Coxsackievirus B3 mutator strains are attenuated in vivo

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Based on structural data of the RNA-dependent RNA polymerase, rational targeting of key residues, and screens for Coxsackievirus B3 fidelity variants, we isolated nine polymerase variants with mutator phenotypes, which allowed us to probe the effects of lowering fidelity on virus replication, mutability, and in vivo fitness. These mutator strains generate higher mutation frequencies than WT virus and are more sensitive to mutagenic treatments, and their purified polymerases present lower-fidelity profiles in an in vitro incorporation assay. Whereas these strains replicate with WT-like kinetics in tissue culture, in vivo infections reveal a strong correlation between mutation frequency and fitness. Variants with the highest mutation frequencies are less fit in vivo and fail to productively infect important target organs, such as the heart or pancreas. Furthermore, whereas WT virus is readily detectable in target organs 30 d after infection, some variants fail to successfully establish persistent infections. Our results show that, although mutator strains are sufficiently fit when grown in large population size, their fitness is greatly impacted when subjected to severe bottlenecks, which would occur during in vivo infection. The data indicate that, although RNA viruses have extreme mutation frequencies to maximize adaptability, nature has fine-tuned replication fidelity. Our work forges ground in showing that the mutability of RNA viruses does have an upper limit, where larger than natural genetic diversity is deleterious to virus survival.

Thirty years ago, our regard of RNA viruses as simple, uniform organisms changed with the demonstration of the genetically heterogeneous composition of Q β -phage populations (1), a concept that has been extended to all RNA viruses. Indeed, as early as 1965, the error-prone nature of RNA virus replication had been observed (2). Since that time, significant progress has been made in describing the highly polymorphic mutational distributions within RNA virus populations, the mechanisms responsible for their generation, and their implication in virus adaptability, evolution, and fitness (3). Important progress was made in recent studies using antimutator strains of RNA viruses presenting higher-fidelity polymerases that generate less diverse populations (4–6). These studies revealed that, although mutation rates can be reduced for these viruses, nature has seemingly selected for error-prone replication to maximize adaptability. This results in virus populations with extreme mutation frequencies approaching a maximum beyond which the likelihood of lethal mutations greatly diminishes virus viability (7). In recent years, the study of lethal mutagenesis as an antiviral approach, based on the accumulation of lethal mutations through treatment with mutagenic compounds, was pivotal in showing that RNA viruses are particularly sensitive to even moderate increases in their already elevated mutation frequencies (8–13). The strong correlation between decreased mutation frequencies and compromised adaptability on the one hand and increased mutation frequency and decreased population fitness on the other hand suggests that nature has fine-tuned polymerase error rates between these two states. However, the potential fitness costs for polymerase variants presenting lower fidelity than WT virus and mutator phenotypes have not yet been described.

The ability to manipulate the intrinsic error rate of RNA-dependent RNA polymerases (RdRPs), a principle source of genetic heterogeneity, and link it with virus fitness in an infected host was first shown for poliovirus and more recently, for chikungunya virus (4–6). In both cases, high-fidelity antimutator strains showed varying degrees of attenuation in vivo. In this study, we sought to extend the observations made in poliovirus to a more natural infection model using another medically relevant picornavirus, Coxsackievirus B3 (CVB3), that has a surface receptor naturally expressed in mice. Coxsackieviruses (type A and B) are the leading cause of viral myocarditis in humans, infections can be fatal for neonates and immunodepressed individuals, and no vaccines or specific antiviral treatments are currently available. During infection of mice with WT virus, viremia is detected from 24 to 48 h after i.p. injection, and peak titers of this systemic infection can be measured 3–4 d post-infection, mainly in the heart, pancreas, small intestine, and spleen (14, 15). In this infection model, virus is rapidly cleared from most organs, with a characteristic onset of viral myocarditis by day 7 and a slower decline of virus titer in the pancreas, where a severe pancreatitis is often observed. Depending on the mouse strain used, viral persistence in the heart and/or spleen can then last up to 5 mo postinfection (16–19).

Our recent studies showing that CVB3 has a naturally higher fidelity than its close relative poliovirus led us to question whether increasing fidelity would be possible for this virus. We, thus, used two strategies based on the rational targeting of select residues involved in or suspected of affecting polymerase activity and fidelity: a network of residues implicated in polymerase fidelity in poliovirus (20, 21) and residues based on predictions of polymerase structure and active site conformational changes during catalysis (22). Here, we report nine RdRp variants that were found to replicate with WT kinetics and presented mutator phenotypes suggestive of decreased replication fidelity in vitro. These newly isolated mutator strains were characterized in vitro and in vivo to determine the potential fitness costs related to increasing the intrinsic mutation frequency beyond natural WT levels.

Results

Rational Targeting of Residues Predicted to Be Involved in Picornavirus Polymerase Fidelity by Site-Directed Mutagenesis. In the search for polymerase fidelity variants of CVB3, we first targeted residues involved in the fidelity checkpoint described for the residue 64 high-fidelity variants of poliovirus (20, 23, 24). Based on bio-

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chemical and structural data (20, 25), residues 1, 64, 239, and 241 participate in a tetrahedral hydrogen bond network that buries the N-terminal glycine (Gly1) residue in a pocket in the fingers domain (Fig. 1A). In turn, this network helps position the Asp238 residue to the active site, where it interacts with the 2' OH of a bound nucleotide in the absence of any bound RNA (21, 25) and the NTP 3' OH and Ser288 (Ser289 in Coxsackievirus polymerase) in the catalytically competent closed conformation of the poliovirus polymerase elongation complex (22). The crystal structures of poliovirus and CVB3 polymerases show that this H-bond network is conserved (25, 26). By site-directed mutagenesis on the CVB3 cDNA clone, we introduced all possible amino acid substitutions at these four positions. The corresponding *in vitro*-transcribed infectious RNA genomes were electroporated into HeLa cells and passaged three times to determine the genetic stability of each mutant. At each passage, if no virus was detected by RT-PCR, the variants were considered nonviable; otherwise, the polymerase gene was sequenced in the progeny population. Four independently generated clones were tested for each mutation. Substitutions of Gly1 were not viable, likely from negative effects on polypeptide processing. The different amino acid changes at positions Glycine 64, Alanine 239, and Leucine 241 that were nonviable, reverted to WT, mutated to another variant, or stable over three passages are summarized in Table 1. For position 64 variants, only G64S, G64A, and G64Q were stable. Although some variants did not yield viable virus at all, for several other mutants, reversion to WT was observed at this position, which was consistently accompanied by either of two new mutations, P48K or S164P (Fig. 1C). Regarding the substitutions at position 239, only A239G and A239S were stable, whereas most other amino acid changes reverted and were accompanied by a new S299T mutation. Interestingly, we had already identified this mutation as decreasing fidelity (27). Among the position 241 substitutions, only L241I was stable, and no reversions or additional mutations were observed.

After this first group of variants, a second group of mutations was generated based on the structure of the homologous poliovirus polymerase elongation complex and the conformational changes that it takes therein to enable catalysis (22). The mutations were predicted to alter polymerase fidelity by affecting one of three groups of interactions within the polymerase (Fig. 1B): the structural change in motif A associated with active site closure, the relative motions of the palm and fingers domains during active site closure, and the positioning of the template RNA and incoming NTP within the active site. A total of 50 mutations were designed at 14 sites to test the functionality of alternative amino acids that were likely to favor or alter different conformational states of the polymerase active site (Table 2). Viruses carrying these 50 mutants were generated and tested for viability and genetic stability as described above. Although the majority of these rationally designed mutations was lethal, seven point mutations at four residue sites resulted in viable, stable variants: I176V, I230F, F232Y, F232L, F232V, Y268H, and Y268W. It should be noted, however, that F232L and F232V were excluded from additional study because of occasional reversion to WT during downstream experiments. The resulting set of viruses sample all three areas targeted in the study; Ile230 and Phe232 are in motif A, which moves on active site closure for catalysis, Tyr268 is in the interface between the palm and fingers domains, and Ile176 is involved in positioning the templating base in the active site (Fig. 1C).

Replication Kinetics of CVB3 Polymerase Mutant Viruses. The kinetics of RNA synthesis and virus production of each variant isolated above were compared with WT virus. The CVB3 variants G64S, G64A, and G64Q were significantly compromised in replication (Fig. S1), which is in contrast to what was observed for poliovirus (23, 24). In addition, variant A239S was also unable to replicate as well as WT ($P < 0.05$, two-way ANOVA) (Fig. 2B and F). These variants were, thus, excluded from additional study. For the other variants from the first group (P48K,

S164P, A239G, and L241I), no significant difference in the production of infectious virus was observed (Fig. 2A and B). Similarly, the second set of polymerase variants presented the same kinetics and final yields of infectious virus as WT (Fig. 2C and D). In addition to infectious virus yield, we quantified total genomic RNA by real-time PCR of the same samples (Fig. 2E–H). All variants except for A239S showed a trend of producing more RNA than WT, with several variants producing significantly higher amounts of RNA at certain time points (e.g., P48K, S164P, and A239G at 24 h). The total RNA synthesized, relative to the corresponding infectious virus, is, thus, exaggerated for these variants, suggesting that these polymerase variants may be introducing more errors (lethal mutations) in their progeny genomes and/or producing more defective virion particles.

CVB3 Polymerase Variants Present Mutator Phenotypes. Next, we examined whether the engineered polymerase mutations affected polymerase fidelity and the resulting virus mutation frequency. For each viral population (passage 3 stocks), a 1.3-kb fragment of the capsid protein-coding region from ~100 individual clones was sequenced and used to determine the average number of mutations per 10^4 nt. This approach was used previously to identify significant differences in mutation frequencies of CVB3 variants that correlated with altered enzyme fidelity in biochemical assays (27). All variants from both sets of generated mutations presented higher mutation frequencies than WT, which presented 4.3 mutations/ 10^4 nt sequenced (Fig. 3A): P48K with 6.8 mutations ($P < 0.05$, two-tailed Mann–Whitney u test), S164P with 6.8 mutations ($P < 0.05$), A239G with 8.2 mutations ($P < 0.01$), and L241I with 6.3 mutations ($P < 0.05$) from the first group and 5.6 mutations for I176V ($P =$ not significant), 11.2 mutations for I230F ($P < 0.0001$), 11.2 mutations for F232Y ($P < 0.0001$), 8.9 mutations for Y268W ($P < 0.0001$), and 9.2 mutations per 10^4 nt for Y268H ($P < 0.0001$) from the second group. Although F232V also presented extreme mutation frequencies (10.2), it was excluded from the study at this point, because Sanger sequencing of the population's consensus sequence revealed that it was a mixed population of F232V and reversions to WT (over 50% of total population). This last mutation frequency estimate may underrepresent the actual mutation frequency of F232V because of a large contribution of WT enzyme in this population. Given these results, we decided to deep sequence the RdRp coding region of some of these populations using Illumina technology. We filtered the analysis to identify any minority variant representing more than 0.5% of the total population (Table 3). The data indicate that, for P48K, S164P, A239G, Y268H, and Y268W, no reversion to WT had occurred, even at the level of background error (0.01% total population). All variants presented three mutations at low frequency (0.5–1.0%; E35G, N37G, and H273R) that seem to constitute the natural mutant spectra of CVB3. The two variants with the highest mutation frequencies, I230F and F232Y, did reveal reversion with 2% and 6%, respectively. A number of other mutations also appeared in significant numbers in these populations. The I230F variant reveals the presence of M145L and S299T with similar frequencies (around 16%), suggesting that these mutations may exist together as a double mutant. For F232Y, evidence of a double mutant is also present with mutations S299T and A372V. Curiously, these mutations as single variants code for a low- (7.0 mutations/10,000) and high- (2.5 mutations/10,000) fidelity versions of our CVB3 Nancy strain, respectively (27). To address whether these mutations were compensating for excessively low fidelity of F232Y and I230F, we generated the corresponding double and triple mutants and examined mutation frequencies. Indeed, all combinations of newly arising mutations resulted in populations with mutation frequencies closer to but not as low as WT (between 6.1 and 8.4 mutations/10,000) (Fig. S2). These data suggest that the most extreme mutators, although viable, are relatively unstable and may indicate an upper limit to the tolerated mutation frequency of CVB3.

Fig. 1. Targeted mutations sites on CV3B polymerase (26) superimposed on the RNA from the homologous poliovirus polymerase elongation complex (22). (A) Details of the buried N terminus (blue sphere) that is locked in place by hydrogen bonds with the backbone carbonyls of residues 64, 239, and 241, positioning Asp238 in the active site for interactions with NTPs. The terminal cytosine of the RNA, which sits in the NTP binding pocket of the postcatalysis poliovirus elongation complex (Protein Data Bank ID code 3OL7), is shown in orange. (B) The three groups of residues comprising 50 separate mutations engineered to alter fidelity by affecting active site closure for catalysis (green; I230, F232, Y234, D238, I306, and L344), the linkage between the palm and fingers domains (orange; S240, F246, Y268, L269, and M287), and the positioning of the template RNA and bound NTP (red; R174, I176, and S289) are shown. (C) Location of the seven stable and viable mutants (blue) from the set of 50 mutants tested in infectious virus studies. The locations of other compensatory mutations commonly observed in the viruses emerging from the mutation studies are shown in dark red.

To confirm the genetic data suggesting mutator status phenotypically, we tested the sensitivity of these variants to the base analog and RNA mutagen ribavirin. Variants with lower-fidelity polymerases (i.e., mutators) should be more sensitive to mutagenic conditions by mistakenly incorporating more ribavirin than correct base and more rapidly accumulating lethal mutations. The result would be a more marked drop in infectivity of the progeny virus population. Thus, WT virus and each variant was subjected to growth in HeLa cells in the presence of either 200 or 400 μ M ribavirin or in its absence, and the relative infectivity of the treated population was determined. At 200 μ M, all variants showed significantly lower percentages of virus progeny surviving ribavirin treatment than WT virus (Fig. 3B). An even more dramatic effect on viral viability was observed at 400 μ M, where the A239G, L241I, Y268H/W, I230F, and F232Y populations were almost extinguished (<0.1% survival). All together, our data show that the nine stable CVB3 polymerase variants are lower-fidelity polymerases that replicate with the same efficiency as WT while being more sensitive to mutagen treatment.

In Vitro Polymerase Fidelity Assays. To confirm the link between polymerase fidelity and mutator phenotype, we set out to analyze the in vitro nucleotide selectivity of purified polymerases. The closure of the picornaviral polymerase active sites for catalysis is, in part, driven by the recognition and proper positioning of the 2' hydroxyl group of the incoming nucleotide triphosphate, because it binds by stacking and base-pairing interactions (22). Based on this finding, we designed an assay to measure how well the mutant polymerases discriminated between CTP and 2'-deoxy-CTP and then correlated the results with the mutation frequencies observed in the infectious virus studies. These studies were carried out on the WT polymerase, the five stable variants from the structure-directed mutagenesis study, and two additional mutations (F232V and F232L) that initially supported virus growth but were not genetically stable.

We examined NTP selectivity using the fluorescence-based PETE assay that detects polymerase elongation activity through changes in the signal from a fluorescein label located at the very 5' end of an extended RNA template strand (28). The assay uses a rapid mixing stopped-flow instrument to measure fluorescence signals as a function of time after mixing preassembled elongation complexes with the NTPs needed for elongation. The result is a kinetic trace consisting of a lag phase as the polymerase replicates through the extended template sequence, which is followed by increases in fluorescence as the polymerase reaches the fifth, fourth, and third nucleotide from the end of the template. In this study, we designed an RNA template with a guanosine base at the fourth position from the end of the template (Fig. 4A). Incorporation of a cytosine opposite of this base then becomes the rate-limiting step for a transition from an ~40% signal change (associated with reaching the fifth nucleotide from the end) to the full 100% signal change (associated with reaching the third nucleotide from the end). The resulting data show the lag phase followed by a sharp increase in signal and then a slower CTP (or dCTP)-dependent transition to the final fluorescence signal (Fig. 4B). Note that, even in the absence of added CTP, there is a slow increase in the fluorescence signal after the initial increase that is caused by misincorporation of uracil opposite the guanosine, and consequently, the UTP concentration was kept low at 1 μ M to minimize this competing reaction.

To determine polymerase specificity for CTP vs. 2'-deoxy-CTP from these data, we titrated the amount of nucleotide in the reaction. The result was a concentration-dependent increase in the observed rate of the second event in the kinetic traces (Fig. 4B), and the rate of this step was determined by fitting that portion of the data to a single exponential curve. A Michaelis-Menten-type analysis was then used to determine the apparent K_m and k_{cat} values for the CTP- and dCTP-dependent changes in fluorescence (Fig. S3 and Table S1). The results show that both nucleotides had essentially the same effects on the shape and k_{cat} associated with the fluorescence change, but they did so over

Table 1. Site-directed mutagenesis on fidelity checkpoint residues 64, 239, and 241

Amino acid 64			Amino acid 239			Amino acid 241		
Desired variant	Status*	New mutation [†]	Desired variant	Status*	New mutation [†]	Desired variant	Status*	New mutation [†]
Arg	Nonviable	—	Gln	Nonviable	—	Ala	Nonviable	—
Asn	Nonviable	—	Phe	Nonviable	—	Arg	Nonviable	—
Glu	Nonviable	—	Pro	Nonviable	—	Asn	Nonviable	—
Iso	Nonviable	—	Trp	Nonviable	—	Asp	Nonviable	—
Leu	Nonviable	—	Tyr	Nonviable	—	Cys	Nonviable	—
Pro	Nonviable	—	Arg	Unstable	R239G	Gln	Nonviable	—
Val	Nonviable	—	Glu	Unstable	E239G	Glu	Nonviable	—
Asp	Reversion	P48K or S164P	Cys	Unstable	C239G	Gly	Nonviable	—
Cys	Reversion	S164P	Asn	Unstable	N239S	His	Nonviable	—
His	Reversion	S164P	Asp	Reversion	—	Lys	Nonviable	—
Lys	Reversion	S164P	Thr	Reversion	—	Met	Nonviable	—
Met	Reversion	S164P	Val	Reversion	—	Phe	Nonviable	—
Phe	Reversion	A239G	His	Reversion	S299T	Pro	Nonviable	—
Thr	Reversion	S164P	Iso	Reversion	S299T	Ser	Nonviable	—
Trp	Reversion	S164P	Lys	Reversion	S299T	Thr	Nonviable	—
Tyr	Reversion	P48K	Met	Reversion	S299T	Trp	Nonviable	—
Ala	Stable	—	Phe	Reversion	S299T	Tyr	Nonviable	—
Gln	Stable	—	Gly	Stable	—	Val	Nonviable	—
Ser	Stable	—	Ser	Stable	—	Iso	Stable	—
Gly	WT	—	Ala	WT	—	Leu	WT	—

Dashes indicate that no additional, compensatory mutations were identified.

*The status indicates whether the mutation was stable over three passages (dark gray), reverted to WT (reversions, lighter gray), changed to another amino acid (unstable, lightest gray), or did not yield viable virus. The wild-type (WT) amino acid at each position is indicated at the bottom of the variant list.

[†]New mutation is acquired by unstable or reverted viruses within the first cycle of replication, and all four independent clones gave rise to the same mutation. For G64D, two clones gave P48K, and two clones gave S164P.

very different concentration ranges. The K_m values for CTP were in the low nanomolar range (~ 25 nM), whereas the K_m values for dCTP were in the micromolar range (~ 20 μ M).

To quantify the nucleotide preference for CTP over 2'-deoxy-CTP of the polymerase mutants, we first calculated the catalytic efficiencies associated with each type of nucleotide as k_{cat}/K_m and then calculated the selectivity as the ratio of these two catalytic efficiencies (Table S1). The results show that the WT Cocksackievirus polymerase had an $\sim 1,200$ -fold preference for CTP over dCTP, whereas the mutants all exhibited reduced selectivity as slow as 650-fold for the F232L mutant. Furthermore, a plot of the observed CTP/dCTP selectivity vs. the actual mutation rates obtained from the deep sequencing of progeny virus genomes shows a clear linear correlation (Fig. 4C), indicating that reduced polymerase nucleotide selectivity measured in vitro is predictive of mutator phenotype viruses in vivo. There is also a correlation between polymerase elongation rates and the RNA genome levels observed in the cell culture-based virus infection studies (Fig. 4D), showing that increased polymerase efficiency can have a direct impact on viral RNA production.

Mutator Variants Are Attenuated in Vivo. As previously shown for the higher-fidelity polymerase variants, G64S of poliovirus and C483Y of chikungunya virus, restricting the ability of a virus to generate a normal mutation frequency attenuates a virus during in vivo infection of mice (4, 24), suggesting that nature has selected for lower fidelity to strike a balance between adaptability and maintaining genetic integrity. This panel of variants then permits us to address whether there is a limit to how low fidelity could be sustained. Although competition assays suggested that these variants had similar or only slightly reduced fitness compared with WT in highly permissive HeLa cells (Fig. S4), we hypothesized that increasing mutation frequency beyond the natural state of a virus would have an even greater negative impact on viral fitness in vivo. To test this hypothesis, we infected mice with each of the nine mutator variants and killed them at day 3 postinoculation, a time when peak titers during infection are reached. We then determined viral titers in the serum, spleen,

heart, and pancreas (Fig. 5). The last two organs are primary targets of CVB3 infection. The panel of nine variants presented a wide range of attenuation with the overall trend of reduced virus titers in all organs tested. Although three variants (S164P, I176V, and L241I) only showed trends of reduced titers that were not statistically significant, six variants (A239G, Y268W, I230F, Y268H, P48K, and F232Y) were significantly attenuated with respect to WT virus in all four organs. The organ titers for these variants were lower than WT virus by 2–4 (serum) (Fig. 5A), 1–4 (spleen) (Fig. 5B), 2–4 (heart) (Fig. 5C), and 2.5–4.5 (pancreas) (Fig. 5D) log. For some variants, such as A239G, Y268W, and I230F, no virus was detectable in the serum and hearts of some or all mice.

We next selected a subset of the most attenuated variants to look at the kinetics of viral infection in more detail (Fig. 5E–I). We infected mice with A239G, I230F, and Y268W variants and examined organ titers (serum, heart, and pancreas) and virus shedding (feces) on days 3, 5, and 7 postinfection. The variants A239G, I230F, and Y268W displayed lower titers in serum that were more rapidly eliminated compared with WT (Fig. 5E). Accordingly, these variants titrated at lower levels in both the serum (Fig. 5F) and pancreas (Fig. 5G), most notably for A239G. Interestingly, none of these variants were able to establish a productive infection in the heart (Fig. 5H), despite this site being one of the principle sites of CVB3 replication during acute infection. A239G, I230F, and Y268W were undetectable from this key organ by day 5, whereas WT titers kept increasing through day 7, indicating the onset of viral myocarditis. Furthermore, shedding of all three variants in feces was no longer detectable 7 d after infection, whereas WT continued to be shed (Fig. 5I).

Finally, because CVB3 is known to establish long-term persistent infection primarily of the heart muscle, we measured virus in the hearts and spleens of mice after 35 d of infection. Although detection of CVB3-specific RNA in the hearts of mice was below the level of detection of our quantitative RT-PCR (qRT-PCR) conditions, we were able to detect high copy numbers of viral RNA in the spleens of mice infected with WT CVB3 (4.7×10^5

Table 2. Site-directed mutagenesis on residues involved in conformational changes during catalysis

Desired variant	Status*
Active site closure for catalysis	
I230A	Nonviable
I230V	Reversion
I230F	Stable
I230W	Nonviable
F232V	Nonviable
F232Y	Stable
F232L	Reversion
F232V	Reversion
F232Q	Nonviable
F232S	Nonviable
Y234W	Nonviable
Y234L	Nonviable
Y234V	Nonviable
Y234A	Nonviable
D238N	Nonviable
D238A	Nonviable
D238S	Nonviable
D238E	Nonviable
I306W	Nonviable
I306F	Nonviable
L344W	Nonviable
L344F	Nonviable
Linkage between palm and thumb	
S240W	Nonviable
S240N	Nonviable
S240Q	Nonviable
S240Y	Nonviable
S240F	Nonviable
F246W	Nonviable
F246L	Nonviable
Y268W	Stable
Y268H	Stable
Y268A	Nonviable
L269W	Nonviable
L269F	Reversion
M287F	Nonviable
M287Q	Nonviable
M287H	Nonviable
M287I	Nonviable
RNA template and NTP positioning	
R174K	Nonviable
R174H	Nonviable
I176L	Nonviable
I176V	Stable
I176Y	Nonviable
S289W	Nonviable
S289Q	Nonviable
S289N	Nonviable
S289D	Nonviable
S289C	Nonviable

*The status indicates whether the mutation was stable over three passages (dark grey), reverted to WT (lighter grey), or did not yield viable virus (lightest grey).

RNA copies/mL) or the strains showing little or no attenuation (Fig. 5J). In contrast, the more attenuated strains (A239G, Y268H, I230F, and Y268W) had fewer than 40, 70, 820, and 3,400 genome copies/mL, and in some mice, virus was not at all detectable. Overall, our data show that increasing mutation frequencies significantly beyond the natural state by lowering the intrinsic fidelity of viral polymerases attenuates a virus and its capacity to establish persistent infection in target organs.

Fig. 2. Viral production (infectious virus and total genomic RNA) of CVB3 polymerase variants in cell culture. (A–D) Production of infectious virus measured by one-step growth kinetics of (A and B) WT, P48K, S164P, L241I, A239G, and A239S viruses and (C and D) WT, I176V, I230F, F232V, F232Y, Y268H, and Y268W viruses. HeLa cells were infected at MOI of 10, and progeny virus was quantified at different hours postinfection by TCID₅₀ assay. Mean titers (TCID₅₀/mL) ± SEM are shown (*n* = 3 independent experiments); no significant difference was found comparing WT and each of the CVB3 variants, except A239S (**P* < 0.05 and ****P* < 0.0001 by two-way ANOVA with Bonferroni posttest). (E–H) Total genomic RNA content of the same virus populations examined above (A–D). The number of RNA genomes was determined by real-time RT-PCR at different times postinfection. Mean values (genome copies per milliliter) ± SEM are shown (*n* = 3 independent experiments); significant differences with respect to WT are indicated by **P* < 0.05 or ****P* < 0.0001 by two-way ANOVA with Bonferroni posttest.

Small Population Size Passage Exacerbates the Fitness Cost of Increasing Virus Mutation Frequency.

Because we did not find a strong enough difference in fitness between WT and low-fidelity variants in competition experiments to explain their attenuation in vivo and because none of the mutants showed a growth defect in tissue culture, we examined whether effects of population size could explain the marked attenuated in vivo phenotypes. Indeed, the previous cell culture experiments were performed with large population sizes [10^7 50% tissue culture infectious doses (TCID₅₀) for one-step growth curves and 10^5 TCID₅₀ for competition assays]. Given the higher mutational burden that is expected of lower-fidelity variants, we hypothesized that, at very small population sizes that are more representative of in vivo infection (at least at early stages of infection or during passage of anatomical bottlenecks), these virus pop-

Fig. 3. CVB3 polymerase variants are mutator strains. (A) Average mutation frequencies of WT and other polymerase variants are shown as the mean number of mutations per 10⁴ nt sequenced by TopoTA cloning of total RNA from passage 3 virus stock populations. Between 75 and 150 clones (78,750–157,500 nt total) were sequenced per population (ns, not significant; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with WT by Mann–Whitney *u* test). (B) The CVB3 fidelity variants are more sensitive to treatment with the RNA mutagen ribavirin. HeLa cells, treated with either 200 or 400 μM ribavirin or mock-treated, were infected at MOI of 0.01; 48 h postinfection, the loss of infectivity in the progeny virus was titrated by TCID₅₀ assay. The percentage of each CVB3 variant surviving the treatment with either 200 or 400 μM ribavirin relative to the untreated control is shown. The mean values ± SEM are shown (*n* = 5; *P* < 0.001 for all samples compared with WT by Student *t* test).

ulations would have a greater tendency to extinguish because of the exacerbated effect of increased deleterious mutation on smaller population sizes. To test this hypothesis in tissue culture, we compared the success of large population size passages of each variant with their ability to survive extreme bottlenecks by plaque-to-plaque transfer of individual viruses. Each variant population was plated on cell monolayers with agarose overlays permitting the isolation of individual plaques originating from single viruses within the population. Three plaques from each population were isolated and immediately replated to isolate a new plaque for each triplicate. This procedure was carried out through nine passages. At each passage, the monolayer was

colored by crystal violet, and each plaque's surface area was measured as a surrogate for possible reductions in virus fitness resulting from the accumulation of detrimental mutations. Virus progeny from the last viable passage for each triplicate was sequenced, and they confirmed that the polymerase retained the engineered mutations and did not fix new mutations; mutations increasing fitness would then be expected elsewhere in the genome, either as single fixed mutations or a constellation of minority mutations affecting aspects of the virus life cycle. For WT virus, no significant loss in virus fitness and infectivity was observed for any of the triplicate passages, and the surface areas of plaques fluctuated tightly around 100 mm² (Fig. 6 A–G). All three triplicates of S164P and two triplicates of P48K did not significantly differ from WT virus (Fig. 6 A and B). All of the remaining variants, however, showed consistent loss of fitness (Fig. 6 C–G). In the most marked cases (e.g., I230F, F232Y, and Y268W variants), the plaque-passaged virus populations were extinguished immediately or after a few passages, presumably because of more rapid accumulation of deleterious mutations. Interestingly, the variants that were most rapidly extinguished presented the highest mutation frequencies (Fig. 3) and the most attenuated phenotypes *in vivo* (Fig. 5). Importantly, when these same variants were passaged as larger populations of 1,000 (Fig. 6H) or 1 × 10⁶ virions (Fig. S5), there were no observable differences in virus fitness (plaque phenotype and extinction) or production (titers and replication kinetics). These results suggest that low-fidelity variants with a genetically more diverse population harbor too many mutated or defective particles and do not have enough viable genomes to ensure survival of the virus population when sizes are very significantly reduced, which might happen during *in vivo* infection and dissemination.

Discussion

The growing number of RNA polymerase fidelity variants that have been described to date (poliovirus, chikungunya virus, and Coxsackievirus) (4, 23, 24, 27) harbors mutations mapping to different regions of the viral RdRp, indicating that replication fidelity is determined by multiple residues that may work alone or in unison as a network that remains to be properly defined. Indeed, the G64S high-fidelity variant of poliovirus helped identify one such network of amino acids participating in fidelity, comprising residues 1, 64, 239, and 241 that are seemingly connected to other residues at the active site through Asp238 (20–22, 25, 29). Although structural studies revealed subtle changes between the poliovirus Wt and high-fidelity G64S polymerases (30), more recent study on enzyme dynamics by NMR suggests that distantly located residues may be working together to affect polymerase fidelity in a functionally dynamic rather than purely structural fashion (31). Our initial intention was to engineer

Table 3. Deep sequence analysis of 3Dpol region

WT	P48K	S164P	A239G	Y268H	Y268W	I230F	F232Y
E35G	E35G	E35G	E35G	E35G	E35G	E35G	E35G
N37G	N37G	N37G	N37G	N37G	N37G	N37G	N37G
						M145L (16%)	
						K170E (2.5%)	I165K
						230L (1.5%), 230I (0.5%)	
							232F (6%)
H273R	H273R	H273R	H273R	H273R	H273R	H273R	H273R
						S299T (17%)	S299T (19%)
						A372V (4%)	A372V (19%)
						E427K	

Passage 3 virus stocks used in these studies were used to generate libraries for deep sequencing; ~4 million Illumina reads per virus were obtained, giving a minimum of 100,000-fold coverage of each nucleotide site. Alignments were used to identify amino acid changes representing at least between 0.5% and 1.5% of total reads (listed in white). In lighter gray, minority variants representing over 1% of the total population are shown. In dark gray, reversions at the originally altered amino acid are shown.

Fig. 4. In vitro fidelity assay used to determine how efficiently the Coxsackievirus polymerases use CTP and 2'-deoxy-CTP as substrates. (A) The 5' fluorescein-labeled primer template RNA was designed with a terminal sequence such that ~40% of the maximal change in the fluorescein signal occurs before the CTP addition, whereas the remaining signal increase to 100% is rate-limited by the CTP or dCTP addition itself. (B) Data from the I176V mutant polymerase showing the similar kinetic behavior on the addition of nanomolar CTP (*Left*) or micromolar dCTP (*Right*). Kinetic parameters (lag phase time, K_m , and k_{cat}) obtained by fitting the data are listed in [Table S1](#). *Insets* show gels with an analogous elongation of a 10 + 1 – 12 RNA (42), where a locked +1 complex yields a +7 product in the absence of CTP and a full-length +13 product in the presence of nanomolar CTP or micromolar dCTP. (C) Plot showing the correlation between the observed infectious virus mutation rates (Fig. 3) and the CTP vs. dCTP discrimination factors based on NTP use efficiencies ([Table S1](#)). F232L and F232V mutants were not stable, and the plotted mutation rates represent a lower limit based on preliminary sequencing from a mixed population of virus in the process of reverting. (D) Plot showing the correlation between polymerase elongation rates and RNA genome production during virus infection (fold change between 3 and 5 h in Fig. 2 E–H). Faster polymerases use less time to replicate through the lag phase sequence indicated in A.

similar high-fidelity variants of CVB3 by manipulating the same conserved sites. Interestingly, whereas mutations at these sites for poliovirus were either nonviable or further mutated to other amino acids at the same position that resulted in high-fidelity variants (24), the same mutations in Coxsackievirus largely reverted at the intended position, and they were accompanied by other mutations that seemingly decreased rather than increased fidelity. Along the same lines, the second batch of mutations engineered around the conformational changes associated with active site closure and catalysis also resulted in only lower-fidelity polymerases. The work by Graci et al. (32) recently showed that the CVB3 polymerase had higher incorporation fidelity than poliovirus in a biochemical assay using purified polymerase. The work by Graci et al. (32) also showed that the same mu-

tational burden added to CVB3 has a greater negative impact than on poliovirus. In other words, CVB3 seems to have lower mutational robustness and thus, may have evolved a relatively higher fidelity because of this lower tolerance to mutation. Previously, in trying to select for a high-fidelity variant of the CVB3 Nancy strain, we obtained the A372V mutant, which turns out to be the native polymerase in the majority of CVB3 naturally occurring strains (26, 27, 33). Given these results, our initial screen for mutagen resistance may not have applied adequately strong selective pressure to isolate variants of even higher fidelity.

It is interesting to note that, for the substitutions performed around the tetrahedral H-bond network that tethers the buried N terminus (residues 1, 64, 239, and 241), the resulting viable variants often contained second site mutations (positions 48, 164, and 299) that also decreased fidelity (Fig. 1C). These results suggest that these sites are linked to this fidelity network and were selected to compensate for a detrimental mutation introduced experimentally. However, only the S299T mutation is close enough in the structure to directly impact the conformation around the N terminus H-bonding network. Ser164 and Pro48 are both 25–30 Å away and located at the top of the fingers domain, and they are more likely to contribute to fidelity through long-range allosteric effects. It is, therefore, not clear from our results whether these second site mutations were selected for two reasons. First, their low fidelity more readily allowed for the reversions to WT to occur at the targeted site. Second, they permitted the stabilization of a polymerase that had been destabilized by the intended substitutions at the H-bond network, thereby rescuing replication and allowing the reversion to follow. We did not observe a similar phenomenon with the second set of variants that localized more closely to the active site. Here, the substitutions were either viable or nonviable/revertants. It is interesting to note that, for these substitutions, the effects on decreasing fidelity were more significant, suggesting that altering the active site may have greater impact in this regard. Considered globally, we found that substitutions that increased the mutation frequency from ~4 to ~8 mutations/10⁴ nt were well-tolerated and fully stable in the context of infectious virus. In contrast, our biochemical assay data indicate that even greater decreases in polymerase fidelity are possible from mutations such as F232L, which has a CTP vs. dCTP discrimination factor of only ~650-fold. Among the stable and viable mutants, the data show a good correlation between virus mutation rates and polymerase NTP selectivity using 2'-deoxy-CTP as a proxy molecule for nucleotide misincorporation (Fig. 4C). Interpolation of the F232L data onto this curve predicts that this mutant would exhibit a very high error rate of 15.5 mutations/10⁴ nt, likely explaining why the virus reverted immediately. Perhaps more interesting, the similar F232V mutation was also not stable; however, its 830-fold nucleotide discrimination factor leads to a prediction of 11.6 mutations/10⁴ nt. This prediction is only slightly higher than the 11.2 mutations/10⁴ nt observed for the viable F232Y and I230V mutants (with low-frequency reversion detected by deep sequencing), and this finding suggests that Coxsackievirus viability may have an upper limit on mutation frequency in the range of 11–12 mutations/10⁴ nt.

Again, these results support our previous data that CVB3 is very sensitive to increases in mutation frequency (32). In this sense, the hypersensitive mutator strains identified in this study may be helpful in screens to identify new mutagenic compounds, which could then be improved to be effective against less-sensitive viruses. Previous studies describing high-fidelity poliovirus polymerase revealed that restricting the genetic diversity of a virus population resulted in moderate to severe attenuation in vivo (5, 6, 24). These studies were performed in a murine model of poliovirus infection involving the transgenic expression of the human poliovirus receptor. More recently, a chikungunya virus high-fidelity variant confirmed a moderate fitness cost of increasing fidelity in both a mouse infection model and infection of its natural mosquito host (4). The isolation of a large panel of

Fig. 5. Low-fidelity CVB3 variants are attenuated in vivo. (A–D) Virus titers (genome copies per milliliter) measured by qRT-PCR of mice infected with 10^5 TCID₅₀ and killed at 3 d after infection are shown. Each panel presents a different organ from the same groups of mice. Box plots show median values $\pm$ SEM ($n = 8$ – 12 ; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by Kruskal–Wallis test). (E–I) In vivo replication kinetics of selected CVB3 variants. Mice were inoculated with WT, I230F, Y268W, and A239G and killed on days 3, 5, and 7 postinoculation ($n = 4$). Virus was quantified as above. (J) Mice were inoculated with each variant or WT virus, and 35 d after infection, virus was quantified in the spleen ($n = 8$; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

low-fidelity variants in this study permitted us to extend the question of fidelity and virus fitness to the other end of the spectrum, where virus populations present larger than natural genetic diversity. In our model of infection, mice naturally express the Coxsackievirus receptor Coxsackie adenovirus receptor, and i.p. infection results in viral myocarditis along with a severe pancreatitis (15, 34). In certain mouse strains, CVB3 can establish a persistent infection in quiescent, differentiated cells of the heart and the B-cell population within the spleen, which is detectable up to 3–5 mo postinfection (35). Because of the relatively mild outcome of infection of the WT CVB3 Nancy strain, we were unable to determine the LD₅₀ values for each mutant. Thus, we examined virus titers in different target organs, particularly at day 3 postinfection when peak titers in all organs are reached. Although not always significant, we show that, in most organs, mutator strains present lower titers, with some variants being extremely attenuated, such as I230F, A239G, or Y268W. Other than P48K and S164P, which presented the most WT-like mutation frequencies, none of the variants were able to establish robust infection of the heart muscle. Importantly, the variants that presented the largest differences in mutation frequency and polymerase fidelity were most attenuated in vivo. Furthermore, in contrast to WT CVB3, these attenuated mutator strains were unable to establish persistent high titer infections. This observation is particularly interesting, because the link between fidelity and pathogenesis has, until now, been examined only in acute virus infection. Future studies using these mutator strains of CVB3 may help better characterize the determinants of persistent infection for chronically infecting viruses. It also raises the possibility that live, attenuated virus vaccines may, indeed, be possible for chronically infecting viruses by manipulating the ability of a virus to establish persistence.

The observed in vivo attenuation is perhaps better understood in light of the large, small, and bottleneck passages carried out in tissue culture. During plaque-to-plaque passaging, an artificial bottleneck was created in vitro to better represent both the early stages of infection, where only a few viruses initiate infection of the host, and later stages, where virus populations undergo genetic bottlenecks while passing through anatomical barriers into new organs. Indeed, only WT virus and the variants showing little attenuation in vivo (P48K and S164P) survived more than nine passages of extreme bottlenecking, whereas most of low-fidelity variants underwent rapid extinction between the second and fifth passages. Virus extinction was accompanied by a progressive decline in plaque size, which is a viable indicator of fitness loss resulting from the accumulation of deleterious mutation. At very small population sizes, mutator strains collapsed under the burden of their presumably excessive intrinsic mutation rates. At larger population sizes, this burden may have been masked, if not reversed, by the frequent recombination that occurs between genomes of picornaviruses (36). Indeed, at large population size and high multiplicity of infection (MOI), no significant fitness costs were observed. Similar population size-related effects are observed when virus mutation frequencies are increased extrinsically, such as by RNA mutagens during lethal mutagenesis (37).

In summary, these results help complete the picture that, for RNA viruses, nature has fine-tuned polymerase fidelity and mutation frequency to strike an optimized balance between adaptability and maintenance of genetic integrity. High-fidelity variants, with moderate reductions in mutation frequency, are unable to rapidly generate the diversity required to adapt to new cell types or avoid the host immune responses. Low-fidelity variants with similar increases, rather than decreases, in mutation frequency are seemingly burdened with too many mutations. The result is a similar degree of attenuation reached by a different mechanism as outlined above.

Methods

Generation of Virus Stocks and Infections. All variants were constructed using the Quikchange XL site-directed mutagenesis kit (Stratagene) and the CVB3-Nancy infectious cDNA; 4 μ g in vitro-transcribed infectious RNA were elec-

Fig. 6. Mutator strains are prone to extinction during extreme bottlenecking in tissue culture. (A–G) WT virus (solid lines) and each polymerase variant (dashed lines) were subjected to extreme bottlenecking by plaque-to-plaque passage on HeLa cells. Triplicate passage of each virus was performed through nine passages (x axis). Some samples extinguished at the first passage (dashed lines along x axis). The mean surfaces are square millimeters of plaques, and SEM values are shown for each triplicate passage series ($n = 20$ –52; $***P < 0.001$ by Mann–Whitney u test). (H) Larger population size passage of polymerase variants; 1×10^6 HeLa cells were infected with 1,000 virus particles of each variant, and 48 h after infection, virus was quantified by qRT-PCR to determine the genome copies per milliliter (y axis) at each passage number (x axis).

troporated into 4×10^6 HeLa cells using previously described conditions (27). At total cytopathic effect (CPE), 250 μ L virus stocks were used to infect fresh HeLa cells monolayers for three more passages. For each passage, virus was harvested by one freeze–thaw cycle. Four independent stocks were generated for each virus. Consensus sequencing of virus stocks used in downstream experiments confirmed the stability of the engineered mutations and did not detect any additional mutations across the genome (although we cannot exclude compensatory mutants existing as minority populations below the level of detection). For ribavirin assays, HeLa cells were pretreated for 1 h with different concentrations of ribavirin and infected at MOI of 0.01 with passage 3 virus; 48 h postinfection, virus titers were determined by TCID₅₀.

Replication Kinetics. For one-step growth kinetics, HeLa cells were infected at MOI of 10, frozen at different time points after infection, and later, titered by TCID₅₀ assay. For qRT-PCR analysis, total RNA from infected cell supernatants was extracted by TRIzol reagent (Invitrogen) and purified. The TaqMan RNA-to-c_t one-step RT-PCR kit (Applied Biosystems) was used to quantify viral RNA. Each 25- μ L reaction contained 5 μ L RNA, 100 μ M each primer (forward 5'-GCATATGGTGATGATGTGATCGCTAGC-3' and reverse 5'-GGGGTACTGTT-CATCTGCTCTAAA-3'), and 25 pmol probe 5'-[6-Fam] GGTTACGGGCTGAT-CATG-3' in an ABI 7000 machine. Reverse transcription was performed at 50 °C for 30 min and 95 °C for 10 min, and it was followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A standard curve ($y = -0.2837x + 12.611$, $R^2 = 0.99912$) was generated using in vitro-transcribed genomic RNA.

Sequencing. Viral RNA from passage 3 virus stocks was extracted and RT-PCR-amplified using the primers sets 878Forward and 2141Rev covering part of the structural proteins. The resulting PCR products were TopoTA-cloned (Invitrogen), sequenced, and analyzed using Lasergene software (DNASTar). The number of mutation frequency was calculated using the total mutations identified per population over the total number of nucleotides sequenced for that population multiplied by 10^4 . For each population, the number of clones presenting zero, one, two, three mutations and so on was quantified and used for statistical inference by Mann–Whitney u test as previously described (38). For deep sequencing, 5×10^8 virion RNA was extracted, and cDNA libraries were prepared by RT (Superscript III) and PCR (Phusion) amplification of the viral RdRp-coding region (3Dpol) that was fragmented (Fragmentase), clustered, and sequenced with Illumina cBot and GAII technology. Over 5 million 75-nt reads were obtained per virus, of which 95–98% passed quality. Quality filtering, adaptor, and unresolved nucleotides (Ns) cleaning were done using fastq-clipper (http://hannonlab.cshl.edu/fastq_toolkit/index.html). Reads were aligned to the CVB3 genome as reference with a maximum two mismatches per read and no gaps using BWA (39). Alignments were

processed using SAMTOOLS (40) to obtain the nucleotide/base calling at each position. The background noise caused by sequencing error was 0.01%, and the limit of detection for our filters to consider a variant to be biologically relevant was set to 0.5% of the total population (50 $\times$ background levels).

In Vitro RdRp Fidelity Assay. WT and mutant CVB3 (Nancy) polymerases containing Val372 and a C-terminal GSSS-6xHis tag were cloned into the pET26b-Ub-3D plasmid and transformed into *Escherichia coli* strain BL21 PCG1 for expression (41). The expression and purification of polymerases were carried out as previously described for poliovirus polymerase (28). Stopped-flow elongation experiments were carried out in an Applied Photophysics SX-20 Stopped Flow instrument, where equal volumes of pre-formed polymerase-RNA elongation complex and NTP solutions were mixed to initiate the reaction. All reactions were carried out at 37 °C and pH 6.5 at final concentrations of 75 mM NaCl, 60 nM polymerase, 10 nM RNA, 20 μ M ATP, 1 μ M GTP, and UTP. Fluorescence excitation was at 492 nm from a monochromator source with bandwidth set to 9.3 nm, and emission from fluorescein was detected using a 515-nm high-pass filter. The kinetic parameters listed in Table S1 are derived from the CTP- and dCTP-dependent changes in fluorescence signals that are caused by movement of the RNA to the polymerase (28) and are not from a direct measure of RNA product formation associated with nucleotide incorporation. The gels in Fig. 4B, Insets show both CTP and dCTP incorporation with a similar $10 + 1 - 12$ RNA (42) obtained under identical experimental conditions.

Fitness Assay. Relative fitness values were obtained by competing each low-fidelity variant with a marked reference virus that contains four adjacent silent mutations in the polymerase region introduced by direct mutagenesis. Coinfections were performed in triplicate at MOI of 0.01 using a 1:1 mixture of each variant with the reference virus; 48 h after infection, two more competition passages were performed. The proportion of each virus was determined by real time RT-PCR on extracted RNA using a mixture of Taqman probes labeled with two different fluorescent reporter dyes. MGB_CVB3_WT detects WT virus (including the fidelity variants) with the sequence CGCATCGTACCCATGG, and it is labeled at the 5' end with a 6FAM dye (6-carboxyfluorescein) and MGB_CVB3_Ref containing the four silent mutations; CGCTAGCTACCCATGG was labeled with a 5' VIC dye. Each 25 μ L-reaction contained 5 μ L RNA, 900 nM each primer (forward primer, 5'-GATCGCATATGGTGATGATGTGA-3'; reverse primer, 5'-AGCTTCAGCGAG-TAAAGATGCA-3'), and 150 nM each probe. The relative fitness was determined by the method described in the work by Carrasco et al. (43). Briefly,

the formula $W = \left[\frac{R(t)}{R(0)} \right]^{\frac{1}{t}}$ represents the fitness W of each mutant genotype

relative to the common competitor reference sequence, where $R(0)$ and $R(t)$ represent the ratio of mutant to reference virus densities in the inoculation mixture and t days postinoculation, respectively. The fitness of the normal WT to reference virus was 1.019, indicating no significant differences in fitness caused by the silent mutations engineered in the reference virus.

Infection of Mice. Mice were kept in the Pasteur Institute animal facilities in biosafety level 2 conditions, with water and food supplied ad libitum, and they were handled in accordance with institutional guidelines for animal welfare. All studies were carried out in C3H/HeOJ male mice between 3 and 6 wk old obtained from Charles River. Mice were infected i.p. with 10^5 TCID₅₀ in 0.25 mL. For tissue tropism studies, we harvested whole organs and sera that were homogenized in PBS using a Precellys 24 tissue homogenizer (Bertin Technologies). Viral RNA was extracted using TRIzol reagent (Invitrogen), and real-time PCR was performed as described above.

Extinction of Viral Populations by Small Population Passaging. HeLa cell monolayers in six-well plates were infected at MOI values of 1 (1×10^6 particles) and 0.001 (1×10^3 particles). To determine MOI, virus was purified from supernatants, RNA was extracted and quantified by qRT-PCR, and viable virus progeny was determined by classic plaque assay. For plaque-to-plaque transfer, passage 3 virus stocks were plated by standard plaque assay under 1% agarose. At 48 h after infection, three individual plaques were

isolated by inserting a pipette tip through the agarose to transfer the agarose immediately covering the plaque to an Eppendorf containing 500 μ L DMEM, vortexing and immediately replating the plaque-purified virus on fresh cell monolayers in serial 10-fold dilution covered with a new agarose overlay; 48 h later, the plaque purification was repeated through nine passages. At each step, after plaque transfer, the agarose overlay was removed, the cells were colored by crystal violet, and the entire cell monolayer was scanned for analysis by computer software. The total surface area of each plaque on each cell monolayer (10–50) was determined in square millimeters using ImageJ freeware (<http://rsbweb.nih.gov/ij/>). The RdRp gene from virus progeny from the last viable passage before extinction or passage nine was sequenced to confirm the presence of the engineered mutations and determine whether new mutations arose.

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