Recovery of Putative Temperature-Sensitive Mutations in Coronavirus Nonstructural Protein 5 Reveals Conserved Determinants of nsp5 Structure

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KEYWORDS. Coronavirus, Nonstructural Protein 5, Temperature-Sensitive Mutations

BRIEF. This study identifies temperature-sensitive mutations as conserved determinants in coronavirus nsp5 proteins of strains HKU1 and OC43.

ABSTRACT. Coronaviruses are positive-strand RNA viruses that cause several upper and lower respiratory diseases, including Severe Acute Respiratory Syndrome (SARS). During coronavirus infection, the genome is initially translated to yield large polyproteins that require proteolytic processing by virus-encoded proteases. Nonstructural protein 5 (nsp5) is an essential cysteine protease present in all coronavirus strains that mediates processing at 11 cleavage sites. Recently, temperature-sensitive (*ts*) mutations in murine hepatitis virus (MHV) nsp5 have been identified that impair protease activity. The previously described *ts* mutations, S133A and V148A, were inserted into chimeric MHV viruses containing nsp5 from human CoV strains, HKU1 and OC43, to reveal insights into the conservation of nsp5. Both HKU1 and OC43 chimeras with V148A showed no apparent growth defects at the non-permissive temperature. However, the OC43 S133A chimera (O5-S133A) had impaired viral replication at the non-permissive temperature of 40°C. This study will help increase the understanding of the structure and function of nsp5 proteases among coronavirus strains and may provide novel targets for pan-coronavirus protease inhibitors since nsp5 is a primary target for coronavirus inhibitor design.

INTRODUCTION.

Viruses are disease-causing agents that can be classified based on their genome. Coronaviruses are a group distinct for causing respiratory diseases of varying severity in humans and having large RNA genomes [1, 2]. There is a persistent threat of diseases caused by coronaviruses, shown most notably by the emergence of severe acute respiratory syndrome (SARS coronavirus) in 2002-2003 [2, 3, 4]. In the last ten years, three new human coronaviruses (HCoV) called HKU1, NL63, and SARS-CoV have been identified emphasizing the potential of new emerging viruses [2, 4, 5]. However, the relationship between the structure and function of the nsp5 protease in different coronavirus strains is not fully understood. This has made inhibitor development difficult [2, 4, 5]. Various approaches are directed targeting the replication mechanisms to develop effective inhibitors to prevent coronavirus infections [2, 4, 5, 6, 7]. If replication of a virus can be prevented, then infection is effectively halted.

The murine hepatitis virus (MHV) is a model system for studying the coronavirus genome [2, Figure 1A]. The MHV genome includes 7 genes designated by their numbers, and two open reading frames, ORF1a and ORF1b, encode gene 1. Gene 1 encodes two long proteins, (polyproteins) which generates 16

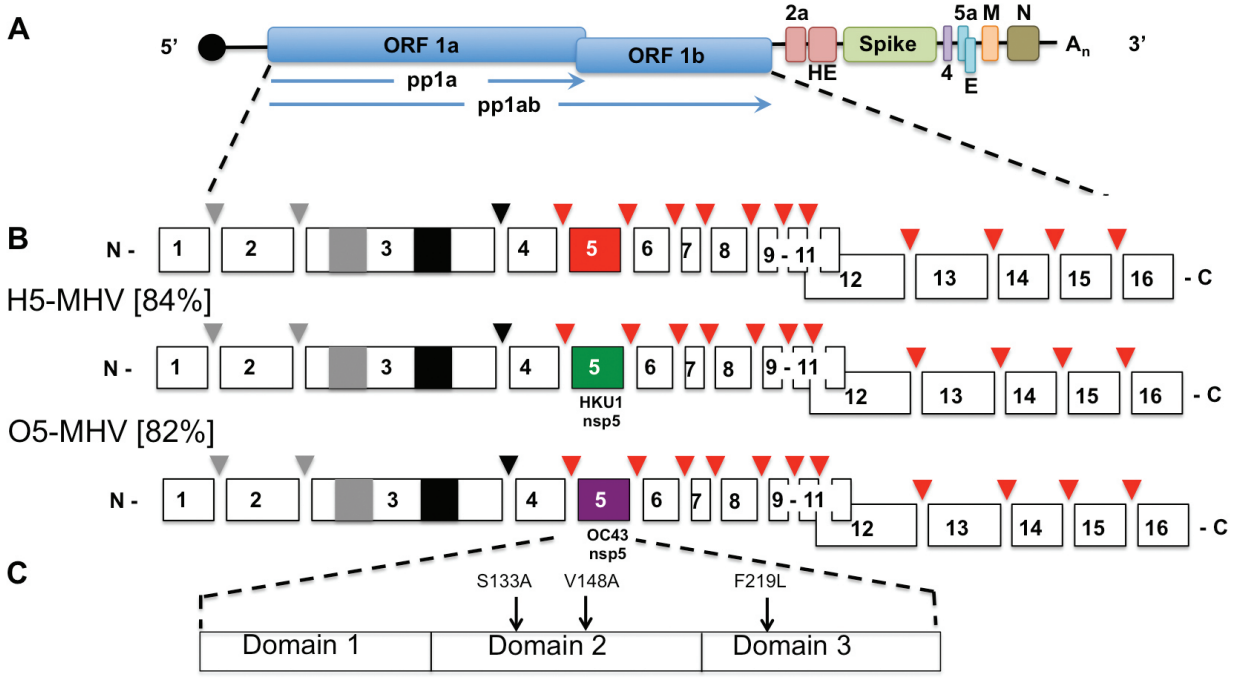

Figure 1. (A) Schematic representation of a murine hepatitis coronavirus (MHV) genome. Gene 1, encoded by ORF1a and ORF1b, contains the replication machinery of the virus. Genes 2 to 7 encode structural and accessory proteins. Protease activity of nsp3 subunits is shown with grey and black arrows. Red arrows indicate cleavage sites of nsp5. (B) Schematic representation of recovered chimeric viruses with nsp5 substitutions from strains HKU1, which shares an 84% sequence identity to MHV nsp5, and OC43 with and 82% sequence identity. (C) Temperature-Sensitive Mutations S133A and V148A are located in Domain 2 and mutation F219L is located in Domain 3.

smaller proteins after posttranslational processing. These 16 proteins are known as nonstructural proteins (nsps) [2, 4, 5, 7-9]. The nsp5 protein is a cysteine protease and is responsible for proteolytic cleavage of nonstructural proteins at 11 distinct locations along the polyproteins. Because of its role in virus replication, the nsp5 protease is the key focus of inhibitor design [4, 5, 10, 11].

The nsp5 protease from HKU1 and OC43 were substituted into an MHV virus based on the close similarity of their sequences to the MHV nsp5 (4). These viruses with human coronavirus proteases were recovered successfully showing little defect in viral growth. The MHV viruses with the nsp5 protease gene were designated as H5-MHV and O5-MHV due to the presence of either the HKU1 nsp5 or OC43 nsp5 in the MHV genome (Figure 1B). This is combined with the knowledge that in coronaviruses, temperature-sensitive (*ts*) mutations have identified key determinants of replication that could be used for potential inhibition of coronavirus infection [4, 5]. An initial study found a profound defect at the non-permissive temperature of 40°C due to the mutation V148A (Valine mutated to Alanine at position 148), located in Domain 2 of the MHV nsp5 [5, Figure 1C]. Another study also identified two *ts* mutations, S133A (Serine mutated to Alanine at position 133) in Domain 2 and F219L (Phenylalanine to Leucine at position 219) in Domain 3 of MHV nsp5 that impaired viral replication [4, Figure 1C].

In order to determine whether these residues are important in other human coronavirus strains, HKU1 and OC43, the putative *ts* mutations were engineered in nsp5 at their respective positions in the chimeric viruses, H5-MHV and O5-MHV [4, 5]. It is hypothesized that the mutations in the backgrounds of HKU1 and OC43 would result in a temperature-sensitive phenotype due to the high level of similarity between the nsp5 protease of these strains.

MATERIALS AND METHODS.

Viruses and Cell Culture.

Recombinant wild-type (WT) murine hepatitis virus (MHV) A59 (GenBank accession no. AY910861) was used as a control in all experiments in this study. Chimeric viruses (H5- and O5- MHV) without any *ts* mutations in nsp5 gene were also used as controls in all experiments to confirm that the viruses did not contain a temperature-sensitive background. Regions of the genome containing nsp5 from HCoV strains HKU1 and OC43 were substituted in to the MHV genome to create chimeric viruses. Murine delayed-brain tumor cells (DBT-9) were utilized, as these cells are highly sensitive to MHV infection. Baby hamster kidney 21 cells expressing the MHV receptor (BHK-MHVR) were used in the genomic assembly of mutant viruses. Cells were cultured using Dulbecco's modified Eagle medium (DMEM) (Gibco) that was supplemented with 10% heat-inactivated fetal calf serum (FCS). BHK-MHVR cells were cultured with medium supplemented with G418 (0.8mg/ml; CellGro). All cell lines were cultured at 37°C until confluence where they were then incubated at temperatures necessary to observe viral growth.

Mutagenesis of OC43 and HKU1 nsp5 in MHV C fragment.

The coronavirus genome was split into seven cDNA fragments; A to G, with the C fragment including the nsp5 coding region. Fragments were inserted into plasmids and ligated together to assemble the viral genome. The temperature-sensitive mutations; S133A, V148A, and F219L, were introduced in to nsp5 cDNA located in fragment C through site-directed mutagenesis PCR. To introduce these mutations, sense and antisense primers were used. The generated construct was confirmed by sequencing, and the C fragment was digested from the plasmid using the BsmBI and BglI restriction enzymes.

Assembly and Recovery of OC43- and HKU1-MHV Chimeras Containing ts-mutations in nsp5 cDNA.

The 6 fragments of the MHV cDNA were digested using appropriate restriction enzymes. They were then gel purified and the six fragments were ligated with mutated C fragments of OC43 or HKU1 overnight at 16°C in a reaction volume of 30 μL. The ligated cDNA genome was extracted and purified using chloroform and isopropanol precipitation. The cDNA was then transcribed to RNA using the mMachine T7 transcription kit (Ambion) and transfected to into BHK-MHVR cells through electroporation. The BHK-MHVR cells were washed twice with phosphate-buffered saline (PBS), trypsinized and resuspended in PBS to get cell titer were of $1x10^7$ cells/ml. In a 4-mm-gap electroporation cuvette, 600 μL of the resuspended BHK-MHVR were added along with MHV transcripts. The cuvette underwent 3 pulses of 850 V at 25 μF using the Bio-Rad Gene Pulser II electroporator. These electroporated cells were added to a flask of DBT-9 cells and incubated at 32°C, the permissive temperature for *ts* viruses, for 36 to 48 hours, or until virus-induced syncytia, a fusion of cells containing multiple nuclei, were evident in the flask visible by a light microscope. Viruses harvested from the culture medium of the original flask were designated as the P0 stock. The viruses were harvested and passaged to obtain P1 and P2 stocks of higher titer for subsequent experiments.

Efficiency of Plating and Titer Analysis.

A plaque assay was used to determine the titer, or the amount of virus per given volume. Viruses taken from the P0 and passage 1 (P1) stock underwent a series of dilutions from 10-1 to 10-6. Subsequently, DBT-9 cells were infected with these dilutions of the chimeric mutant viruses. The cells were grown with an overlay of agar growth medium. The viruses were incubated at three temperatures, 32°C, 37°C, and 40°C and were grown until plaques form, an indication of viral infection. The plaques were then preserved with a 4% formaldehyde solution. The gel overlays and the formaldehyde solution were discarded before the plaques were visualized and ready for counting.

Viral Growth Analysis.

Growth assays were conducted for mutant chimeric viruses by infecting DBT-9 cells at a multiplicity of infection (MOI) 1 PFU/mL. After a 30-minute absorption period, the medium with the viruses was removed and cells were washed three times with PBS. Prewarmed 10% FCS-DMEM was added to the infected cells, and they were then incubated at either 32°C, 37°C, and 40°C. Samples of media were collected at various time points. Plaque assays were conducted for the virus samples collected from 1 to 32 hours post infection (h.p.i). Wild type and mutant viruses were grown at known titers under temperature conditions of 32°C and 40°C.

RESULTS.

Recovery of Mutant Chimeric Viruses.

In order to address the question of the importance of specific residues in nsp5 among closely related coronavirus strains, this study attempted to incorporate 3 mutations (S133A, V148A, and F219L) into 2 chimeric backgrounds (H5 and O5) for a total of 6 viruses. Using the MHV infectious clone to assemble these mutant chimeric viruses, four of the six viruses attempted were successfully recovered with the primary mutation present in the nsp5 coding region from human coronavirus strains HKU1 and OC43. Mutations S133A and V148A were successfully engineered in both chimeric viruses. However, viruses with mutation F219L in nsp5 could not be recovered in the chimeric backgrounds. Virus cDNA was sequenced, and it confirmed the presence of the designated mutations in the nsp5 coding region.

Identification of Temperature-Sensitive Viruses.

To assess the phenotype of the mutations, plaque assays were performed at both the permissive and non-permissive temperatures. An indicator of viral growth is the formation of plaques, or a clearing, in a cell monolayer after infection of cells. To determine if a virus is temperature-sensitive, the measure of efficiency of plating (EOP) was used. The EOP is ratio of the non-permissive temperature titer to the permissive temperature titer. An EOP of 10-1 or lower demonstrates growth impairment and temperature-sensitivity (4). The EOP was calculated for all recovered viruses, chimeric viruses, and the WT-MHV virus (Figure 2). In this study, the EOP was averaged from 4 biological replicates, and the standard error of mean (SEM) is also reported.

Virus	Virus Titers (PFU / mL)*			EOP	
	32°C	37°C	40°C	40°/ 32°	40°/ 37°
WT-MHV	5.1×10^7 ($\pm 0.3 \times 10^7$)	8.3×10^7 ($\pm 1.5 \times 10^7$)	7.2×10^7 ($\pm 0.8 \times 10^7$)	1.4	0.9
H5-MHV	2.0×10^7 ($\pm 0.7 \times 10^7$)	1.2×10^8 ($\pm 0.1 \times 10^8$)	1.1×10^8 ($\pm 0.08 \times 10^8$)	5.7	1.0
O5-MHV	1.1×10^7 ($\pm 0.1 \times 10^7$)	2.0×10^7 ($\pm 0.2 \times 10^7$)	2.0×10^7 ($\pm 0.3 \times 10^7$)	1.7	1.0
H5-V148A	3.1×10^6 ($\pm 1.1 \times 10^6$)	2.0×10^7 ($\pm 0.4 \times 10^7$)	8.7×10^6 ($\pm 1.7 \times 10^6$)	2.8	0.4
H5-S133A	1.7×10^7 ($\pm 0.4 \times 10^7$)	2.5×10^7 ($\pm 0.6 \times 10^7$)	3.1×10^7 ($\pm 0.4 \times 10^7$)	1.8	1.2
O5-V148A	1.2×10^7 ($\pm 0.2 \times 10^7$)	3.7×10^7 ($\pm 0.6 \times 10^7$)	2.1×10^7 ($\pm 0.7 \times 10^7$)	1.7	0.6
O5-S133A	1.5×10^7 ($\pm 0.09 \times 10^7$)	7.5×10^6 ($\pm 1.1 \times 10^6$)	8.8×10^4 ($\pm 1.2 \times 10^4$)	0.006	0.01

Figure 2. Identification of Temperature-Sensitive Viruses. DBT-9 cells were infected with recovered viruses at the permissive temperature (32°C) and the non-permissive temperature (40°C). To determine if a virus is *ts,* the non-permissive temperature titer is divided by the permissive temperature titer, which finds the efficiency of plating (EOP). An EOP value below than 10-1 is considered *ts*.

The EOP data demonstrates that WT-MHV at the non-permissive temperature has an approximately 1.4-fold increase in titer compared to the permissive temperature, which is typical of a virus that is not temperature-sensitive (Figure 2). Compared to the WT-MHV EOP, O5-MHV with mutation S133A was temperature-sensitive and impaired at 40°C, with a 0.006-fold decrease in viral titers at the non-permissive temperatures (Figure 2). This is further demonstrated by the lower titer of this virus at 40°C compared to WT MHV, H5-MHV, and O5-MHV. Viruses H5-MHV with mutations V148A or S133A and O5-MHV with V148A did not have EOP values less than 10-1, having similar titers at the permissive and non-permissive temperatures.

Viral Growth Analysis.

To observe viral replication impairments, DBT-9 cells were infected with virus and titers were calculated from different time points, measured as hours post infection (h.p.i). In order to obtain the titer of each virus at every time, plaque assays were conducted for every time point for each virus. Results of sequencing from virus samples indicated a contamination in virus stock of H5-S133A with virus O5-S133A, causing results of H5-S133A for growth analysis to be removed.

Viral growth at the permissive temperature was similar for all viruses (Figure 3). All viruses reached similar maximum peak titers at 16 to 24 h.p.i., which is typical for virus replication. At 40°C, O5-MHV with S133A had apparent growth defects compared to WT MHV and chimeric viruses. There is a clear growth defect of virus O5-S133A illustrated by the delay in reaching its maximum peak titer. O5-S133A had a sudden increase in viral titer after 12 h.p.i, however its maximum peak titer at 16 h.p.i was lower than WT MHV and chimeric virus peak titers (Figure 3B). All other recovered mutant chimeric viruses had maximum peak titers similar to WT MHV (Figure 3B).

DISCUSSION.

The identification of a temperature-sensitive mutation in the nsp5 cysteine protease of a human coronavirus strain provides a tool for assessing the conservation of nsp5 structure and function. We identified the first temperature-sensitive mutation, S133A, in the human coronavirus strain OC43 based on the EOP data and viral growth analysis. However, the EOP does not suggest a similar level of temperature-sensitivity compared to previously identified temperature-sensitive mutations in nsp5 protease of MHV [4, 5]. One study reported an EOP of approximately 3 x 10-5 for the MHV virus containing mutation S133A in nsp5 [4, Figure 2]. This differs from the EOP of S133A in OC43 nsp5 of approximately 6 x 10-3, which suggests less temperature-sensitivity compared to S133A in MHV (Figure 2). The EOP for H5-S133A, H5-V148A, O5-S133A does not suggest impaired growth at the non-permissive temperature. The growth analysis at the permissive temperature indicates that all viruses were functional and were able to replicate similar to wild-type MHV. At the non-permissive temperature, viruses H5-V148A and O5-V148A did not show impaired viral growth, suggesting that the primary mutation, V148A, is not temperature-sensitive and did not debilitate nsp5 protease function. The virus O5-S133A appears to have growth defects compared to WT-MHV and chimeric viruses until approximately 16 h.p.i., where virus titers increase (Figure 3B). At this point, the virus may have reverted back to the original residue allowing virus titers to increase or it may have adapted second-site suppressors to help compensate for the debilitation caused by the primary mutation.

Interestingly, a previously identified second-site suppressor in MHV, H134Y, is present as residue Y134 in the nsp5 of HKU1 and OC43 The second-site suppressor H134Y was identified using reversion analysis in the MHV virus containing primary temperature-sensitive mutation, S133A [4, 5]. Previous reports also showed that a mutation at H134Y partially compensates for the mutation S133A in MHV. This leads to the hypothesis that the presence of Y134 in the genetic background may be compensating for the growth defects caused by the engineered mutations S133A in the nsp5 of HKU1 and OC43. It is expected that there may be compensating mechanisms in the chimeric mutant viruses constructed in this study, evidenced by the increase in viral titer for virus O5-S133A.

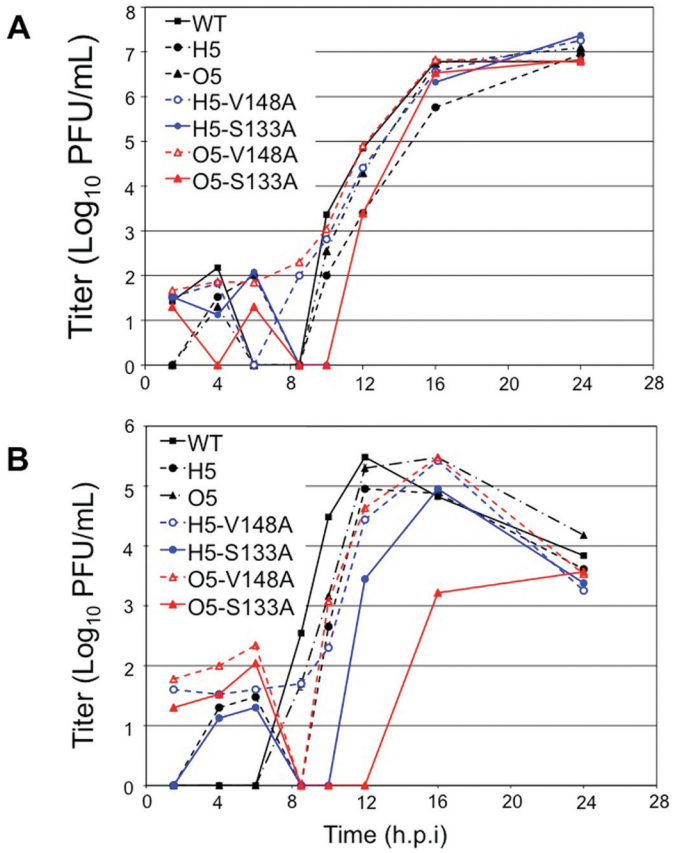

Figure 3. (A) Growth analysis of chimeric mutant viruses at 32°C. DBT-9 cells were infected with recovered viruses at 32°C to identify characteristics of viral growth. Plaque assays were performed on all time points for all viruses. At 32°C, viruses reach similar peak titers between 16 h.p.i. and 24 h.p.i., the range for WT-like viral growth. (B) Growth analysis of chimeric mutant viruses at 40°C. DBT-9 cells were infected with recovered viruses at 40°C to observe any growth impairments of the viruses at the non-permissive temperatures.

The identification of a conserved mutation indicates the overall importance of nsp5 functionality for virus replication because it results in the same phenotype in different CoV genetic backgrounds. Furthermore, the temperature-sensitive mutation demonstrates the conservation of functionally important residues in multiple nsp5 proteases from different CoV strains. It is possible that the viruses have adapted specific mechanisms that may be unique to the genetic background that the mutation has been engineered. The residues not involved with nsp5 substrate binding site may be necessary for nsp5 function and are unique to specific virus strains. On the other hand, these viruses may adapt with similar mechanisms demonstrated in the MHV nsp5 protease to compensate for the temperature-sensitive phenotype suggesting that the long-distance communication by specific residues that regulate nsp5 function may in fact be a conserved characteristic among closely related coronaviruses [4].

CONCLUSION.

Mutations in nsp5 that result in a *ts*-phenotype are likely to be vital for replication of coronaviruses. By characterizing these *ts*-mutations, the replication processes of coronaviruses will be better understood. We have identified one temperature-sensitive mutation in the nsp5 of a human coronavirus strain, giving insight into the conservation of important residues for the function of nsp5 proteases. The identified residue(s) appear to associate with conserved residues of nsp5 proteins and is a potential target for drug design.

More information on reversion mechanisms, essential residues for nsp5 and coronavirus function, and the structure of the protein with these new mutations can also shed light on how this protein can be targeted by inhibitors. In other words, when identified and characterized, these residues may be used as well-characterized targets for inhibitors.

To further the findings of this study, reversion analysis should be conducted to identify second-site suppressors and compensatory mutations. This would require infection of cells with the virus and subsequent extraction of virus and its genome for sequencing analysis. To further understand the relationship between any identified compensatory mutations and nsp5 structure and function, these mutations would be engineered in the nsp5 coding region to characterize solely the effect of the mutation. Similarly, the mutation Y134H should be made in the nsp5 coding region of HKU1 and OC43 with mutations S133A and V148A since Y134 is already present in the HKU1 and OC43 nsp5 genetic background. This will determine if the Y134 residue is compensating for the temperature-sensitive phenotype of these mutations. These experiments will help support the idea of long distance communication between residues in different domains of nsp5 is important for the regulation of nsp5 activity. Finally, the identified residues appear to associate with conserved residues of nsp5 structure and can be potentially identified as targets for inhibitor studies for multiple coronavirus strains.

ACKNOWLEDGMENTS. We would like to thank the members of the Denison Lab; Xiaotao Lu, Megan Culler, Nicole Sexton, Clint Smith, Dia Beachboard, and Michelle Becker as well as Angela Eeds for their support. The project described was supported by Award Number R25RR024261 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health.

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