Quasispecies theory is a case of mutation-selection balance for evolution at high mutation rates, such as those observed in RNA viruses. One of the main predictions of this model is the selection for robustness, defined as the ability of an organism to remain phenotypically unchanged in the face of mutation. We have used a collection of vesicular stomatitis virus strains that had been evolving either under positive selection or under random drift. We had previously shown that the former increase in fitness while the latter have overall fitness decreases (I. S. Novella, J. B. Presloid, T. Zhou, S. D. Smith-Tsurkan, B. E. Ebendick-Corpus, R. N. Dutta, K. L. Lust, and C. O. Wilke, J. Virol. 84:4960–4968, 2010). Here, we determined the robustness of these strains and demonstrated that strains under positive selection not only increase in fitness but also increase in robustness. In contrast, strains under drift not only decreased in fitness but also decreased in robustness. There was a good overall correlation between fitness and robustness. We also tested whether there was a correlation between fitness and thermostability, and we observed that the correlation was imperfect, indicating that the fitness effects of mutations are exerted in part at a level other than changing the resistance of the protein to temperature.
greater thermostability than clones with low robustness (41). Work on microRNA is controversial, and while the experimental results seemed to show uncorrelated genetic robustness and thermal tolerance (19), there is disagreement on the interpretation of data (42).

In the present report, we used unmutagenized VSV strains to test whether increased or relaxed selection led to changes in genetic robustness and whether there is a correlation between robustness and thermostability. We analyzed a collection of strains that we had generated by plaque-to-plaque passages (43, 44) or by large-population passages (29). Plaque-to-plaque passages minimize selection, while large-population passages maximize selection. Our results indicated that, as predicted, in the absence of selection there was a loss of robustness, while in the presence of selection robustness improved. We also found that robustness does not need to covary with thermostability in VSV; however, in the absence of selection, the VSV genetic architecture seems to impose a moderately strong relationship between robustness and thermostability. Finally, unexpectedly, thermostability increased during plaque-to-plaque passages, even though robustness decreased.

**MATERIALS AND METHODS**

**Cells and viruses.** We used BHK-21 cells for all infections. For cell growth, we used minimal essential medium (MEM) with Hank’s salts supplemented with 0.6% PP3 (Difco) and 7% bovine calf serum (BCS). The same medium was used for plaque assays, except that PP3 was omitted. For viral infections, we used MEM with Hank’s salts supplemented with 7% fetal bovine serum (FBS). We used agarose (0.2%) to make semisolid overlays, and all incubations were done at 37°C. We worked with vesicular stomatitis virus, Indiana serotype, Mudd Summer’s strain, obtained from John Holland (UCSD). The wild type (wt) is sensitive to 11 monoclonal antibody (Mab). MARM U is an 11 MAb-resistant mutant with a single substitution in amino acid 256 of the G glycoprotein (45). We had previously subjected the wt to a continuous regimen of positive selection in BHK-21 cells by repeated-large-population passages (2 × 10⁶ PFU/passage), as previously described (29). Three replicates were randomly selected for the present study, and these were labeled w25A (fitness of 3.1 × 0.08). All except MRa, MRg, MRo, and MRz were sequenced (44). We also previously subjected the wt to a continuous regimen of positive selection in BHK-21 cells by repeated-large-population passages (43, 44) or by large-population passages (29). Plaque-to-plaque passages minimized selection, while large-population passages maximize selection. Our results indicated that, as predicted, in the absence of selection there was a loss of robustness, while in the presence of selection robustness improved. We also found that robustness does not need to covary with thermostability in VSV; however, in the absence of selection, the VSV genetic architecture seems to impose a moderately strong relationship between robustness and thermostability. Finally, unexpectedly, thermostability increased during plaque-to-plaque passages, even though robustness declined.

**Robustness measurements.** We used resistance to mutagenesis as a surrogate of robustness. We seeded T-25 flasks with BHK-21 cells, and after overnight growth we treated the resulting semiconfluent monolayers with 5 ml of 5-fluorouracil (FU) solution dissolved in MEM plus FBS at a final concentration of 0 (control), 10, 35, or 100 μg/ml. After 6.5 h of incubation at 37°C, during which the drug concentration inside cells equilibrated, the monolayers were infected with approximately 10⁴ PFU, and the infections were monitored for cytopathic effect (CPE), which developed more slowly with increasing concentrations of mutagen, so in the absence of mutagen it was complete at 24 h postinfection, in the presence of 10 μg/ml it took 48 h, and in the presence of 35 or 100 μg/ml it took 72 h. All strains were recovered at the same time in the presence of a given amount of drug. When CPE was complete, we recovered the viral yields and determined titers by plaque assay. We then normalized titers by dividing them by the control titer.

To quantify robustness, we regressed log-transformed, normalized titers against the square root-transformed FU concentrations. The data were nearly linear after this transformation for all mutants (see the figures in the supplemental material). The regression line was fit without an intercept, since the log-transformed, normalized titer has to be zero by definition. We used the slope of the line as our measure of robustness. All slope values are negative, and a more negative slope indicates more sensitivity to FU and, thus, less robustness.

**Mutant frequency determinations.** For wt and derived populations, we determined the frequency of I1-resistant mutants. Samples from frozen stocks were diluted as needed and titrated in triplicate plaque assays without I1 to calculate total virus concentration. In parallel, we used more concentrated samples (typically 3 to 4 orders of magnitude more concentrated) to perform plaque assays in the presence of excess concentrations of I1 in the agarose overlay. Importantly, we did not treat virus with antibody prior to plating, because this treatment would lead to significant underestimation of mutant frequencies due to phenotypic mixing and hiding (50, 51). The plaque numbers under these conditions would represent the number of MARM mutants in the stock. To calculate mutant frequency, we determined the MARM averages divided by wt averages.

**Quantitation of physical particle-to-infectious-particle ratios.** Total genome copy number in virus stocks was measured using a tagged reverse transcription (RT) primer system in order to increase strand specificity (54). For RNA standards, we used RNA transcribed from pVSV, a plasmid containing the full-length sequence of VSV (unpublished results). pVSV was linearized with SpeI, and T3 polymerase (Promega) was used in vitro to produce a full-length, negative-sense RNA transcript identical to the VSV genome. The transcribed RNA was cleaned using an RNase Minikit (Qiagen). RNA transcript concentrations were determined spectrophotometrically, and sizes and concentrations were verified via formaldehyde gel. The RNA transcripts were then serially diluted. Total RNA from each dilution and from virus samples that was isolated using the QIAamp viral RNA kit (Qiagen). Reverse transcription was performed using an RT primer (5′GGCAGTATGGTTAAGGATCTCGTCTCAAGGCCC 3′) containing viral sequence recognizing VSV genomic nucleotides (nt) 4664 to 4683 and a nonviral tag sequence (in boldface). Briefly, 2 μl of RNA was mixed with 20 μM of RT primer and 8 μl of RNase-free water. RNA was denatured at 70°C for 5 min and then allowed to rest on ice for 1 min. Superscript III (Invitrogen) and RNase OUT (Invitrogen) were added, and the reaction was carried out at 50°C for 30 min. The enzyme was inactivated at 94°C for 5 min, and all samples were diluted 100-fold. Real-time PCR cycling was then performed using as the forward primer the tag

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For standard curves only an RNA copy range of 4 to 10^6 RNA transcripts is on the scale of 1,000 copies in the RT reaction, although Iowa Black FQ at the 3′ end. The sensitivity of this assay when reading melting curves. Copy numbers for viral RT reactions were calculated based on the melting temperature of 74.5°C, which was verified using SYBR green extension step. This amplification protocol yields a 167-bp product with a thermostability. We ruled out a potential contribution of aggregation and adhesion to the tube wall in all of the bottleneck strains with mutations in the G ORF, which resulted in values that were not significantly different from those from assays in plastic tubes (P = 0.243 by paired t test).

RESULTS
Robustness increases during adaptive evolution. One of the predictions of quasispecies theory is that adaptation will produce populations of increased robustness. We tested this hypothesis by determining sensitivity to FU in VSV populations generated after 25 large-population passages (w25A, w25D, and w25E). Under these conditions, selection for increased fitness is the predominant evolutionary force, and viral fitness increases consistently (29, 56). Figure 1A shows that the populations under selection had a significantly higher resistance to FU than the wt progenitor (P = 0.006 by one-sample t test; n = 3). (We calculated robustness/resistance to FU as the slope in a regression of the log-transformed, normalized viral titer against the square root of the FU concentration [see Fig. S1 in the supplemental material]. More-negative numbers imply lower robustness.)

Plaque assays measure live virus and cannot give estimates of lethal mutations. We tested whether differences in FU sensitivity correlate to changes in the frequency of mutants carrying lethal mutations by determining the physical particle/infectious particle ratios. We used quantitative reverse transcription-PCR to measure the number of genomic RNA copies, which represents the number of physical particles in VSV. The RNA molecule/PFU ratios of strains under selection (w25A, w25D, and w25E) were significantly lower than that of the wt progenitor (P = 0.01 by one-sample t test; n = 2) (Fig. 1B), suggesting that the deleterious mutation rate was lower under selection, providing additional evidence that these viral populations have higher robustness.

We tested whether the increased resistance to FU was due to differences in the number of mutations incorporated during mutagenesis. For instance, more resistance could be the result of higher polymerase fidelity or of decreased affinity for the drug. If that had been the case, we would expect an inverse correlation between mutant frequency and resistance to FU. We infected BHK-21 monolayers in the presence of 10 μg/ml FU, and we measured the frequency of I1-resistant mutants among the progeny. We found that mutant frequency increased with robustness; however, this trend was not significant (Fig. 1C), and none of the strains under selection had lower mutant frequencies than the wt. Therefore, we can rule out increased polymerase fidelity as a contributor to increased resistance to FU.

Robustness is lost during random drift. There are several environmental factors that decrease the effectiveness of selection. Selection in viral populations may be minimized by coinfection because of complementation, which leads to the overall loss of robustness in the RNA phage phi-6 (26). Another means to minimize selection is through the chance transmission of individual viral particles. This type of regimen represents the most extreme bottleneck, and it can be performed easily in the laboratory by randomly picking individual plaques (46). Under these conditions, random drift dominates and there is overall fitness loss (46). We hypothesized that robustness would evolve by following the same pattern, so there would be an overall loss of robustness.

We tested 16 VSV strains generated from MARM U by 20 plaque-to-plaque passages. As we did before, we first determined their sensitivity to FU (see Fig. S2 in the supplemental material) and compared it to that of their MARM U progenitor. Figure 2A shows the results, which supported our hypothesis. All 16 strains were more sensitive to FU than the MARM U progenitor. This result was highly significant (P = 0.000015 by one-sample t test; n = 16).

The next experiment consisted of determining physical particle/PFU ratios. There were significant variations among strains
(Fig. 2B), but overall there were no changes in the ratios ($P = 0.15$ by one-sample $t$ test; $n = 16$).

Finally, we investigated the possibility that the differences in sensitivity to FU were due to differences in polymerase accuracy in the presence of the mutagen. Once again, we calculated mutant frequency in these populations after replication in the presence of $10 \mu g/ml$ FU. Because they are all progeny of MARM U, they are already fully resistant to I1 antibody, thus we tested resistance to I14 antibody, which recognizes a different antigenic site in the G glycoprotein (52, 53). We did not find any correlation between mutant frequency and robustness ($r = -0.014; P = 0.96; n = 13$), so we could not explain lower robustness as a result of higher mutation rates (Fig. 2C). This result is consistent with our previous finding that MRr and MRb have genomic mutant frequencies that do not differ from that of their MARM U progenitor (29).

A correlation between fitness and robustness. Quasispecies theory predicts that selection affects two phenotypic traits: fitness and robustness. We found that a regimen that minimizes selection, such as plaque-to-plaque passages, overall resulted in both fitness loss and robustness loss (Fig. 3; also see Fig. S2 in the supplemental material). In contrast, we found that a regimen that maximizes selection, such as large-population passages, produced strains with increased fitness and increased robustness (Fig. 3; also see Fig. S1). Furthermore, taking together the results for all strains, we found an excellent and statistically significant correlation between fitness and robustness (Fig. 3), which is consistent with the theoretical predictions under consideration.

An imperfect correlation between robustness and thermostability. The theory of plastogenetic congruence posits that genetic robustness and thermostability will have a positive correlation (38). We tested whether this was the case by testing the thermostability of strains under selection or random drift. The wt strains under selection showed increased robustness (see above), but there was no overall change in their thermostability ($P = 0.60$ by one-sample $t$ test; $n = 3$) (Fig. 4A). In contrast, the strains under random drift showed reduced robustness (see above), yet their thermostability was increased both at $37°C$ ($P = 0.01$ by one-sample $t$ test; $n = 16$) (Fig. 4A) and at $39°C$ ($P = 0.008$ by one-sample $t$ test) (Fig. 4B). Strains under selection that had MARM U as the progenitor behaved the same as the wt under selection, so there were no significant changes in thermostability ($P = 0.075$ by one-sample $t$ test). There was a positive correlation between thermostability and robustness for strains evolved under random drift ($r = 0.58; P = 0.018; n = 16$) (Fig. 4, dotted line), yet there was no such correlation when we considered all strains of this study, irre-
spective of their origin or evolutionary history ($r = -0.05; P = 0.84; n = 21$) (Fig. 4). In aggregate, these results show that thermostability and robustness can evolve independently in VSV, yet the genetic architecture of VSV imposes a moderate link between thermostability and robustness when selection is absent.

**DISCUSSION**

We have shown that the mutational robustness of VSV can change substantially during evolution, even when robustness itself is not under selection. As theory predicts, simple selection for increased fitness leads to the evolution of increased robustness; it is likely that the virus maximizes fitness at least in part by reducing the deleterious effect of mutations. In contrast, under random drift, robustness declines significantly. This finding again supports the theoretical argument that robustness is an evolved property (16, 38, 57).

The results presented here are consistent with previous work in which we demonstrated low MRr robustness using fitness distributions (29). Here, we used sensitivity to FU to come to the same conclusion. Furthermore, MRr was one of the most sensitive strains, with only MRz and Jerry having lower values (see Fig. S2 in the supplemental material). In contrast, using fitness distributions, we were unable to find an adaptability defect in MRb, and only after a period of adaptation was there evidence of low robustness (29). However, using sensitivity to FU, we found a difference between the robustness of MRb ($1.84 \pm 0.099$) and its progenitor, MARM U ($1.55 \pm 0.035$). This result demonstrates that measuring resistance to mutagenesis is a more sensitive method than the time-consuming alternative of measuring the fitness of individual plaques to produce fitness distributions.

Interestingly, all of the strains under random drift had some loss of robustness, even those that had increased in fitness (MRa and MRy). There was no correlation between robustness and number of mutations accumulated during drift (not shown), and we identified loss of robustness in strains that differed from the MARM U progenitor in only two mutations (MRb, MRe, and MRI). This result is not surprising because of the mounting evidence of widespread epistasis throughout the genomes of RNA viruses (44, 58–61).

There was no difference between the robustness of MARM U ($1.55 \pm 0.03$) and the wt ($1.57 \pm 0.04$), but surprisingly, we found significant differences in thermostability. MARM U was significantly more thermostable than the wt ($P = 0.015$ by two-sample $t$ test; difference in means, 0.12). The fact that the difference between the two strains is a single mutation in the glycoprotein that results in a change in antibody recognition (53) raises the possibility of a correlation between antigenicity and thermostability, which we are currently exploring.

Work on RNA (38) and proteins (62) has led to the notion that mutational robustness and thermostability should be correlated. In these simple macromolecules, most mutations are deleterious because they induce unfolding or misfolding. The more thermostable an RNA molecule or protein is, the more it is protected from the destabilizing effects of mutations and, hence, the more robust. To what extent this reasoning transfers to higher biological levels, such as the fitness of an entire RNA virus, remains unclear, but there are mathematical models that connect the fitness effects of mutations in virus and bacteria with their effect on thermostability (63, 64). Results from phage phi-6 are consistent with the predictions of these models (65), while our results are not always so. Perhaps this difference in the behavior of the two systems is due to the presence of a bilipid membrane in the eukaryotic virus but not in the phage. We found a moderate correlation between thermostability and robustness in strains propagated under genetic drift. This correlation shows that the underlying genetic architecture of VSV does impose a relationship between these two quantities. However, the correlation disappeared when we considered all strains in the study, not just the ones evolved under drift. In particular, and unexpectedly, thermostability in strains subjected to drift was much higher than thermostability in either the wt or strains evolved for high fitness. It seems that in VSV, an increase in fitness leads to a decrease in thermostability, even though it tends to be accompanied by an increase in robustness. The molecular basis for this relationship remains unknown.

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