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Coronavirus replicase nsp4 is critical for virus-induced membrane modifications. An nsp4 mutant (N258T) of murine hepatitis virus (MHV) has been reported to be temperature-sensitive (ts) and to alter membrane targeting. We engineered and recovered all four possible codon variants of N258T in the cloned MHV-A59 background. All mutant viruses demonstrated impaired replication compared to wildtype MHV, but no nsp4 N258T mutant virus was ts, and all variants colocalized with viral protein markers for replication complexes, but not with markers for mitochondria. This study emphasizes that complete genome sequencing may be necessary, even with directed and confirmed reverse genetic mutants.
permissive temperature (30°C) divided by the titer at the permissive temperature (30°C). When WT and N258TACA viruses were compared for EOP, N258TACA demonstrated an EOP similar to WT, and without a ts phenotype (Fig. 1).

The finding that N258TACA was not ts by EOP lead us to the questions: why our engineered mutant virus was different than the one reported by Clementz et al.; whether there were additional changes in their virus that led to the observed phenotypes; and if the phenotype was codon-specific. Therefore, we engineered viruses containing the TACC, TACG and TACT codon variants. All three Thr258 codon variant viruses, N258TACA, N258TACC, and N258TACT, were recovered at 30°C, consistent with a stable replication defect and likely decreased fitness compared with WT (Fig. 1).

We then tested growth following a temperature shift from 30°C to 40°C at 6 h.p.i., with supernatants sampled at 0.6, 2, 4, 6, 8, 10, 12, and 16 h.p.i. WT virus growth kinetics demonstrated an initial decrease in titer immediately following the temperature shift, but recovered quickly, and achieved peak titers by 12 h.p.i. All four codon mutants grew indistinguishably from each other, and achieved WT-like peak titers at 16 h.p.i. Similar to growth at 30°C, between 8 and 16 h.p.i., the codon mutants exhibited a lag in exponential growth and decreased viral titers compared to WT (Fig. 1). These data demonstrate that while the N258T substitution within nsp4 exhibited impairment in growth, it did not confer temperature-sensitivity, contrary to what has been previously reported.

The N258TACA virus reported by Clementz et al. (2008), was concluded to have altered localization of nsp4 to mitochondrial membranes at 39.5°C. To determine the localization of our mutant nsp4 proteins, DBT cells were infected with WT, N258TACA, N258TACC, N258TACG, and N258TACT on glass coverslips at an MOI of 0.1 PFU/cell for 16 h at 30°C or for 7 h at 40°C. Infected cells were then fixed and permeabilized with methanol, immunostained with antibodies specific to nsp4 and nsp8 or pyruvate dehydrogenase (PDH), a mitochondrial matrix protein. Cells were imaged using a Zeiss LSM510 confocal microscope.

At both 30°C and 40°C, for WT and all N258T codon substitutions, nsp4 and nsp8 extensively colocalized to punctate perinuclear foci (Fig. 2 and data not shown). In contrast, both WT and
mutant viruses display non-colocalization of nsp4 and PDH. Nsp4 localized to punctate perinuclear foci, whereas PDH localized to foci dispersed throughout the cytoplasm that were adjacent to but distinct from nsp4 foci. Within the same fields of view, there were infected cells that had not formed syncytia as well as syncytial cells and the pattern of colocalization were consistent between both sets of infected cells as well as within a z-stack. In order to quantify colocalization, Pearson’s correlation coefficient was calculated using the JACoP plugin for ImageJ (Bolte and Cordelieres, 2006; Schneider et al., 2012). To avoid bias, colocalization was quantified for the entire field and the entire z-stack of five images per condition. At both 30 °C and 40 °C, WT and N258TACA nsp4 and nsp8 had Pearson’s correlation coefficients of 0.71 and 0.79, respectively, consistent with colocalization. Nsp4 and PDH displayed Pearson’s correlation coefficients of 0.45 to 0.52 (p < .002), respectively, demonstrating non-colocalization (Fig. 2). These results demonstrate that the nsp4 N258T substitution did not result in altered localization of nsp4 to the mitochondria at either 30 °C or 40 °C.

Conclusions

Our results demonstrate that the nsp4 N258T substitution is not responsible for either the ts phenotype, or for the altered localization of nsp4 to the mitochondria reported by Clementz et al. Although the nsp4 N258T codon variant viruses were not ts, they displayed decreased titers and delayed growth, demonstrating that N258 or loop 1 of nsp4 is likely important for replication. Interestingly, this residue is highly conserved among beta-coronaviruses, including bovine coronavirus, human coronavirus OC43 and SARS-CoV as an aspartic acid, with MHV being the exception. The conservation of this residue suggests that it may be important. Our lab has previously reported two nsp4 mutant viruses, with mutations located in luminal loop 1. Nsp4 contains two glycosylation sites at N176 and N237 (Fig. 1), that when substituted with alanine, demonstrate delayed growth and decreased viral titers similar to those of nsp4 N258T, as well as altered DMV formation (Gadlage et al., 2010). The nsp4 E226A/E227A mutant virus (Fig. 1) is debilitated for growth and viral RNA synthesis (Sparks et al.,

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**Fig. 2.** (A) Nsp4 N258TACA codon variant localizes to the replication complex. DBT cells were infected at an MOI of 5 PFU/cell for 16 h at 30 °C or 7 h at 40 °C. Cells were fixed in methanol, probed for nsp4 (red) and nsp8 (green) or PDH (green) and imaged on a Zeiss LSM510 confocal microscope. Yellow pixels represent colocalization of overlapping red and green pixels. The scale bar in the bottom right corner of merged images represents 10 μm. (B) Pearson’s correlation coefficient was calculated for nsp4-nsp8 or nsp4-PDH for both WT and N258TACA at 30 °C and 40 °C (n = 5). Error bars represent standard deviation.
Together, these mutations suggest that loop 1 of nsp4 is important for viral replication, RNA synthesis, and formation of DMVs.

We are unable to explain the results reported by Clementz et al. because the virus was not available for direct comparison. However, our EOP results were confirmed by the Baker lab (data not shown). The reverse genetics system uses seven cDNA fragments that are ligated for transcription of genomic RNA that is then electroporated into cells for virus recovery. There is the possibility that mutations arose during amplification or transcription of the cloned fragments. Our lab and the Baker lab have the same original source for the cDNA fragments. In order to account for changes during amplification, we obtained all seven cDNA fragments from the Baker lab and attempts to recover virus were unsuccessful. We sequenced the cDNA fragment containing nsp4 and identified the N258TACA substitution, as well as a single nucleotide deletion at nt 8582 that resulted in a possible stop codon (UGA at nt 8644 to 8646). The virus reported by Clementz et al. was difficult to recover (personal communication), leading to the possibility of multiple adaptive changes. The virus was not available for sequencing; therefore, we could not test for additional mutations. The experiments in this study were performed in MHV-A59, and it is important to consider polymorphisms within different strains of virus when analyzing the importance of specific residues. The results of our study strongly suggest that sequencing of the entire genome of mutant coronaviruses derived from the reverse genetics approach may be necessary. Several studies have documented mutations that arise during the process of mutagenesis or propagation of cDNA clones, as well as adaptive mutations that may occur in genes not thought to have any relationship. We demonstrated that the original MHV infectious clone had WT-like replication in culture, but was attenuated in vivo (Sperry et al., 2005). Complete genome sequencing found mutations in other fragments that arose during propagation of the clones that were confirmed to be responsible for the attenuating phenotype. Hurst et al. (2010) showed that impairment in MHV replication by mutations in the nucleocapsid gene resulted in compensating second-site mutations in the replicase protein nsp3. Thus coronaviruses may have unexpected linked functions or epistatic relationships that might be missed by partial sequencing. Fortunately, the cost and time of genome sequencing is rapidly improving. Establishment and availability of validated primer sets may allow for more rapid sequencing in a 96 well format or by deep sequencing, further reducing the cost and time associated with complete genome analysis, and may identify novel and important new relationships among coronavirus proteins.

Acknowledgments

This work was supported by Public Health Service award RO1 AI50083 (M.R.D.) from the National Institute of Allergy and Infectious Disease. D.C.B. was supported by Immunobiology of Blood and Vascular Systems through the Vanderbilt University School of Medicine (T32HL697659). This work was also supported by the Elizabeth B. Lamb Center for Pediatric Research.

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