

Exponential Fitness Gains of RNA Virus Populations Are Limited by Bottleneck Effects

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Fitness is a parameter that quantitatively measures adaptation of a virus to a given environment. We have previously reported exponential fitness gains of large populations of vesicular stomatitis virus replicating in a constant environment (I. S. Novella et al., Proc. Natl. Acad. Sci. USA 92:5841–5844, 1995). In this paper, we report that during long-term passage of such large viral populations, fitness values reached a high-fitness plateau during which stochastic fitness variations were observed. This effect appears likely to be due to bottleneck effects on very high fitness populations.

RNA virus populations, even when derived from a clone, are extremely heterogeneous mixtures of closely related genomes termed quasispecies (8, 9, 13–15). With mutation rates of the order of 10^{-4} substitutions per nucleotide and round of copying, and with a genome size of about 10 kb, an average of approximately one mutation is incorporated each time a genome is copied. Such high mutation rates together with short replication times and large population sizes confer upon RNA viruses an enormous capability for adaptation and rapid evolution (reviewed in references 4 to 9 and 19). Vesicular stomatitis virus (VSV) is a nonsegmented negative-strand RNA virus that has been used as a model in studies on RNA virus population dynamics and fitness evolution (2, 10–12, 16, 17, 23–25, 27). Fitness can be defined as the overall replicative capability of the virus in a given environment, and relative fitness can be quantitated in growth competition experiments by competition with a reference virus (8, 20). We have previously described the evolution of VSV under conditions of large population passages in a constant environment. We have shown that in this case natural selection predominates, and this is reflected by rather constant exponential fitness increases (24). In this paper, we extend our studies and show that upon prolonged passage the rate of fitness increase declines and can reach a plateau. In this period of slower fitness gains, considerable variations in fitness are seen. Such variations are consistent with previous measurements (25) that indicated that very large population transfers are needed to maintain fitness of viral populations that have already reached high fitness values. Extrapolation from previous results (25) suggests that the population size used in these transfers (2×10^5 infectious units) may be near the level which favors bottleneck effects for populations with very high fitness.

We have employed BHK-21 cells and wild-type VSV (Indiana serotype, Mudd-Summers strain) in this study. Sandfly LL-5 (28) and mouse L-929 cells were also used to provide alternative biological environments during virus replication.

Methods for cell culture and virus passages have been previously described in detail (20). Passages were done by using a population transfer size of 2×10^5 infectious particles infecting about 2×10^6 cells. Larger numbers of infectious particles may result in selection of defective interfering particles (DIPs). In 24 h, the viral yields were of the order of 10^{10} infectious particles per ml in BHK-21 cells, 10^9 per ml in L-929 cells, and 10^8 per ml in LL-5 cells. Fitness assays were done with neutral MARM (monoclonal antibody-resistant mutant) U as the reference virus (20). Virus obtained after each passage was mixed with a known amount of reference virus (MARM U), and the initial ratio of wild type (wt) to MARM was determined by triplicate plaque assay in the presence and absence of monoclonal antibody I1 (MAb I1) in the agarose overlay. The use of MAb I1 in the agarose overlay and not in direct neutralization reactions is critical to avoid phenotypic mixing and hiding (21, 29). The same mixture was employed to initiate the first competition passage in BHK-21 monolayers at a multiplicity of infection of 0.1. After complete cytopathology, virus yields were recovered and used to start serial competition passages under the same conditions. Typically, for fitness values under 3.5, competitions were carried out for several passages (three to six) and wt/MARM ratios were calculated at each point by triplicate plaque assays in the presence and absence of MAb I1. For higher fitness values, competitions cannot be carried out for several passages, because wt soon overcomes MARM, and accurate determinations are difficult to make. In these cases, original mixtures are subjected to two independent determinations of the wt/MARM ratio, and the first competition passage is done in triplicate. In all cases (double determination of the original mixture and triplicate competition passage), ratios are determined by triplicate plaque assay in the presence and absence of MAb I1. Initial mixtures of high-fitness viruses were prepared at wt/MARM ratios approximately between 0.5 and 2.0 to minimize frequency-dependent selection effects. Variations in the fraction of the original wt/MARM ratio are plotted versus passage number, and the fit produces a fitness vector. The slope of the fitness vector is the fitness value. For more details on this method, see the work of Holland et al. (20).

Four series (A to D) of large-population passages in BHK-21 cells were monitored and analyzed over time (Fig. 1). Initially, fitness increased exponentially, as previously observed with other VSV populations (24). Between passages 80 and

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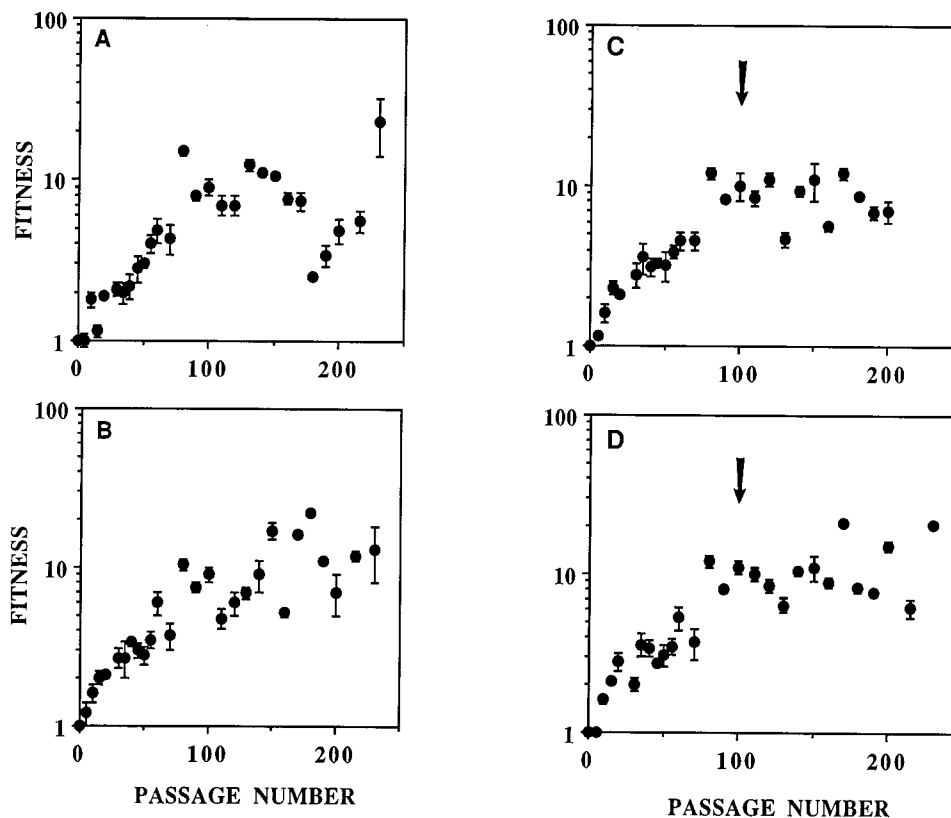


FIG. 1. Kinetics of fitness gains during viral replication in BHK-21 cells. (A to D) Results of independent series A to D, respectively. Arrows in panels C and D indicate the points at which alternative environments (mouse L-929 and sandfly LL-5 cells) were unsuccessfully used to rescue viral populations.

100, a plateau was reached; afterwards, fitness values drifted for the duration of the experiment.

To explain the results presented here, there are several possibilities that can be ruled out. The first and most obvious is that our fitness assays are not reliable and/or depend on factors other than the fitness of our virus populations. We have previously shown that fitness assays are reproducible (12). High fitness values can render variation upon repeated measurement due to sampling effects during the preparation of wt-MARM mixtures (different subpopulations are used in each measurement) (10), but this variation is not enough to explain the sudden changes of fitness reported here. In addition, determinations of fitness corresponding to the four series were not always done on the same day. When they were done on the same day, on some occasions fitness values were similar for all of the series, but on others, there were drastic differences. For instance, all determinations of passage 180 were done on the same day and the four fitness values ranged from 2.5 in series A (the lowest value since passage 40) to 22 in series B (the highest throughout the B series). Frequency-dependent selection has been reported to occur in viral quasispecies (3, 17) and theoretically could be affecting our fitness determinations. These effects have been minimized by two means: in the first place, competition mixtures in high-fitness populations (initial population fitness, >3.5) have all been prepared at similar wt/MARM ratios (approximately between 0.5 and 2.0); in the second place, only one competition passage has been carried out (although in triplicate), which limits the possibility of unexpected interactions between wt and MARM populations. It should be reminded that during competition both populations are evolving and gaining fitness (2).

Another possible factor to be considered is the presence of DIPs. We did not specifically test the presence of DIPs, although there are facts that argue against a significant role in the fitness variations reported in this article. As previously reported, our passages and assays are designed to minimize the effect of DIPs (20). DIP interference is translated into decreased viral titers and less or slower cytopathic effect. We obtained titers of our populations every 5 to 10 passages for preparation of MARM-wt mixtures, and drops in titers were never observed. Also, all passages were recovered after cytopathic effect was complete, and, again, in no instance was complete cytopathic effect delayed. These two facts indicate that DIPs did not accumulate to the level at which they could cause interference.

The results can be best interpreted in terms of walks in fitness landscapes (30) and viral population numbers required to maintain fitness values. Initially, natural selection is the main force driving evolution, as a consequence of the experimental design. Passages were initiated with a population of neutral relative fitness, with a large population size, and with massive replication and competition favored. In this scenario, the populations move uphill to nearby fitness peaks. The limitation of fitness gains once a plateau is reached might be attributed to limitations of further exploration of sequence space (15), but high mutation rates and the large population sizes reached during our experiments make genetic homogeneity extremely unlikely. Alternatively, bottleneck effects might constitute a perturbing factor for the maintenance of fitness values. Bottleneck effects are due to random drift (fixation of mutations independently of their effect on fitness) when the size of the population involved is not large enough to carry a

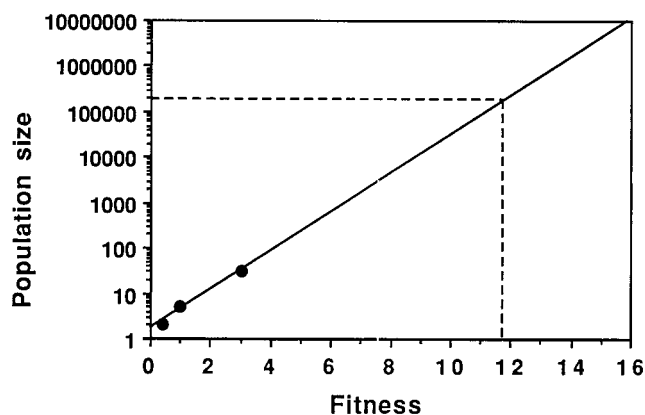


FIG. 2. Initial fitness values determine the effective size of a bottleneck regarding the maintenance of fitness values. We used published data from the work of Novella et al. (25) to obtain the plot shown. To fit our data, the best regression value ($R = 0.981$) was obtained by using an exponential fit $E = 1.675 \times 10^{0.433W}$, where E is the effective size of a bottleneck and W is the initial fitness of the population. With a population size of 2×10^5 infectious units, bottleneck effects are expected when fitness reaches values of about 12 (broken lines), and our present data fit this model. Bottleneck sizes were calculated by averaging the results from several replicas. For instance, after 20 consecutive 5-plaque-to-5-plaque passages of MARM U ($W = 1.0$), the resulting fitness values of the six replicas ranged from 0.81 to 2.03. Overall, there were no significant fitness changes, but particular populations may show an increase, a decrease, or no change in fitness. This means that in a particular large population, bottleneck effects might be observed at fitness values lower or higher than 12.

good representation of the initial population. In most cases, the consequence of this bottleneck is the operation of Muller's ratchet, which leads to overall fitness decrease (22). Muller's ratchet has been shown to be operational in small RNA virus populations (1 to 30 infectious particles per passage) by several groups (1, 12, 18, 25), but such effects were never observed in our initial neutral population passages involving 2×10^5 infectious particles. However, at the top of the peaks, relative fitness values are around 10 to 15, and bottleneck effects can be expected despite the large population size involved in each infection. This is due to the effective size of a bottleneck depending on the prior evolutionary history (i.e., initial fitness) of the population (25). In fact, we were able to estimate the fitness at which bottleneck effects are expected in a 2×10^5 population from previous work involving pools of virus from up to 30 plaques (individual virus particles), which constitute a limited effective population size regarding quasispecies complexity (25). The best fit of our data at lower fitness values predicts bottleneck effects when fitness values reach approximately 12 (Fig. 2), a value consistent with our present results.

Random drift allowed release of the population in series A from the fitness peak that was initially climbed. At passage 180, a relatively low fitness value was reached, and, from there, a second phase of exponential gains was observed (Fig. 1A). The very high fitness value reached at the last passage (passage 230) suggested that a second and higher fitness peak was climbed by the population.

An alternative way to rescue populations trapped in a fitness peak would be to change the environment and thus the fitness landscape. Genomes that are very fit in a particular environment can be deadapted in another environment and move to other areas of sequence space (13, 30). We tested this possibility by using as alternative environments either another mammalian cell line (mouse L-929 cells) or an insect cell line (sandfly LL-5 cells). We took viral populations from series C and D at passage 100 and carried out five large population passages in

the alternative cell line. After that, passages in BHK-21 were resumed and fitness changes were monitored. No drastic changes in fitness were observed after passages in L-929 or LL-5 cells, and the populations did not behave significantly different than their counterparts in a constant environment (data not shown). This result suggests that the adaptive environment of these three cell lines offers overlapping fitness peaks for our VSV populations. Other work carried out in our laboratory supports this hypothesis. We have shown that VSV populations that have been replicating exclusively in BHK-21 or LL-5 cells show increasing fitness in both cell lines (26).

The implication of the observations reported here is that when a virus population reaches high fitness values, depending on the population size involved in the infection process, an area of uncertainty regarding fitness evolution is reached. Such an indeterminacy may be also favored by the increased probability of occurrence of deleterious mutations when viruses have reached high fitness values (5). Classical models of population genetics cannot explain our findings. Theoretically, a loss of beneficial mutations by genetic drift occurs when $1 \geq sNe$, where s is the selective advantage and Ne is the effective population size. Therefore, mutations lost by drift would have a minor beneficial effect. On the other hand, high-fitness viruses that are present at such a low frequency as to be easily lost would be expected to have little or no effect on the overall fitness of the population. It may seem strange that several hundred thousand infectious particles can constitute a genetic bottleneck, but earlier work showed that even a clone of very high fitness is composed mainly of virions having lower fitness than that of the total population (10). Also, the results are fully consistent with previous quantitations (25) in that the population size used could not ensure a steady gain or even the maintenance of fitness values when those became very high. Muller's ratchet operating in viral populations can be envisioned as a process during which beneficial mutations are not necessarily lost but are voided by the continuous generation of new mutations (10). A specific deleterious mutation will not be fixed, but each high-fitness genome will produce during replication a progeny that will carry an average of one mutation per genome, most of which will be deleterious (10). Viral populations are subjected to noncanonical Darwinian evolution, as proposed by Eigen and the theory of quasispecies (13–15). The whole population, and not the individual virion, is the unit of selection (i.e., group selection), and this is not considered in current models of population genetics. Therefore, the observations reported in this article suggest that different models are needed to explain the behavior of RNA virus populations, supporting the quasispecies model of viral evolution. Alternatively, if DNA-based organisms should behave like VSV populations under similar circumstances, new models are needed to understand general evolutionary processes.

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