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Genetic Bottlenecks and Population Passages Cause Profound Fitness Differences in RNA Viruses

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Repeated clone-to-clone (genetic bottleneck) passages of an RNA phage and vesicular stomatitis virus have been shown previously to result in loss of fitness due to Muller's ratchet. We now demonstrate that Muller's ratchet also operates when genetic bottleneck passages are carried out at 37 rather than 32°C. Thus, these fitness losses do not depend on growth of temperature-sensitive (*ts*) mutants at lowered temperatures. We also demonstrate that during repeated genetic bottleneck passages, accumulation of deleterious mutations does occur in a stepwise (ratchet-like) manner as originally proposed by Muller. One selected clone which had undergone significant loss of fitness after only 20 genetic bottleneck passages was passaged again in clone-to-clone series. Additional large losses of fitness were observed in five of nine independent bottleneck series; the relative fitnesses of the other four series remained close to the starting fitness. In sharp contrast, when the same selected clone was transferred 20 more times as large populations (10^5 to 10^6 PFU transferred at each passage), significant increases in fitness were observed in all eight passage series. Finally, we selected several clones which had undergone extreme losses of fitness during 20 bottleneck passages. When these low-fitness clones were passaged many times as large virus populations, they always regained very high relative fitness. We conclude that transfer of large populations of RNA viruses regularly selects those genomes within the quasispecies population which have the highest relative fitness, whereas bottleneck transfers have a high probability of leading to loss of fitness by random isolation of genomes carrying debilitating mutations. Both phenomena arise from, and underscore, the extreme mutability and variability of RNA viruses.

There is increasing evidence that RNA virus replicases are extremely error prone, that mutation frequencies at most genome sites are very high, and that RNA virus clones quickly generate complex virus populations (quasispecies populations). Several reviews and recent articles discuss the mutability, heterogeneity, and evolution of RNA viruses (3, 4, 6, 7, 9–11, 14–17, 19, 22, 25, 27, 28, 30, 34, 41, 44–46, 49, 50, 53–55, 57). The mutability and consequent adaptability of riboviruses and retroviruses naturally focuses attention on the biological advantages of very high mutation frequencies. However, extreme base misincorporation frequencies must also generate a considerable genetic load of biologically unfavorable mutations. The rather high virion particle/PFU ratio of most riboviruses and retroviruses must be due in part to lethal mutations, and a much larger fraction of quasispecies genome populations must carry one or more deleterious mutations. Because of extreme mutation rates, high genetic loads of lethal or unfavorable mutations must inevitably be present, but they may or may not be readily detectable depending on methods of analysis and virus replication strategy. For example, highly mutated, hypermutated, and defective or partially defective genomes are observed in lethal human brain infections with measles virus (5), in chronic infections with hepatitis B virus (49), human immunodeficiency virus type 1 (HIV-1) (36), and feline leukemia virus (42), and in murine AIDS due to murine leukemia virus (8).

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Muller (38) suggested that whenever mutation rates were high, deleterious mutations would accumulate in asexual organisms by an irreversible ratchet mechanism (21, 38). Sexual reproductive processes, however, can allow recombinational repair of damaged genomes (7, 21, 29, 39, 40). Bell provided evidence for Muller's ratchet hypothesis by using senescence in nonmating populations of protozoa (2). Some models of Muller's ratchet theory have been questioned, but Chao (6) recently provided strong evidence for the operation of Muller's ratchet in the RNA bacteriophage $\phi 6$ under conditions which greatly minimize or eliminate sexual reassortment of the three segments of the tripartite phage genome. Chao showed that 40 consecutive plaque-to-plaque transfers produced a significant decrease in mean fitness. Fitness was measured by paired growth competition experiments using a genetically marked competitor phage as an internal standard. Restoration of fitness by phage segment reassortment was avoided by transfer of phage populations diluted to produce independent plaques after each passage (6). We recently demonstrated that Muller's ratchet also operates during repeated genetic bottleneck (clone-to-clone) transfers of a nonsegmented animal RNA virus, vesicular stomatitis virus (VSV) (18). Because VSV does not undergo recombination at a detectable level (23, 46), we were able to employ direct competition assays for fitness (18, 26). In these assays, a genetically marked test virus population was mixed with wild-type virus (the internal standard), and the two populations were allowed to compete directly in liquid cell cultures (and often within the same cell). Our results confirmed for an animal RNA virus the observations of Chao

(6) that Muller's ratchet operates during repeated genetic bottleneck transfers. This Muller's ratchet effect on RNA viruses might frequently occur during virus transmission in nature whenever only one or a small number of infectious particles are transmitted (from one host to another or from one cell to a distant cell in an infected individual). Genetic bottlenecks must occur frequently during natural transmission via respiratory droplets because virus concentrations are very low, and the majority of droplets are so small that most of them should contain no infectious virions, with only one or a few per droplet when they are present (1, 12, 13, 24). Regardless of the transmission mode, genetic bottlenecks should occur frequently whenever virus yields are extremely low or when infectious virions are released slowly and infrequently from infected cells. For example, transmission of HIV-1 by infectious virions might often involve only one or several infectious particles, whereas transmission by infected cells might involve numerous infectious genomes. In fact, HIV-1 sequences in newly infected individuals are highly homogeneous compared with heterogeneous populations in presumed donors; this suggests bottleneck transmission or initial selection of a restricted subset of all genomes present in donors (43, 56). In this report, we show that a cumulative ratchet-like mechanism operates during repeated genetic bottleneck transmissions of VSV, that Muller's ratchet operates at 37 as well as at 32°C, and that repeated passages of large quasispecies populations of VSV regularly select virus of high relative fitness even from clones which are greatly debilitated by genetic bottleneck-induced loss of fitness.

MATERIALS AND METHODS

Cells and virus. The BHK_{21} hamster kidney cell line and the HeLa human cervical carcinoma cell line were propagated in Eagle's minimal essential medium (MEM) containing heat-inactivated (60°C, 30 min) bovine calf serum, the entire lot of which was pretested to ensure the absence of antibody against VSV (or other inhibitors of VSV). Heat-inactivated calf serum was added at a level of 5 to 7% for cell growth, virus growth, and virus plaque assays. The BHK_{21} cells were originally obtained from American Type Culture Collection and were cloned in our laboratory. For growth of BHK_{21} cells, the MEM was supplemented with 0.06% Difco Proteose Peptone no. 3 to increase growth rates and to form optimal monolayers for plaque assays of virus. Proteose Peptone was not added to medium used for virus replication or for virus plaque assays, nor was it added to MEM used to propagate HeLa cells. HeLa cells were originally obtained from Bert Semler (University of California, Irvine). The wild-type VSV employed as an internal standard for relative fitness was the Mudd-Summers strain of VSV Indiana, which was originally obtained from Donald Summers (37) and then replicated for 2 decades in our laboratory exclusively on BHK_{21} cells at low multiplicity of infection to avoid defective interfering particle interference. It is a clonal pool stored in aliquots at −70 to −90°C. This clonal pool was prepared from a multiply cloned stock. The monoclonal antibody (MAb)-resistant (MAR mutant) VSV used as a genetically marked test virus was also prepared from our Mudd-Summers virus stocks by plaque selection under I1 MAb-containing plaque overlay medium.

Mouse hybridoma cells and MAbs. The mouse hybridoma cell line and the corresponding I1 MAb used in this study were derived and characterized by Lefrancois and Lyles (31–33), and reactive viral epitope sites were mapped by Vande Pol et al. (52). The I1 antibody-producing mouse hybridoma cells were kindly provided by Leo Lefrancois of The Upjohn Co., Kalamazoo, Mich. These hybridoma cells were propagated as loosely adherent or nonadherent cells in 950-ml glass culture bottles under Eagle's MEM supplemented with a high concentration (20%) of heat-inactivated bovine calf serum. The hybridoma cells were stored frozen at −70 to −90°C when not in use. For production of multiliter quantities of I1 MAb, the hybridoma cells were expanded during rapid growth to dozens of glass culture bottles. They were then allowed to reach maximum cell density, and the crowded nondividing or slowly dividing cells were allowed to secrete MAb for several days. Spent medium containing I1 MAb was removed and pooled, fresh MEM was added, and the cultures were incubated for several more days; this process was repeated until many liters of cell culture medium containing high MAb levels had been produced, pooled, and frozen in aliquots at −20°C.

Assays of virus relative fitness. Virus relative fitness (W) was quantitated exactly as described previously (18, 26). An I1 MAb-resistant (genetically marked) test virus was mixed with larger amounts of wild-type virus clone used as an internal standard for relative fitness. The I1 MAR mutant virus C was used as a nearly neutral test virus. It has an Asp→Ala amino acid substitution at position 259 within the I1-reactive epitope of the VSV surface glycoprotein (31, 32, 52). In some experiments (where indicated below), we used MAR mutant virus X61 which had acquired extremely high fitness (W = 2.5) in BHK_{21} cells following 61 sequential low-multiplicity passages on BHK_{21} cells (26). Mutant X61 has an Asp→Val amino acid substitution at position 257 within the I1-reactive epitope of the G protein (52).

Genetic bottleneck transfers for Muller's ratchet and large population transfers for selection. Genetic bottleneck passages of I1 MAR mutants were carried out by repetitive plaque-to-plaque transfers as described previously (18). Passages of large virus populations were carried out by repetitive low-multiplicity virus transfers as follows. Virus produced at each passage was recovered in the medium after cytopathic effect was complete (usually following overnight incubation) and then diluted 1/10,000 (or 1/1,000 where indicated) before transfer to uninfected cells for the next passage. To avoid problems due to MAR mutants reverting to the wild type, I1 MAb was added during every 5th to 10th passage and during the penultimate passage of any series. MAb was never present during the final passage because it could affect the results of the later fitness competition assays.

RESULTS

Genetic bottleneck passages at 37°C lead to Muller's ratchet fitness losses. In our initial study of Muller's ratchet effects on bottlenecked VSV, we carried out all clone-to-clone passages of virus at 32°C in order to avoid loss of temperature-sensitive (*ts*) mutants which might be present (18). It was important to learn whether lowered temperature was required for Muller's ratchet to operate during bottleneck transmissions of VSV. Figure 1 shows that Muller's ratchet operated at least as effectively when clone-to-clone passages (and all fitness assays) were performed at 37°C. A high-fitness clone of VSV (genetically marked by resistance to I1 MAb) was transferred plaque to plaque 20 consecutive times on BHK_{21} cell monolayers (Fig. 1A). The resulting passage 20 population was then mixed with wild-type (internal standard) VSV; these viruses were passaged together on BHK_{21}

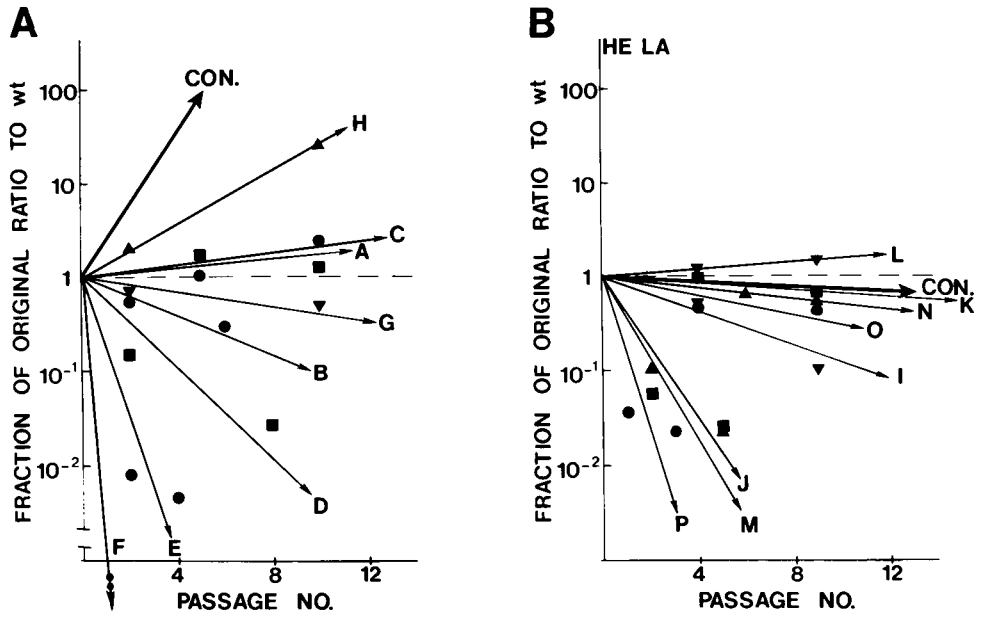

FIG. 1. Fitness vector diagrams of individual VSV clones following 20 consecutive plaque-to-plaque transfers at 37°C. The genetically marked virus (MAR mutant) was tested for relative fitness in direct replicative competition during passages of mixtures with wild-type (wt) virus. Fitness assays and plaque-to-plaque transfers were carried out as described previously (18), except that each independent series of plaque-to-plaque transfers was carried out at 37°C. All fitness assays were also performed at 37°C. The starting fitness vectors of control MAR mutant virus are indicated by thick arrows (CON). The final fitness vector of each test virus following 20 plaque-to-plaque transfers during each test passage series is shown with a thin arrow and identified with a letter (A to P). (A) Relative fitness after each series of clone-to-clone transfers (A to H) on BHK_{21} cells at 37°C, starting with a high-fitness clone which is well adapted to BHK_{21} cells after 61 consecutive transfers of large virus populations on BHK_{21} cells at 37°C. This high-fitness clone is clone X61 and was derived as described previously (26). The fitness of this clone is 2.5 (not 1.25 as mistakenly reported earlier [18]). (B) Result of experiment that was similar, except that the starting MAR clone had a fitness nearly equal to that of the wild-type virus, all plaque-to-plaque transfers were carried out at 37°C on HeLa cells, and subsequently competition passages of each passage 20 clone (I to N) mixed with wild-type virus were done at 37°C on HeLa cells.

cells at 37°C in relative fitness competition assays, and fitness vectors were plotted in Fig. 1A. These fitness vectors represent the changing ratios of test versus wild-type (internal standard) virus during direct competition of each transfer (18, 26), and the slope of each vector line provides the quantitative relative fitness values (W) of the test virus (18). It can be seen in Fig. 1A that all eight of the bottlenecked clones lost fitness after only 20 plaque-to-plaque transfers. One clone (clone F) was extremely debilitated, having undergone a loss of relative fitness exceeding 30,000-fold! This extremely unfit clone was later used to study restoration of fitness as described below. Figure 1B shows similar results obtained after a genetically marked clone with a fitness approximately equal to that of wild-type virus was passaged clone to clone 20 times at 37° on a "new" host cell line (HeLa cells). Even during adaptation to a new host, approximately half of these genetically bottlenecked clones exhibited fitness losses. We conclude that bottleneck transfers at 37°C lead to fitness losses. This agrees with the original results of Chao's transfers of an RNA phage at 37°C (6). In all subsequent studies described below, we carried out all bottleneck transfers and fitness vector assays at 37°C, and all plaques were chosen randomly (regardless of size) during plaque-to-plaque transfers.

Fitness losses during genetic bottleneck transfers proceed in a cumulative (ratchet-like) manner. Because Muller's ratchet hypothesis postulates gradual accumulation of deleterious mutations during (asexual) reproduction (38), it was important to determine whether these fitness losses can occur by stepwise accumulation of mutations or only by large, low-probability stochastic jumps in fitness. To examine this, we used a VSV clone which had already been passaged clone to clone 20 times and in which a moderate loss of relative fitness versus the wild type had resulted because of Muller's ratchet (W = 0.78). Nine aliquots of this clone were subjected to an additional 20 plaque-to-plaque bottleneck passages on BHK_{21} cells (each in independent clonal passage series). The results are presented as relative fitness vector plots in Fig. 2A. It can be seen that in five of the additional bottleneck transfer series, the already less-fit clone underwent further losses of relative fitness (series C, D, F, H, and I), while the other four (series A, B, G, and E) remained close to the fitness of the starting clone. It is clear that fitness changes can proceed in a stepwise or ratchet-like manner and that subsequent changes in fitness are not necessarily predetermined by initial changes (i.e., subsequent changes vary randomly even for an already ratcheted clone).

Transfer of large virus populations can profoundly increase fitness of bottlenecked low-fitness clones. It is well established that RNA viruses are biologically very adaptable and that they can increase fitness in changing or constant environments (9, 14, 16, 19, 27, 50, 53, 57). It might be inferred from the results of genetic bottleneck transfers that although isolation of individual RNA virus genomes frequently encounters lower-fitness representatives of complex quasispecies populations, transfer of large virus populations might do the opposite. To compare the effects of transfers of large virus populations, we used the same clones of moderate fitness (W = 0.78) which we had used for the cumulative bottleneck transfers whose results are shown in Fig. 1A.

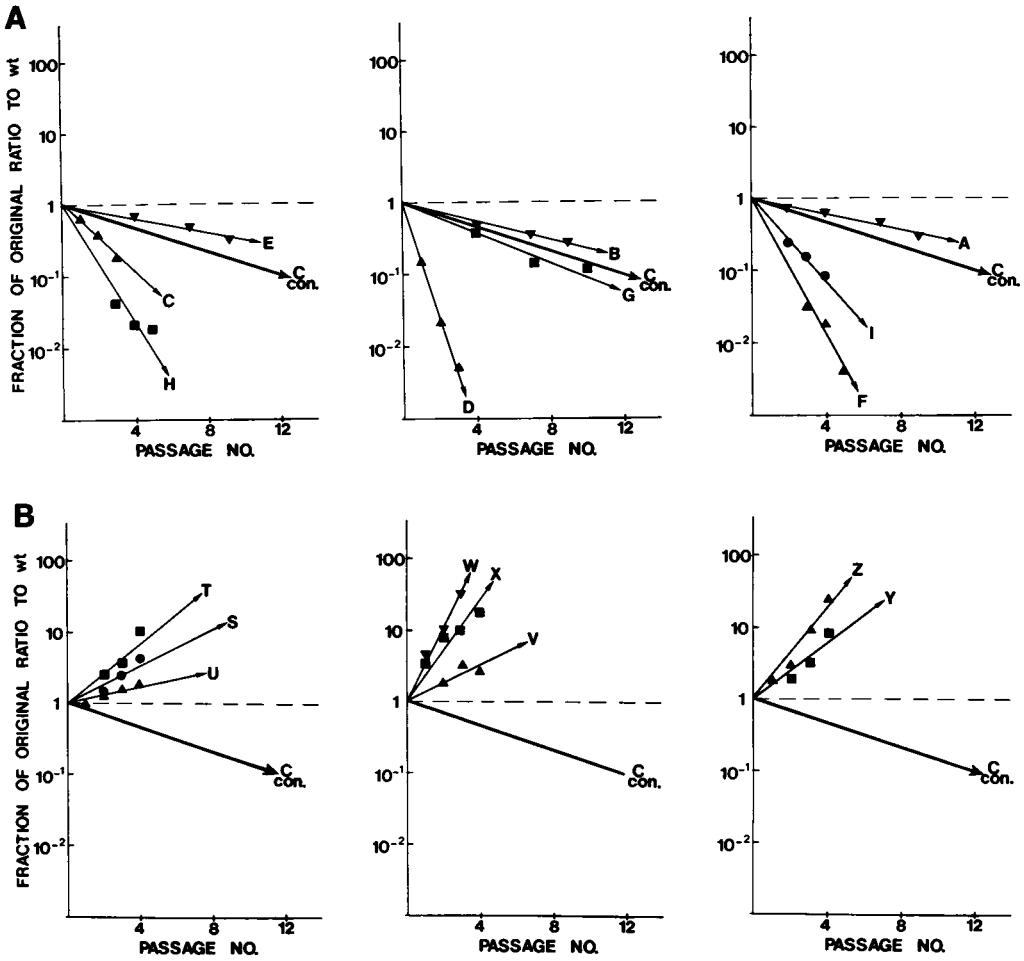

FIG. 2. Changes in virus relative fitness after a series of 20 consecutive plaque-to-plaque transfers on BHK_{21} cells of a low-fitness clone (A) contrasted with changes after the same clone was passaged in 20 independent transfer series as large virus populations (B). The starting MAR mutant clone (thick arrow) in all series was the low-fitness clone C (C con.). This low-fitness mutant was obtained after an earlier series of plaque-to-plaque transfers of a nearly neutral MAR mutant on BHK_{21} cells (see clone C vector in Fig. 1 [top left panel] in reference 18). Conditions for large-population transfers of virus are described in Materials and Methods. All transfers and fitness assays were done at 37°C on BHK_{21} cell monolayers. wt, wild type.

However, in this case (Fig. 2B), we examined the effects on fitness following 20 transfers of large virus populations. After each passage on BHK_{21} cells, the virus yield was diluted 10^4-fold so that between 10^5 and 10^6 PFU was transferred at each of the 20 passages. These diluted passages avoided problems due to defective interfering particle interference while transferring a broad spectrum of the quasispecies mutant swarm. The results shown in Fig. 2B show that all eight series of virus population passages led to large increases in relative fitness following 20 transfers. It is clear from Fig. 2 that clonal transfers and population transfers exert profoundly different effects on RNA virus fitness.

We further examined the effects of virus population transfers by starting with two bottlenecked clones of much lower fitness. The results are presented in Fig. 3. Figure 3A and B show the starting fitness vector of an extremely unfit clone obtained previously (F clone of Fig. 1A) after 20 plaque-to-plaque passages ($W < 0.0001$). We transferred this debilitated clone in seven independent large-population passage series. One series was transferred 61 times (again 10^5 to 10^6 PFU was transferred at each passage). This conferred (vector Ff pop. 61) extremely high relative fitness ($W = 2.8$; $\Delta W > 30{,}000$-fold). A smaller number of population passages (22 passages) also restored very high fitness (vectors Fa, Fb, Fc, Fd, and Fe in Fig. 3A and B). Results of a similar experiment are shown in Fig. 3C, but in this case the starting bottlenecked clone had previously been transferred plaque to plaque on HeLa cells ($W = 0.36$), and the subsequent transfers of virus populations were repeated 30 times on HeLa cells. Figure 3C demonstrates again that a virus population greatly increased its fitness ($W = 1.7$; $\Delta W = 4.7$-fold) after 30 population passages on a new cell type.

The results presented above were all subjected to statistical analysis. The slopes of control and mutant vectors were compared by means of covariance analysis (51) with the ANCOVA program of the BIOM package (47). These covariance analyses employed individual ratio values rather than the mean ratio values used for datum points in the fitness vector plots. In Fig. 1A, all eight fitness vector slopes (A to H) are very significantly below starting control (highly fit clone) fitness values. In Fig. 1B, all eight fitness slopes are significantly different from starting control (near-neutral

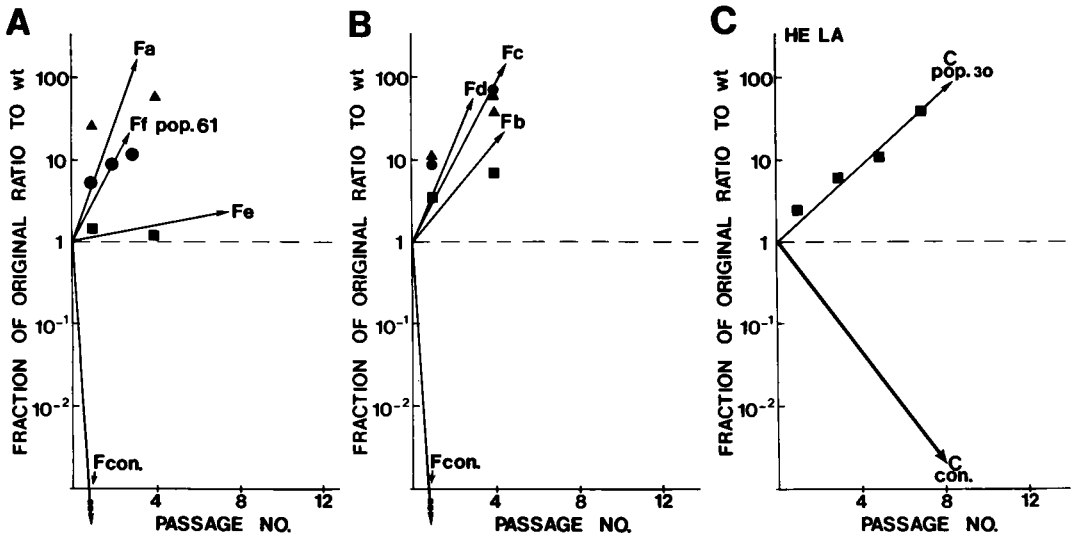

FIG. 3. Profound increases in relative fitness of clones following repetitive transfers of large virus populations at 37°C. (A and B) The starting virus clone (F con.) was the extremely low-fitness clone F (Fig. 1A, fitness vector F), which was isolated after 20 consecutive plaque-to-plaque transfers on BHK_{21} cells at 37°C. This extremely debilitated clone ($W < 0.0001$) was transferred as large virus populations (diluted 1/1,000 at each passage) as described in Materials and Methods. One series (Ff pop. 61) was transferred 61 consecutive times as a large virus population. In five other transfer series (Fa to Fe), this F clone was transferred only 22 consecutive times on BHK_{21} cells at 37°C before replicative competition fitness assays were carried out on BHK_{21} cells at 37°C. All exhibited profound increases in fitness. (C) Results of an experiment that was similar, except that the starting virus clone (C con.) was a low-fitness clone isolated after 20 repeated plaque-to-plaque transfers of a MAR mutant on HeLa cells (see clone C of Fig. 2 [bottom row] in reference 18). This debilitated clone was transferred 30 consecutive times as large virus populations on HeLa cells at 37°C. This resulted in a profound increase in relative fitness on HeLa cells (C pop. 30). wt, wild type.

clone) fitness values with the exception of clone L, the slope for which is not significantly different (at the $P = 0.05$ level) than the starting control clone slope. In Fig. 2A, the slopes for clones A, B, and I are not significantly different from the slope of the starting control (C clone). The other six clones (C to H) are significantly different. In Fig. 2B, all eight clones tested have significantly greater fitness than the starting control (C clone). In Fig. 3, all clones tested have a highly significant elevated fitness compared with the starting control (F or C) clone. We conclude that repeated genetic bottleneck transfers and population transfers usually lead to significant, but opposite, changes in virus fitness.

DISCUSSION

Our results show striking contrasts between the effects of bottleneck passages and population passages for an RNA virus and are in general agreement with population genetics theories concerning the operation of Muller's ratchet (2, 7, 21, 29, 37, 39) and the evolutionary effects of genetic bottlenecks as proposed by Mayr (35). In vitro cell culture studies such as these can only provide a model for virus behavior in natural hosts. However, we demonstrated earlier by fitness vector assays that VSV does increase relative fitness for mice during virus population passages in mice and that it loses fitness for replication in mice during many highly adaptive passages on BHK_{21} cells (26). So, although selective environments in vivo are more complex and impose different requirements for virus adaptation, the basic principles are probably similar.

The very high and very low fitness values reported here for some virus populations resulting from large-population passages or from bottleneck passages are extreme changes. In particular, the loss of relative fitness of clone F due to Muller's ratchet (Fig. 1A) was profound (over 30,000-fold). Even more remarkably, only 22 additional population passages of this debilitated F clone reproducibly restored all (or most) of the original virus fitness. In population biology, a fitness value of 1.2 represents a highly significant reproductive advantage, and a value of 0.8 represents an equally significant disadvantage. The more extreme changes described above further attest to the great mutability, heterogeneity, and adaptability of RNA viruses. Finally, the data above suggest that Muller's ratchet appears to cause more rapid declines in fitness when the starting virus population exhibits very high fitness. This was also observed in our previous study (18) of Muller's ratchet effects. In retrospect, this is not surprising because genetic bottleneck transfers of highly fit populations are less likely to isolate clones of even greater fitness. Conversely, bottleneck transfers of extremely unfit populations might be expected to isolate clones which are not greatly reduced in fitness; otherwise, they might not be capable of plaque formation. At some point, Muller's ratchet effects should cause extinction, but our plaque assays are capable of detecting only those mutants which produce visible plaques, so they are not capable of detecting extremely debilitated (or lethal) mutants.

In conclusion, our results provide further support for Chao's observation (6) that Muller's ratchet operates during bottleneck passages of RNA viruses, and we have shown that transfers of large virus populations have an equally profound but opposite effect on virus fitness. We have initiated experiments in which genetic bottleneck passages are alternated with large-population passages. Such alternate transmission patterns must occur often in nature, and it will be important to learn whether the biological adaptability associated with amplification to large populations can overwhelm the stochastic negative fitness effects of Muller's

ratchet. The fact that even severe losses of fitness can eventually be restored by population passages suggests that similar accumulation of deleterious mutations during natural transmission of animal viruses need not lead to extinction of the bottlenecked strain.

Finally, it is obvious that different passage conditions might sometimes inadvertently influence laboratory manipulation of RNA viruses. For example, repeated isolation of virus clones from plaques during the process of virus attenuation for live virus vaccines (20, 48) could involve Muller's ratchet effects, and too-extensive population passages of vaccine seed stocks might sometimes reverse attenuation. As might be expected, we have observed that only several passages of our MAR mutant stocks (or our wild-type standard stocks) can slightly alter fitness vectors.

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