, characterization and neuropathogenic properties, Virology, 127:393.

Leibowitz, J.L., DeVries, J.R., and Haspel, M.V., 1982, Genetic analysis of murine hepatitis virus strain JHM, J. Virol., 42:1080.

Pringle, C.R., 1982, The genetics of vesiculoviruses, Arch. Virol., 72:1.

Robb, J.A., Bond, C.W., and Leibowitz, J.L., 1979, Pathogenic murine coronaviruses. III. Biological and biochemical characterization of temperature-sensitive mutants of JHMV, Virology, 94:385.

Siddell, S., 1983. Coronavirus JHM: coding assignments of subgenomic mRNAs. J. gen Virol. 64:113

Skinner, M.A., Ebner, D., and Siddell, S.G., 1985, Coronavirus MHV-JHM mRNA 5 has a sequence arrangement which potentially allows translation of a second, downstream open reading frame. J. gen. Virol., 66:581.

Skinner, M.A., and Siddell, S.G., 1985, Coding sequence of coronavirus MHV-JHM mRNA 4, J. gen Virol., 66:593.

Sturman, L.S., and Holmes, K.V., 1983, The molecular biology of coronaviruses, Adv. Virus Res., 28:35.

Sturman, L.S., Ricard, C.S., and Holmes, K.V., 1985, Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments, J. Virol., 56:904.