Homology-based identification of a mutation in the coronavirus RNA-dependent RNA polymerase that confers resistance to multiple mutagens

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ABSTRACT

Positive-sense (+) RNA viruses encode RNA-dependent RNA polymerases (RdRps) essential for genomic replication. With the exception of the large nidoviruses, such as coronaviruses (CoVs), RNA viruses lack proofreading and thus are dependent on RdRps to control nucleotide selectivity and fidelity. CoVs encode a proofreading exonuclease in nonstructural protein 14 (nsp14-ExoN), which confers a greater-than 10-fold increase in fidelity compared to other RNA viruses. It is unknown to what extent the CoV polymerase (nsp12-RdRp) participates in replication fidelity. We sought to determine whether homology modeling could identify putative determinants of nucleotide selectivity and fidelity in CoV RdRps. We modeled the CoV murine hepatitis virus (MHV) nsp12-RdRp structure and superimposed it with solved picornaviral RdRp structures. Fidelity-altering mutations previously identified in coxsackie virus B3 (CVB3) were mapped onto the nsp12-RdRp model structure and then engineered into the MHV genome with [nsp14-ExoN(+)] or without [nsp14-ExoN(-)] ExoN activity. Using this method we identified two mutations conferring resistance to the mutagen 5-fluorouracil (5-FU): nsp12-M611F and nsp12-V553I. For nsp12-V553I we also demonstrate resistance to the mutagen 5-azacytidine (5-AZC) and decreased accumulation of mutations. Resistance to 5-FU, and decreased number of genomic mutations, was effectively masked by nsp14-ExoN proofreading activity. These results indicate that nsp12-RdRp likely functions in fidelity regulation and that, despite low sequence conservation, some determinants of RdRp nucleotide selectivity are conserved across RNA viruses. These results also indicate that, with regards to nucleotide selectivity, nsp14-ExoN is epistatic to nsp12-RdRp, consistent with its proposed role in a multi-protein replicase/proofreading complex.
IMPORTANCE
RNA viruses have evolutionarily fine-tuned replication fidelity to balance requirements for genetic stability and diversity. Responsibility for replication fidelity in RNA viruses has been attributed to the RNA-dependent RNA polymerases, with mutations in RdRps for multiple RNA viruses shown to alter fidelity and attenuate virus replication and virulence. Coronaviruses (CoVs) are the only known RNA viruses to encode a proofreading exonuclease (nsp14-ExoN), as well as other replicase proteins involved in regulation of fidelity. This report shows that the CoV RdRp (nsp12) likely functions in replication fidelity, that residue determinants of CoV RdRp nucleotide selectivity map to similar structural regions of other unrelated RNA viral polymerases, and that for CoVs, the proofreading activity of the nsp14-ExoN is epistatic to the function of the RdRp in fidelity.
RNA virus replication results in the incorporation of a relatively high number of mutations, ranging from $10^{-4}$ to $10^{-6}$ mutations per site per round of replication (1-5). It is thought that low-fidelity replication is largely responsible for the capacity of RNA viruses to evolve rapidly and adapt to new host species and ever changing environmental pressures (6-8). The RNA-dependent RNA polymerase (RdRp) is central to the replication of RNA viruses and is a key regulator of nucleotide selectivity and fidelity (9, 10). Recent studies of CVB3, polio, HIV-1 and other viruses demonstrated that viable viruses are only recoverable within a four-fold range of RdRp fidelity (11-14). In most cases altered RdRp fidelity decreases fitness relative to WT viruses; this has been demonstrated for changes as small as a 1.2 fold difference in the accumulation of mutations (12, 14-16). Despite having as low as no amino acid identity outside of conserved motifs (11-14, 17-19), all described polymerase structures (including RdRps) resemble a “cupped right hand” with fingers, palm and thumb domains (20). The fingers form a channel that allows entry of the template RNA and ribonucleotide triphosphates (rNTPs) and assist in proper positioning of incoming nucleosites in the active site (21). The palm contains the active site, and the thumb functions in contacting exiting nascent RNA (21-23). However, there is diversity in the viral proteins that encode for RdRps; additional domains are often present that encode a variety of functions such as methyltransferase, endonuclease, polyribonucleotidyl-transferase, guanylyltransferase, membrane targeting, protein-protein binding or protein-RNA binding activities (24-26).

Coronaviruses (CoVs) infect a wide array of species and have emerged as highly pathogenic human pathogens twice in this century, first with Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) in 2003 (27) and second with Middle East Respiratory
Syndrome coronavirus (MERS-CoV) in 2012 (28). CoVs, and other large nidoviruses, replicate with higher fidelity than all other known (+)-RNA viruses (29, 30). CoVs also encode for the largest known RNA virus genomes ranging from 27-34 kb (31, 32), and increased fidelity in CoVs is likely required for maintenance of these large genomes (14). CoV genomes encode 16 non-structural proteins (nsp1-16), several of which are known or predicted to function in fidelity regulation, including nsp14-ExoN, a 3’-5’ exoribonuclease, and nsp10, a modulator of nsp14-ExoN activity (33, 34). Mutating the DE of the nsp14-ExoN active site to AA inactivates the exoribonuclease, yielding nsp14-ExoN(-) viruses, and nsp14-ExoN(-) viruses exhibit a greater-than 10-fold increase in mutation frequency (29, 35-37). Recent evidence demonstrates that nsp14 directly interacts with the CoV RdRp encoded in nsp12 (nsp12-RdRp) (38), but the effect of this interaction on nucleotide selectivity and overall fidelity regulation is not known. There are no solved structures for any CoV nsp12-RdRp, but the presence of conserved RdRp motifs and modeling of the C-terminal half of nsp12 predicts an RdRp domain that is structurally similar to other RNA viruses (39, 40).

The demonstrated function of nsp14-ExoN in high fidelity CoV replication raises the question of whether and how nsp12-RdRp participates in fidelity regulation. We sought to determine whether nsp12-RdRp can modulate nucleotide selectivity independently or in association with the proofreading nsp14-exonuclease. We modeled the RdRp domain of CoV nsp12 on coxsackievirus virus B3 (CVB3) and poliovirus polymerase structures and predicted residues important for fidelity based on prior result from those virus systems. Substitution mutations at these residues were introduced in the isogenic recombinant genome of the β-CoV, murine hepatitis virus (MHV-A59). We demonstrate that two of these mutations, nsp12-V553I and nsp12-M611F, confer resistance to the mutagen 5-fluorouracil (5-FU) and one, nsp12-
V553I, also results in resistance to the mutagen 5-AZC and demonstrates a decreased accumulation of mutations. Increased mutagen resistance and decreased accumulation of mutations was only observed in viruses with an inactivated ExoN, demonstrating that nsp14-ExoN proofreading activity is epistatic to the nucleotide selectivity of nsp12-RdRp. In this paper we define epistasis as a situation where the phenotype of one gene or viral protein masks the phenotype of genetic variants of another viral protein. This result is consistent with a primary role for nsp14-ExoN in error recognition and removal. However, introduction of RdRp mutations within the WT-MHV background decreased fitness relative to WT. Together the results suggest that nsp12-RdRp shares common determinants of nucleotide selectivity with RdRps from other RNA virus families. Further, the CoV RdRp has likely evolved to function in cooperation with nsp14-ExoN rather than independently.
MATERIALS AND METHODS

Virus and cell culture. Murine delayed brain tumor (DBT) cells (41) and baby hamster kidney 21 cells expressing the murine hepatitis virus (MHV) receptor (BHK-R) (42) were maintained at 37°C in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% FBS (Invitrogen), penicillin, streptomycin (Gibco) and amphotericin B (Corning). BHK-R cells were further supplemented with 0.8mg/mL of G418 (Mediatech). All virus work was performed using recombinant WT-MHV strain MHV-A59 (GenBank accession number AY910861 (42)).

Sequence analysis and homology modeling of CoV MHV nsp12-RdRp. The MHV RdRp domain structure was generated using Phyre2 online program (43) using nsp12 residues 385-887 that correspond to the reported SARS-CoV nsp12-RdRp model (40). The structural model was compared to the X-ray crystal structures of coxsackievirus B3 (CVB3) and poliovirus (PDB accession numbers 3DDK and 1RA7 respectively) using the Pymol Molecular Graphics System (Schrödinger, LLC). ClustalX multiple-sequence alignments were generated using the program MacVector.

Cloning, recovery and verification of mutant viruses. Quick-change mutagenesis was used to generate point mutations in individual MHV genome cDNA fragment plasmids using the previously described MHV infectious clone reverse genetics system (42). Mutant viruses were recovered in co-cultured BHK-R and DBT cells following electroporation of in vitro transcribed genome RNA in BHK-R cells. All viruses that included nsp14-ExoN(-) mutations were generated using the F fragment previously described (35). Before use in viral recovery all mutagenized plasmids were fully sequenced (GenHunter Corporation, Nashville, TN) to assure
no additional mutations were introduced. We also sequence verified engineered mutations in recovered viruses. Viruses in the nsp14-ExoN(-) background took between 84 and 96 hours to reach around 80 percent involvement in syncytia for a P0 stock in contrast to viruses in the WT background which were frozen at 24-48 h p.i. P1 working stocks were made by infecting D9s at an MOI 0.01 and freezing when 80 percent involved in syncytia, approximately 24 h p.i for WT viruses and 36 h p.i for nsp14-ExoN(-) viruses (2-3 rounds of replication).

Compounds and drug sensitivity studies. 5-fluorouracil (5-FU) was obtained from Sigma, and prepared as 200mM stock solutions in DMSO. 5-azacytidine (5-AZC) was also obtained from Sigma and prepared as 50mM stocks solutions in water. Sub-confluent DBT cells were pre-treated for 30min with DMEM with the indicated concentrations of 5-FU or DMSO, 5-AZC or media alone. Treatment was removed, and inocula added and allowed to adsorb for 1hr at 37°C. Inocula was then removed and media with drug or DMSO was returned. Infection proceeded for 24hrs for WT or 32hrs for nsp14-ExoN(-) viruses, when supernatants were acquired, frozen and titered by plaque assay as previously described (35).

Virus replication and RNA synthesis assays. Sub-confluent DBT cell monolayers in triplicate were infected at an MOI of 0.01 PFU/cell. Virus was allowed to adsorb for 30min when inocula were removed and the cells were washed 2X with PBS, followed by addition of pre-warmed media. For replication kinetics assays samples were taken at various time points post infection. Titering was performed by plaque assay as previously described (35). For analysis of RNA synthesis, total infected-cell RNA was obtained by TRIzol reagent (Invitrogen) at various times post infection and two-step RT-qPCR was performed as previously described (44).
**Determination of Specific Infectivity.** Sub-confluent DBT cells were pre-treated for 30 min with DMEM with indicated concentrations of 5-FU or DMSO alone. Treatment was removed, and inocula added and allowed to adsorb for 1 hr at 37°C. Inocula was then removed and media with drug or DMSO was returned. Infection proceeded for 20 hrs for WT or 24 hrs for nsp14-ExoN(-) viruses then supernatants were acquired, frozen and titered by plaque assay as previously described (35). Supernatants were also used for RNA genome isolation by adding 100 μL supernatant to 900 μL TRIzol reagent (Invitrogen), chloroform extracting by phase separation and using the aqueous layer in the PureLink Mini RNA kit (Ambion) as per the manufacturers protocol. One-step RT-qPCR was performed as below and the ratio of PFU to genomes of the supernatant was determined.

**One-step RT-qPCR for determining supernatant genome copies for specific infectivity assay.** An RNA standard was generated using MHV A fragment (42) to generate a 931 nucleotide RNA. First cDNA was generated by PCR using the primers: forward 5’-TAATACGACTCACTATAGGGGGCTATGTGGATTGTTGG-3’ which begins with a T7 promoter and reverse 5’-AATTCTTGACAAGCTCAGGC-3’. RNA for the standard curve was then generated using mMessage mMachine T7 kit (Ambion). An agarose gel with 1% bleach was run and a ~900 nt band was observed. RNA was purified using RNeasy Mini kit (Qiagen).

Dilutions of the standard curve were made from 10^3 to 10^8 genome equivalents for use in assay as needed. Primers and probes for one-step RT-qPCR were purchased from BioSearch Tech. Probe is 5’ FAM labeled and 3’ BHQ-1 labeled with the sequence 5’-TTCTGACAACGGCTACACCCAACG-3’ and made up to 5 μM in nuclease free water. The
184 primers used were forward 5’-AGAAGGTTACTGGCAACTG-3’ and reverse 5’-TGTCCACGCTAAATCAAAC-3’. Reactions were set-up on ice with enzyme added last.

185 Final volume for reactions was 20uL with 150nM probe, 900nM each primer, 2uL sample RNA and 10uL 2X ToughMix, one-step, low ROX enzyme mix (Quantas) used per reaction. Samples were plated in duplicate and run on the Applied Biosciences 7500 Real-Time PCR System with the conditions 55C for 10min, 95C for 5min, 95C for 30sec and 60C for 1min with the last two steps repeated 40X. The standard curve was graphed and genomes per mL determined.

191 Competitive fitness of mutant viruses. Sub-confluent DBT monolayers were co-infected at a total MOI of 0.01 PFU/mL with RdRp mutant viruses in the nsp14-ExoN(-) background and nsp14-ExoN(-) at either a 1:1, 1:9 or 9:1 ratio. When 50-70% of the monolayer was involved in syncytia, total RNA was harvested. RNA was then reverse transcribed using SuperScriptIII (Invitrogen) per the manufactures protocol and amplicons were generated using primers designed to cover the region including the codons for both the V553 and M611 residues. Amplicons were sent for sequencing and electropherograms were analyzed using MacVector.

199 Passage reversion analysis. Triplicate monolayers of sub-confluent DBT cells were infected with an initial MOI of 0.01 PFU/mL of nsp12-V553I and nsp12-M611F viruses in both the WT and nsp14-ExoN(-) background. Viruses were then blind passaged in triplicate for 5 passages. Total RNA was sequenced across a 1.7kb region of nsp12-RdRp that included both nsp12-RdRp mutations. Electropherograms were analyzed using MacVector.

206 Preparation of amplicons for deep sequencing of full viral genomes. Sub-confluent DBT
cells were infected at an MOI of 0.01 PFU/mL with nsp12-V553I or nsp12-M611F in either the WT or nsp14-ExoN(-) background, nsp14-ExoN(-) alone or WT alone. Infections were allowed to progress for 20 hours then RNA was isolated. RNA was reverse transcribed using SuperScriptIII (Invitrogen) per the manufactures protocol, and 12 amplicons were generated to cover the whole genome and processed as described previously (44).

Deep sequencing sample preparation and analysis. Amplicons were subsequently purified via a nucleospin PCR purification kit (Macherey-Nagel), quantified by picogreen, fragmented (Fragmentase) and prepared using the Illumina NextSeq500 Mid Output 150 cycle kits following the standard protocols. Sequences were obtained with an Illumina NextSeq500 machine. Sequencing runs were analyzed using the previously published ViVan bioinformatics pipeline (45). Briefly, the pipeline performs quality filtering and adaptor cleaning was done using fastq-clipper (http://hannonlab.cshl.edu/fastx_toolkit/index.html). The 150-nt reads were aligned to the reference sequence with a maximum 2 mismatches per read, using BWA (46) and processed using SAMTOOLS (47) to obtain the nucleotide/base calling at each position. The ViVan pipeline then identifies statistically significant variants above the background noise due to sequencing error, calculated for each nucleotide site: for each position throughout the viral genome, base identity and their quality scores are gathered. Each variant allele's rate is initially modified according to its covering read qualities based on a maximum likelihood estimation, and tested for significance using a generalized likelihood-ratio test. Additionally, an allele confidence interval is calculated for each allele. In order to correct for multiple testing, Benjamini-Hochberg false-discovery rate of 5% was set. In all experiments, a minimum coverage of 3000X reads was obtained and the background error frequency at every nucleotide site was always below 0.0001.
For analysis we use a conservative frequency cut off of 0.01 consistent with previous studies (48-50).

Statistical analysis. Statistics were applied as described in figure legends using GraphPad Prism 6 software (La Jolla, CA). The number of replicates performed for each experiment is similarly listed in each respective figure legend. Finally, some of the data was normalized to controls; the GraphPad Prism 6 software also performed this analysis.
RESULTS

Homology modeling of MHV nsp12-RdRp polymerase core domain predicts putative fidelity determinants. Mutations that alter nucleotide selectivity have been identified across multiple RNA virus RdRps (3, 11, 51-53); however, whether these residues are conserved across virus families is unknown. We sought to determine whether residues within nsp12-RdRp that are structurally homologous to known RNA virus fidelity determinants would have similar effects on nucleotide selectivity when introduced into the MHV background. To do this, we modeled the structure of MHV nsp12-RdRp using Phyre2 software (43). A series of nsp12-RdRp truncations was assessed, and the highest confidence model was used for further study. This region corresponded with a published model for the SARS nsp12-RdRp (40) and included residues 385-887 of the MHV nsp12 protein, referred to here as the RdRp core domain (Fig. 1a,b). Deletion of the CoV specific domain (residues 1-384), and a small C-terminal portion of the thumb domain (888-928), was required to establish this high-confidence model (Fig. 1a). The model was resolved by highest probability similarity to human rhinovirus serotype 1b (PDB ID: 1XR5a), rabbit hemorrhagic disease virus (PDB ID: 1KHV) and EV71 (PDB ID: 3N6M). The Phyre2 confidence, i.e. the probability of true homology, for the RdRp core domain to these structures was >99% while the percent identity was only 14-20%. Having generated a structural model for the MHV nsp12-RdRp core domain we next sought to predict residues involved in nucleotide selectivity. The nsp12-RdRp core domain model was aligned with the solved structure of coxsackie virus B3 (CVB3, PDB ID: 3DDK) using PyMol (Fig. 1c). A series of CVB3 RdRp mutations have been shown to result in decreased fidelity (10, 11, 54). The CVB3 fidelity determinants were compared with the MHV nsp12-RdRp core domain model. Those that aligned well structurally and by amino acid similarity were further investigated: MHV nsp12-V553,
M611, W613, A621, Y649 and K794 (Fig. 1). Finally, the nsp12 aa sequences of 27 different α−, \( \beta^- \) and \( \gamma \)-CoVs were aligned, including SARS-CoV and MERS-CoV. All six identified residues were conserved across these CoVs (Fig. 1d). Analysis of similarity and types of residues in the picornaviruses were then used to determine the specific amino acid changes that would be introduced at the identified MHV residues. The resulting substitution mutations were engineered in the isogenic cloned MHV genome: nsp12-V553A/I, M611F, W613Y, A621G, Y694H/W and K794R (Table 1).

**Recovery of mutant viruses in the MHV nsp14-ExoN(+) and nsp14-ExoN(-) isogenic backgrounds.** We next tested whether viable viruses could be recovered with substitutions at the identified residues. Virus recovery was attempted a maximum of 3 times, resulting in recovery of 6 of the 8 mutant viruses in the WT background: nsp12-V553I, M611F, W613Y, A621G, Y649H, and K794R. The time required for recovery of mutant viruses in the WT background ranged from 24 to 48 hours. No other mutations were identified across nsp12 sequences in recovered viruses. The A621G mutant was not further studied as it demonstrated rapid primary reversion even in the recovery (P0) supernatant. Since our goal was to understand the relationship of nsp12-RdRp and nsp14-ExoN in fidelity regulation we additionally attempted recovery of the WT background viable mutants in the setting of inactivated nsp14-ExoN (nsp14-ExoN(-)). In contrast to mutant viruses recovered in the WT background, we only recovered 2 of the 5 mutants in the nsp14-ExoN(-) background: nsp12-V553I/nsp14-ExoN(-) and nsp12-M611F/nsp14-ExoN(-) (Table 1). The time to recovery for nsp12-V553I/nsp14-ExoN(-) was 84 hours and for nsp12-M611F/nsp14-ExoN(-) was 96 hours. Working stocks of all viruses were made by infecting D9s with an MOI of 0.01 and recovering stocks at around 24 h p.i. for WT
viruses, or between 32 and 48 h p.i. for mutant viruses in the nsp14-ExoN(-) background.

Therefore 4-10 replication cycles were required to generate stocks. Working stocks were sequenced to verify that the introduced mutations were still present.

**Resistance of recovered mutant viruses to the base analog 5-fluorouracil.** We next tested our panel of recovered mutant viruses for resistance to the RNA mutagen 5-fluorouracil (5-FU). 5-FU has been used with picornaviruses, influenza viruses, vesicular stomatitis viruses and others to reflect changes in fidelity based on increased or decreased sensitivity to incorporation and virus inhibition (11, 30, 49, 55). WT CoVs (MHV, SARS-CoV) are resistant to 5-FU, while nsp14-ExoN(-) mutants are profoundly sensitive to 5-FU inhibition, consistent with nsp14-ExoN-mediated removal of misincorporated 5-FU. The effect of 5-FU on DBT cell viability was previously tested with no effect observed up to 400uM (30). We compared WT-MHV with the nsp12 mutants in both the WT and nsp14-ExoN(-) backgrounds. There was no significant change in sensitivity to 5-FU compared to WT for any of the nsp12-RdRp mutant viruses in the WT background at up to 120uM 5-FU although the mutation Y649H does appear to decrease resistance slightly (Fig. 2a). In contrast, the nsp12-M611F/nsp14-ExoN(-) and nsp12-V553I/nsp14-ExoN(-) mutant viruses both were significantly less sensitive to 5-FU than nsp14-ExoN(-) alone, with both populations persisting when treated with 120uM 5-FU where nsp14-ExoN(-) was not detectable beyond 80uM 5-FU (Fig. 2b). These data demonstrate that both nsp12-RdRp mutations V553I and M611F confer resistance to 5-FU. This suggests a couple possibilities: that it is not possible to increase the exclusion of 5-FU beyond the high level dictated by nsp14-ExoN or that selectivity for native nucleotides over 5-FU is in fact increased by nsp12 mutations, but at a low level that is not detectable by changes in virus titer.
Three of the mutations (nsp12-Y649H, W613Y, and K794R) were viable in the nsp14-ExoN(+) background, but failed to grow in the absence of proofreading. The observation that they only grew in an nsp14-ExoN(+) background indicated that the mutations retained sufficient polymerase function to support virus replication, but it was critically dependent on having proofreading functionality, suggesting these mutations may have given rise to low fidelity variants. Unfortunately, the low titer from the nsp14-ExoN(-) background precluded direct sequencing analysis and we therefore cannot definitively show this is the case.

Replication kinetics of nsp12-V553I and nsp12-M611F mutant viruses in the WT and nsp14-ExoN(-) backgrounds. Since we were interested in mutations that potentially confer altered fidelity, we prioritized the nsp12-V553I and nsp12-M611F mutant viruses for further analysis. We next sought to determine how the nsp12-V553I and nsp12-M611F viruses replicated in comparison to their isogenic background (Fig. 3). In the wild-type background both mutant viruses had slightly delayed exponential replication, but eventually reached similar peak titers comparable to WT. In contrast, in the nsp14-ExoN(-) background both nsp12-V553I and nsp12-M611F mutant viruses displayed similar replication kinetics to the isogenic nsp14-ExoN(-) background. We also assessed RNA synthesis for nsp12-V553I and nsp12-M611F by RT-qPCR. Measured genomic RNA levels were consistent with the virus replication kinetics data, and we observed delayed and decreased genome RNA synthesis in the WT background after multiple rounds of replication (Fig. 3E). However, at early time points there was no difference in RNA accumulation suggesting that decreased RNA is a result of steps post RNA synthesis (Fig. 3C). In the nsp14-ExoN(-) background, RNA synthesis levels were indistinguishable from the nsp14-ExoN(-) background for both nsp12-V553I and nsp12-M611F, with no additional RNA
synthesis defects detectable (Fig. 3D and F). The results, along with the ability to recover several
of the nsp12-RdRp mutants only in the nsp14-ExoN(+) background, support the hypothesis that
nsp14-ExoN and nsp12-RdRp may have an epistatic relationship. However, for replication
kinetics the effects of nsp12-V553I and nsp12-M611F are not observable in the presence of an
inactive nsp14-ExoN, demonstrating that the replication phenotype of an inactive nsp14-ExoN is
epistatic to replication variants encoded in nsp12-RdRp.

Specific infectivity of nsp12-V553I and nsp12-M611F. Having identified two mutant viruses
with resistance to 5-FU we wanted to further test whether resistance was due to decreased
incorporation of the mutagen. Measurement of specific infectivity has been useful for
determining lethal mutagenesis for MHV and other RNA viruses (30, 56). We tested the nsp12-
M611F and nsp12-V553I mutants, in both the WT and nsp14-ExoN(-) backgrounds, for changes
in specific infectivity when infected at an MOI of 0.01 and treated with 5-FU (Fig. 4). Both
nsp12-M611F and nsp12-V553I resulted in an increased ratio of infectious particles (PFU/ml) to
total particles (RNA genomes) in the nsp14-ExoN(-) background (Fig. 4b). Similarly, in the WT
background nsp12-M611F demonstrated an increase in the ratio of infectious particles to RNA
genomes (Fig. 4a). Thus specific infectivity may be a more sensitive measure of lower level
changes in nucleotide selectivity in the setting of nsp14-ExoN(-).

Fitness cost of nsp12-V553I and nsp12-M611F in WT and nsp14-ExoN(-) backgrounds. In
multiple RNA viruses, including CoVs, both increased and decreased fidelity have been reported
to have a fitness cost (14). We therefore sought to determine whether nsp12-V553I or nsp12-
M611F conferred any cost in fitness, defined as the ability to directly compete during co-
infection. In the WT background, both nsp12-V553I and nsp12-M611F demonstrated delays in
replication and impaired RNA accumulation. However, since there were not any observed
additional defects in replication or RNA synthesis for nsp12-V553I and nsp12-M611F when
introduced in the nsp14-ExoN(-) background (Fig. 3b, d), we tested for any additional fitness
cost of nsp12-V553I/nsp14-ExoN(-) or nsp12-M611F/nsp14-ExoN(-) viruses compared with
nsp14-ExoN(-) alone. When co-infected with nsp14-ExoN(-) at ratios from 1:9 to 9:1, nsp12-
V553I/nsp14-ExoN(-) maintained the input ratio compared with nsp14-ExoN(-) (Fig. 5a). A
small advantage to nsp12-V553I/nsp14-ExoN(-) was observed when the co-infected cultures
were treated with 60uM 5-FU, consistent with a conferred advantage for 5-FU resistance (Fig.
5a). Thus there appeared to be no additional fitness cost of nsp12-V553I/nsp14-ExoN(-)
compared to nsp14-ExoN(-) alone. In contrast, nsp12-M611F/nsp14-ExoN(-) was not able to
compete with nsp14-ExoN(-) at any ratio (Fig. 5b). Treatment with 60uM 5-FU again favored
nsp12-M611F/nsp14-ExoN(-); however, even then the percent of the population made up of
nsp12-M611F/nsp14-ExoN(-) only remained at around 30% of the population when initially
given a 9-fold advantage (Fig. 5b).

We next tested whether the relative differences in fitness cost resulted in selective pressure for
reversion of nsp12-V553I and nsp12-M611F (Fig. 6). DBT cells were infected with nsp12-
V553I, nsp12-V553I/nsp14-ExoN(-), nsp12-M611F, or nsp12-M611F/nsp14-ExoN(-) at an
initial MOI of 0.01. After 5 passages the viruses were analyzed for retention of original
mutations using di-deoxy (Sanger) sequencing. The nsp12-V553I mutation was stable after
passage in both WT and nsp14-ExoN(-) backgrounds, maintaining the mutated AUU codon (Fig.
6a), suggesting minimal selective pressure on that nucleotide, codon, or amino acid. In contrast,
the nsp12-M611F mutation demonstrated significant change over passage in both the WT or the
nsp14-ExoN(-) backgrounds. The original mutation, UUC, was no longer the majority codon in the nsp14-ExoN(-) background and was less than 52% of the population in all WT background lineages (Fig. 6b). The nsp12-M611F/nsp14-ExoN(-) population resulted in a mix of two nucleotide changes resulting in reversion to AUG (methionine) (≤68% of the population), single nt changes that result in mutation it to Leu (≤55% of the population), a somewhat smaller but still hydrophobic residue, or retaining a Phe substitution (≤27% of the population). Thus the fitness cost of nsp12-M611F results in significant selective pressure for changes at that residue during passage in absence of 5-FU.

Resistance of recovered mutant viruses to the base analog 5-azacytidine. Having shown that both nsp12-M611F and nsp12-V553I mutations conferred resistance to 5-FU in the nsp14-ExoN(-) background but that nsp12-M611F reverted quickly and was at a fitness disadvantage in both the WT and nsp14-ExoN(-) background, we next wanted to test whether the mutations conferred resistance specifically to 5-FU or broadly to base analogs and therefore were likely determinants of fidelity. A study by Arias et al demonstrated that for foot-and-mouth disease virus (FMDV) resistance to a specific mutagen can result from a point mutation while conferring the opposite overall fidelity (57). We therefore tested for resistance to the additional mutagen 5-azacytidine (5-AZC). Similar to the 5-FU results, the nsp12-V553I/nsp14-ExoN(-) mutant virus was more resistant to 5-AZC than nsp14-ExoN(-) alone, maintaining approximately one log higher titers from 20-50uM 5-AZC (Fig. 7). However, the nsp12-M611F/nsp14-ExoN(-) mutant virus showed no difference in resistance to 5-AZC compared with nsp14-ExoN(-) alone (Fig. 7). These data suggest that nsp12-V553I is likely a fidelity determinant where as nsp12-M611F
The nsp12-V533I mutation results in a decrease in the accumulation of mutations. Having shown that the nsp12-V553I and nsp12-M611F mutations resulted in resistance to 5-FU, and that nsp12-V553I additionally conferred resistance to 5-AZC, we sought to directly determine whether either of these mutations resulted in a change in the number of mutation accumulated in viral RNA. DBT cells were infected with wild type, nsp12-V553I, nsp12-M611F, nsp14-ExoN(-), nsp12-V553I/nsp14-ExoN(-) or nsp12-M611F/nsp14-ExoN(-) at an MOI of 0.01 and RNA was collected at 20 h. pi. These samples were then prepared for Illumina next generation sequencing (NGS) across the full genome and analyzed using the ViVan analysis pipeline (45). Mutations present as 1% or more of the population were graphed by frequency and position in the genome. Engineered mutations are depicted with colored dots. Most non-engineered new mutations were present at 10% or less of the population and were distributed across the genome with no detectable “hot-spots” (Fig. 8a-f). For nsp12-V553I in the WT background, no difference was observed in the number of mutations accumulated to 1% or greater of the population compared to WT alone. In contrast, in the nsp14-ExoN(-) background, nsp12-V553I was associated with a 1.7-fold decrease in the frequency of mutations compared to nsp14-ExoN(-) alone (Fig. 8g), again with no change in the distribution of mutations across the genome (Fig. 8c, d). These results are consistent with both the 5-FU and 5-AZC data in suggesting increased fidelity. Results from the nsp12-M611F mutant viruses were more complicated. To our surprise, the nsp12-M611F/nsp14-ExoN(-) virus fully reverted at both engineered nsp12-M611F nucleotides during the low MOI infection, resulting in a viral population that was nsp14-ExoN(-)
alone (Fig. 8f). This reversion made the results for the nsp12-M611F/nsp14-ExoN(-) virus interpretable. However, since accumulation of mutations to over 1% of the population is a combination of all replication cycles from initial recovery to the final sample (roughly 11 total for nsp12-M611F/nsp14-ExoN(-)) we included this data in Figure 8g and 8h. In the WT background the nsp12-M611F mutations were still present as 100% of the population and resulted in a 1.93 fold increase in the total number of accumulated mutations. We observed only a slight increase in the number of mutations accumulated in the nsp12-M611F/nsp14-ExoN(-) sample over those of nsp14-ExoN(-), which was not surprising seeing as the M611F mutation was no longer present. Neither sample appeared to have mutations concentrated in specific locations across the genome suggesting the accumulation of mutations was due to random generation of mutations rather than strong selection in particular locations or proteins (Fig. 8e and f). Of note, one mutation in nsp3 of the nsp14-ExoN(-) sample and a mutation in nsp3, nsp13 and the E proteins of nsp12-V553I/nsp14-ExoN(-) reached nearly 100% of the sample population. None of these mutation were present in the fragments used for recovery and the nsp3 mutation which arose in the nsp14-ExoN(-) population was not present prior to the final low MOI infection. It is possible that these mutations provide some benefit to these viruses since they were fixed so rapidly in the population. We next determined whether either of the mutations resulted in a change in the types of mutations occurring during replication (Fig. 8h). Consistent with our previous studies, there were differences in the types of mutations incorporated when comparing WT and nsp14-ExoN(-) backgrounds (30). However, the addition of nsp12-V553I or nsp12-M611F did not alter these patterns in either WT or nsp14-ExoN(-) backgrounds. Thus, nsp12-V553I results in an overall decrease in the accumulation of mutations over passage while nsp12-M611F seems to increase the number of mutations accumulated. These results confirm by
sequence analysis the results from the 5-FU and 5-AZC resistance experiments for nsp12-V553I, specifically that the effects of nsp12-V553I are dependent on inactivation of nsp14-ExoN for their detection and that nsp12-V553I likely confers broad resistance to the incorporation of incorrect nucleotides. In contrast, these results further increase the complexity of the nsp12-M611F mutation in relation to incorporation of nucleotides and their analogs. We conclude that nsp12-M611F confers resistance to 5-FU but that this resistance is not likely to be due to a broad resistance to the incorporation of alternate nucleotides.
RdRp structures of divergent RNA viruses are structurally conserved and likely have common determinants of activity in the finger, palm and thumb domains. Positive strand RNA virus polymerases appear to utilize a common palm-domain based mechanism for active site closure (23) and associated molecular determinants of fidelity in different RdRp domains have been proposed based on biochemical and mutagenesis studies (10). To date there are no solved crystal structures of any CoV RdRp and thus direct comparison with other virus RdRp structures has not been possible. Further, regulation of CoV fidelity is likely dependent on multiple proteins, including the RdRp and proofreading ExoN. Thus it was not clear that a CoV would phenotypically exhibit effects from mutating fidelity-determining residues located in the RdRp itself. In this study we sought to determine whether we could use structure and mutagenesis data from distantly related RNA viruses to identify determinants of CoV nsp12-RdRp fidelity. Our results suggest that CoV RdRps do in fact participate in fidelity regulation, at residues orthologous to those in the picornaviruses, and likely in other RNA virus RdRps. The results also define, for the first time, a CoV RdRp determinant that increases resistance to multiple mutagens, decreases the accumulation of mutations over time and so, likely increases overall fidelity. Additionally, the data in this paper suggest that CoV RdRp-mediated increased fidelity is only detectable when nsp14-ExoN is inactive, and is only partially compensating for the loss of nsp14-ExoN high fidelity. Finally, both the nsp12-V553I and M611F mutations confer a replication cost in the WT background but only nsp12-M611F confers a fitness disadvantage in the nsp14-ExoN(-) background. Together the results suggest that nsp14-ExoN proofreading activity is epistatic to nsp12-RdRp fidelity but that in contrast the replication defects of an inactive nsp14-ExoN are epistatic to replication defects in nsp12-RdRp.
Determinants of nucleotide selectivity and fidelity in CoVs may be conserved with other RNA viruses. Picornavirus functional RdRps contain only RdRp domains (58, 59). This is not the case for many viral RdRps, CoVs included (26, 39). In addition to the predicted RdRp core domain, all CoV nsp12 proteins contain a “CoV-specific” domain of over 350 amino acids at the N terminus of the protein. A nucleotidyltransferase activity was identified in this CoV specific domain and has been shown to be important in SARS-CoV replication, but the specific function in replication remains to be determined (39). The N-terminal CoV specific domain could not be modeled due to lack of evolutionary homologues of known structure but, the predicted RdRp core domain could be modeled with high confidence using bioinformatic approaches. The structures which provided the best models for the CoV RdRp were from picornaviruses, including enterovirus 71, foot and mouth disease virus and coxsackie virus B3. This allowed for direct alignment and comparison of the known fidelity determinants in CVB3 with the MHV nsp12 core domain, across both fingers and palm domains. The recovery of mutations in these structurally conserved residues and their participation in CoV nucleotide selectivity supports the hypothesis that there are determinants of base specificity conserved between CoVs and distantly related RNA viruses, specifically the picornaviruses. This supports the idea that all RdRps function similarly due to their structural conservation and despite the low level of sequence similarity and attached domains (54). It also suggests that the CoV RdRp domain folds in a manner similar to other RdRps, likely separate from the CoV-specific domain. RdRp fidelity and nucleotide selectivity has been investigated extensively in picornaviruses, especially poliovirus (12, 15, 51, 53, 60, 61). However, even between picornaviruses, the impact on fidelity of changes at identical or similar residues can vary.
dramatically (10). Mutations at the same residues in the RdRp of poliovirus and CVB3 affect fidelity differently; poliovirus mutations generally result in increased fidelity and CVB3 mutations in decreased fidelity (11). Specifically, the structurally orthologous residue to nsp12-M611 in poliovirus is F230 and in CVB3 it is I230; when F230 is mutated to an Ile in poliovirus it results in an increase in fidelity, but when I230 in CVB3 is mutated to a Phe, Trp or Val it results in a decrease in fidelity (10). Similarly, the orthologous residue to nsp12-V553 in CVB3 is I176; when I176 is mutated to a Val in CVB3 it results in a decrease in fidelity (10, 11).

Although the specific substitutions differ, when nsp12-V553I and nsp12-M611F are introduced into the MHV genome the resulting 5-FU resistance, and for nsp12-V553I a likely increase in fidelity, mimics results seen in poliovirus rather than CVB3. However, nsp12-M611F seems to accumulate more mutations overtime than controls and many of the predicted mutations were only recoverable in the WT background, therefore changes in nucleotide selectivity and fidelity were not tested, so it remains possible that the mutations that were non-viable in the nsp14-ExoN(-) background had decreased fidelity and that nsp14-ExoN(-) treated with 5-FU defines the error threshold for CoVs.

**Coronavirus nsp12-RdRp and nsp14-ExoN cooperate to optimize both fidelity and replication kinetics.** In addition to nsp12-RdRp, CoVs encode nsp14-ExoN, which functions as a proofreading enzyme (29, 30, 33, 35). Beyond nsp14-ExoN, there are additional CoV-encoded nsps that potentially contribute to overall fidelity, such as: the small molecule modulator of nsp14-ExoN encoded in nsp10 (33, 62), nsp7 and nsp8 that together function as an elongation factor (63) and a primase (64, 65), and nsp13 that functions as a helicase (66). These proteins interact with each other and likely function as a multi-protein replication/fidelity complex (34,
The results of this study show that while determinants in nsp12-RdRp are likely capable of increasing fidelity, those changes are only detectable in the setting of loss of nsp14-ExoN proofreading, and do not completely compensate for the impaired fidelity associated with nsp14-ExoN(-). If altering nucleotide selectivity determinants within nsp12-RdRp no longer significantly affect the overall nucleotide selectivity of the WT virus, then the evolutionary pressure on nsp12-RdRp may be more heavily weighted toward other aspects of replication, such as speed. Recent evidence suggests that RdRp fidelity and speed have an inverse relationship (10, 67). So, if fidelity regulation by nsp14-ExoN occurs more rapidly than correct nucleotide selectivity by the RdRp then CoV nsp12-RdRp may have been selected specifically for replication speed. Our data may support this hypothesis, as in the WT background both nsp12-V553I and nsp12-M611F resulted in increased replication lag phases (Fig. 3A and E), though early RNA synthesis was not observably different in our system (Fig. 3C). However, this was not seen in the nsp14-ExoN(-) background which could be explained by our data indicating that replication is already slowed when nsp14-ExoN is inactivated. One possible mechanism for this could be that the nsp14-ExoN(-) protein is trying to remove incorrect nucleotides and stalling replication due to its inability to do so. In this case nsp14-ExoN(-) would be epistatic to nsp12-RdRp in relation to speed, thereby obscuring decreases in replication speed caused by mutations in nsp12-RdRp itself.

**Conclusion.** Our results support the hypothesis that determinants of nucleotide selectivity are conserved across viral orders, identify the first likely increased fidelity determinant for CoV nsp12-RdRp, and demonstrate that nsp14-ExoN proofreading activity is epistatic to nsp12-RdRp nucleotide selectivity. Knowing that some fidelity determinants may be conserved across viral
orders is an exciting discovery, as many fidelity determinates identified so far have resulted in attenuation (11, 15, 54, 68-70). It would be interesting to determine whether nsp12-V553I is also attenuated. We would predict that it would be, based on the fitness cost *in vitro*. However, there is also no clear increase in nucleotide selectivity or fidelity in the WT background. This may be due to a very minimal change that is just not measureable even with the deep sequencing technology used in this study. Alternatively, it could be that any change is simply overwhelmed by the high fidelity of intact ExoN proofreading. In any case, these mutations or other changes at these residues may allow for selection of viruses that replicate with normal kinetics *in vitro* and *in vivo*, yet confer attenuation in an animal setting. We know that the nsp14-ExoN(-) mutations confer genotypically and phenotypically stable attenuation *in vivo* (36). However, the concept of a high level mutator as a mechanism for attenuation in live viruses may be problematic. The identification of increased fidelity mutations in the RdRp that can partially, or potentially completely, compensate for the fidelity impairment of nsp14-ExoN(-) viruses, may allow for development of approaches that can benefit from the stability of the nsp14-ExoN(-) mutator phenotype while allowing more stability to the input genomes. Finally, these results combined with those from previous work (33, 44) suggest that CoVs encode at least three proteins involved in fidelity (nsp12-RdRp, nsp14-ExoN and nsp10) supporting the assembly of a multi-protein replicase / fidelity complex as described previously (38). This increases the importance of establishing a biochemical model of the multi-protein complex to directly test the interactions of fidelity determinants as well as potential inhibitors of each or all of these functions.
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FIGURE LEGENDS

Figure 1. Homology modeling of CoV nsp12-RdRp and identification of residues that potentially regulate fidelity based on CVB3 structure. Phyre2 software was used to model A) a subsection of the MHV nsp12-RdRp core domain (expanded from nsp12-RdRp full schematic). This B) modeled MHV RdRp structure was aligned with the C) solved CVB3 RdRp structure. Residues chosen for site-directed mutagenesis (A and B) were selected by comparing previously determined fidelity altering mutations of picornavirus RdRps. Amino acid alignments D) across CoVs show all residues are completely conserved.

Figure 2. Resistance of MHV nsp12-RdRp mutant viruses to 5-fluorouracil in the WT and nsp14-ExoN(-) background. Domain location of mutations is indicated by: fingers (blues) and palm (reds). DBT cells were pre-treated with different concentrations of 5-FU for 30 min. Treatment was removed and cells were infected with indicated viruses in the A) WT background or B) nsp14-ExoN(-) background at an MOI of 0.01. Media containing 5-FU was replaced 30 min p.i. Virus samples were taken at 24 (WT) or 32 (nsp14-ExoN(-)) h p.i. and titer was determined by plaque assay. Data represents 3 independent experiments, each with 2 replicates. Error bars represent SEM (*P < 0.05 by Wilcoxon test).

Figure 3. Replication kinetics of MHV nsp12-RdRp mutant viruses. Mutation location is indicated by: fingers (blues) and palm (red). DBT cells were infected with the viruses indicated in the A, C and E) WT background or B, D and F) nsp14-ExoN(-) background at an MOI of 0.01 PFU/cell. Supernatant aliquots were taken at indicated times p.i. and titer determined by plaque
assay. Total RNA was taken at indicated times p.i. and RT-qPCR was performed. Data represents 3 independent experiments. Error bars represent SEM.

Figure 4. Specific infectivity is increased in both nsp12-V553I and nsp12-M611F mutants. DBT cells were pre-treated with increasing concentrations of 5-FU for 30 min. Treatment was removed and cells were infected with indicated viruses in the A) WT background or B) nsp14-ExoN(-) background at an MOI of 0.01. Media containing 5-FU was replaced 60 min p.i. Virus samples were taken at 20 and 24 h p.i. respectively. Titer was determined by plaque assay and number of supernatant genomes were determined using one-step RT-qPCR. Data represents 2 independent experiments, each with 3 replicates. Error bars represent SEM (*P < 0.05 by 2way ANOVA using the Bonferroni correction for multiple comparisons).

Figure 5. Competitive fitness analysis in the WT and nsp14-ExoN(-) Background. DBT cells were pre-treated with media alone or containing 60uM or 300uM 5-FU for 30 min. Treatment was removed and cells were co-infected, at total MOI of 0.01, with WT and A) nsp12-V553I or B) nsp12-M611F at a ratio of 1:9 or nsp14-ExoN(-) and C) nsp12-V553I/nsp14-ExoN(-) or D) nsp12-M611F/nsp14-ExoN(-) at a ratio of 9:1, 1:1 or 1:9. Media alone or containing 60uM or 300uM 5-FU was replaced 30 min p.i. Total RNA was taken at 20 h p.i. for WT competition (A and B) or 24 h p.i. for nsp14-ExoN(-) competition (C and D). Sequencing was performed across a 1.7kb region of nsp12-RdRp that included both mutations. Data represents 3 independent experiments, each with 2 replicates. Error bars represent SEM.
Figure 6. The nsp12-V553I mutation is stable across passage; however, nsp12-M611F is vulnerable to reversion. DBTs were infected with an initial MOI of 0.01 then blind passaged in triplicate for 5 passages. Total RNA was taken and sequencing was performed across a 1.7kb region of nsp12-RdRp that included both mutations. Percentage of each nucleotide present in each of the triplicate lineages after 5 passages is shown. Mutant viruses in the WT (solid bars) and nsp14-ExoN(-) (slashed bars) backgrounds are both shown. The original mutation for each of the viruses is shown above the graph and the likely majority, secondary and tertiary codons present in the population are shown below the graph.

Figure 7. Resistance of MHV nsp12-RdRp V553I and M611F mutant viruses to 5-azacytidine in the nsp14-ExoN(-) background. Domain location of mutations is indicated by: fingers (blues) and palm (reds). DBT cells were pre-treated with different concentrations of 5-AZC for 30 min. Treatment was removed and cells were infected with indicated viruses at an MOI of 0.01. Media containing 5-AZC was replaced 30 min p.i. Virus samples were taken at 32 h p.i. and titer was determined by plaque assay. Data represents 5 independent experiments, each with 2 replicates. Error bars represent SEM (*P < 0.05 by ratio paired t test).

Figure 8. The nsp12-V553I mutation confers decreased accumulation of mutations in the nsp14-ExoN(-) background with no bias toward the exclusion of specific nucleotides. DBTs were infected with an MOI of 0.01 and total RNA collected. Deep sequencing was performed on these samples. The statistically significant mutations present as greater than or equal to 1% of the total population are shown for wild type, nsp14-ExoN and nsp12-V553I or nsp12-M611F in both backgrounds. These were graphed (A-F) according to their distribution across the genome with...
intentionally introduced mutations (B-F) shown with circles colored blue (nsp12-V553I), red (nsp12-M611F) or green (nsp14-ExoN(-)), as (E) the total number of mutations present in the population and as (F) the percent of specific mutations present.
Table 1. Recovery of mutant viruses using site-directed mutagenesis.

<table>
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<tr>
<th>nsp12-RdRp Region</th>
<th>CVB3 engineered substitutions (nsp12) (8)</th>
<th>MHV engineered substitutions (nsp12)</th>
<th>Recovery: WT (nsp14-ExoN(+))</th>
<th>Recovery: nsp14-ExoN(-)</th>
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