Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet

(RNA virus mutation/virus populations/replicative competition of virus clones/vesicular stomatitis virus)

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ABSTRACT   Muller's ratchet is an important concept in population genetics. It predicts that when mutation rates are high and a significant proportion of mutations are deleterious, a kind of irreversible ratchet mechanism will gradually decrease the mean fitness of small populations of asexual organisms. In contrast, sexual recombination may stop or reverse this mutational ratchet by recombinational repair of genetic damage. Experimental support for Muller's ratchet has previously been obtained in protozoa and in a tripartite RNA bacteriophage. We now show clear evidence that Muller's ratchet can operate on a nonsegmented nonrecombining pathogenic RNA virus of animals and humans. We did genetic bottleneck passages (plaque-to-plaque transfers) of vesicular somatitis virus (VSV) and then quantitated relative fitness of the bottleneck clones by allowing direct replicative competition in mixed infections in cell culture. We document variable fitness drops (some severe) following only 20 plaque-to-plaque transfers of VSV. In some clones no fitness changes (or only insignificant changes) were observed. Surprisingly, the most regular and severe fitness losses occurred during virus passages on a new host cell type. These results again demonstrate the extreme genetic and biological variability of RNA virus populations. Muller's ratchet could have significant implications for variability of disease severity during virus outbreaks, since genetic bottlenecks must often occur during respiratory droplet transmissions and during spread of low-yield RNA viruses from one body site to another (as with human immunodeficiency virus). Likewise, the lower-probability generation of increased-fitness clones during repeated genetic bottleneck transfers of RNA viruses in nature might also affect disease pathogenesis in infected individuals and in host populations. Whenever genetic bottlenecks of RNA viruses occur, enhanced biological differences among viral subpopulations may result.

Muller (1) suggested that accumulation of deleterious mutations in asexual (2) organisms occurs by "a kind of irreversible ratchet mechanism;" in contrast, sexual reproduction allows repair of genetic damage by recombination (2–4). Bell (5) used senescence in protozoa to provide experimental support for Muller's ratchet hypothesis, but some models of Muller's ratchet have been questioned recently (6). Chao (7) recently reported that 40 consecutive plaque-to-plaque (genetic bottleneck) transfers of the tripartite RNA bacteriophage φ6 led to a significant decrease in mean fitness. Such clone-to-clone transfers of a virus repeatedly reduce virus population size to one infectious particle and should increase the probability for Muller's ratchet to become manifest if mutation rates are high and sexual recombination is absent. Chao (7) tested fitness of his repeatedly bottlenecked φ6 phage clones in paired-growth experiments in which each transferred clone was mixed at a known ratio with a genetically marked parental phage clone.

These mixtures were then diluted and plated on lawns of bacterial host cells to form independent plaques during each growth competition transfer. This precluded genetic reassortment of the three genome segments of this tripartite bacteriophage while allowing internally controlled comparisons of the relative replication rates of wild-type phage versus repeatedly bottlenecked clones. We have now quantitated the relative fitness of repeatedly bottlenecked clones of vesicular stomatitis virus (VSV) a single-stranded RNA virus of animals, humans, and insect vectors. VSV is the prototype model for negative-stranded animal RNA viruses (which include a large number of medically important human pathogens). Because VSV has only a single, nonsegmented genome, which does not undergo recombination at a detectable level, we are able to quantitate relative fitness directly in mixed-infection competition experiments. We can allow the parental VSV clone and the genetically marked monoclonal antibody (mAb)-resistant (MAR) clones to replicate and compete directly in liquid medium cell cultures (and even in the same cells late during each passage). We previously described quantitative relative fitness assay methods for determining virus fitness during direct replicative competition experiments (8, 9), and these are employed here.

RNA viruses generally should be ideal for testing Muller's ratchet theory because most or all of them have extremely high mutation frequencies. RNA viruses apparently lack the mechanisms of proofreading and mismatch repair that are available for high-fidelity DNA replication. Their replicase error frequencies per single base site are of the order of 10^{-4} to 10^{-5}, so that even clones are composed of complex quasispecies populations of related mutants (reviewed in refs. 10–17). A significant fraction of genome replication events produces at least one mutation, many of which must be deleterious. Thus, it might be expected that the Muller's ratchet effect might occur in a number of animal RNA viruses. We have carried out three different sets of experiments involving genetic bottleneck, plaque-to-plaque passages with VSV. Twenty plaque-to-plaque passages were carried out at 32°C to prevent loss of temperature-sensitive mutants. Then, relative fitness was tested by growth competition between marked mutant and wild-type virus. Our data show for a nonsegmented animal RNA virus the Muller's ratchet effect reported for an RNA bacteriophage by Chao (7). This could have important implications for RNA virus populations and the diseases they cause.

MATERIALS AND METHODS

Cells and Virus. BHK-21 cells and HeLa cells were grown and infected as cell monolayers under Eagle's minimum

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essential medium (MEM) containing 5% bovine calf serum (which had been heat-inactivated for 30 min at 60°C prior to addition to MEM). The BHK-21 baby hamster kidney cells were originally obtained from the American Type Culture Collection and the HeLa cell human cervical carcinoma line was originally obtained from Bert Semler (University of California, Irvine). All virus quantitation was done by plaque assays on nearly confluent BHK-21 cell monolayers in T25 flasks under MEM solidified with 0.3–0.4% agarose. Differential quantitation of genetically marked MAR mutant virus compared with total virus (MAR mutant virus plus wild-type parental virus) was done with or without mAb in the plaque overlay agarose medium, respectively (8), usually with triplicate plaque assays for each point (range, 1–6). The Mudd–Summers strain of VSV Indiana serotype was employed for all studies below. It was obtained from Donald Summers more than two decades ago and has been repeatedly replicated (and plaque-purified) only on BHK-21 cells during low-multiplicity passages (or clone-to-clone propagation) to minimize interfering and selective effects of defective interfering particles. The unmarked parental wild-type virus was from large clonal pools amplified directly from a plaque by virus transfer to BHK-21 cell monolayers.

Mouse mAb and MAR Mutant Virus. The mouse mAb employed was the I1 mAb produced and characterized by Lefrancois and Lyles (18, 19) and VandePol *et al.* (20). Leo Lefrancois (Upjohn) kindly provided the hybridoma cells that produce this mAb. We propagated these hybridoma cells in MEM containing 20% bovine calf serum in large culture vessels to produce large quantities (many liters) of high-titer neutralizing I1 mAb, which was stored frozen at −20°C until used. The MAR mutant virus employed was derived from parental wild-type VSV by plaque selection under I1 mAb-containing agarose. The neutral genetically marked MAR virus employed here was the mutant C virus clone (8). This MAR clone has an Asp → Ala amino acid substitution at position 259 of the VSV surface (G) glycoprotein (8, 20), and it is able to form normal-size plaques in 24 hr under agarose medium containing I1 mAb added at levels that are maximally neutralizing for unmarked wild-type virus and that prevent wild-type plaque formation (8). mAb was always added within plaque assay overlay medium after virus attachment and penetration were completed. This prevented inaccuracies that would have resulted from phenotypic mixing and phenotypic masking if antibody had been present before or during virus attachment and penetration (8, 21, 22). The mutant C virus is of approximately equal fitness with wild-type parental virus (8), but its relative fitness varies on different host cells as shown below. In one set of bottleneck transfer experiments, we used MAR mutant X virus, which had gained very high relative fitness following 61 consecutive passages at low multiplicity on BHK-21 cells (8). This MAR mutant X has an Asp → Val amino acid substitution at position 257 of the VSV G glycoprotein, and it, too, produces normal size plaques under agarose medium containing I1 mAb.

Genetic Bottleneck Transfers. All genetic bottleneck transfers were done with genetically marked virus (either MAR mutant C or MAR mutant X as indicated below). In each set of experiments, 20 consecutive plaque-to-plaque transfers were done at 32°C, and the resulting bottlenecked (passage 20) clones were tested for relative fitness compared with wild-type parental virus. In each set of experiments, eight well-isolated plaques were picked during the first bottleneck transfer of MAR mutant C or MAR mutant X; then these were plaque-to-plaque transferred daily (19 more times) in completely separate passage series (labeled A–H in Fig. 1). Virus was carefully recovered each day from well-isolated plaques and dispersed in MEM, and appropriate dilutions were seeded onto new monolayers to produce new isolated clones. These plaque-to-plaque transfers were continued until 20 consecu-

tive bottleneck transfers had been done for each of the eight transfer series in each set of experiments. I1 mAb was sometimes added to agarose overlays to eliminate any revertants. Great care was taken to pick virus from plaques selected randomly. Only representative-size plaques were picked, because control experiments (data not shown) demonstrated that deliberate and repeated picking of very small (or very large) plaques could sometimes lead to greatly decreased (or greatly increased) fitness of resulting passage 20 clones. After the 20th bottleneck plaque-to-plaque passage, the virus recovered from each passage series was mixed with a larger amount of parental wild-type virus and the mixture was subjected to a series of low-multiplicity replicative competition passages to quantitate relative fitness (8).

Quantitation of Relative Fitness by Fitness Vector Graphs. Quantitation of relative fitness of each 20-times-bottlenecked clone versus parental wild-type virus was performed as described (8) and as presented in Fig. 1. Each bottlenecked clone of marked virus was mixed with a known amount of the wild-type clone and the initial ratios of each were determined by triplicate plaque assays with and without I1 mAb in the agarose overlay medium (8). These virus mixtures were then used to initiate serial competition passages in the indicated cell line. After each competition passage (when viral cytopathology was complete), the resulting mixed virus yield was diluted 1:10,000 and used to initiate the next competition transfer in monolayers of the same cell line. After a number of competition passages, the ratio of MAR mutant to parental (non-bottlenecked) wild-type virus was quantitated with and without I1 mAb in the overlay medium (usually with triplicate plaque assays for each point). Then the fraction of the original MAR mutant ratio to wild-type was plotted versus the competition passage numbers to derive the fitness vector plots of Fig. 1. If the MAR mutant/wild-type ratio does not change during the competition passages, a zero slope (dotted line) would result, indicating approximately equal replicative fitness of wild-type and the MAR mutant. A positive slope indicates that the MAR mutant has greater replicative fitness than wild-type parental virus, and a negative slope indicates lower fitness (8). Relative fitness values per competitive transfer can be obtained from the slope of each vector line. However, because the starting (control) clones of each MAR mutant exhibit a positive fitness slope in each of the three experimental series, determination of fitness changes due to the 20 plaque-to-plaque bottleneck transfers requires comparison of the final MAR mutant fitness vector with the fitness vector of the starting (non-bottlenecked) MAR mutant clone. Note that the control vectors in Fig. 1 (large arrowheads) show the competitive fitness (versus wild-type parental virus) of the original marked MAR clone (prior to 20 plaque-to-plaque transfers), whereas the smaller arrowheads mark the fitness vectors for each of the final MAR clones (after 20 bottleneck passages). Thus, wild-type virus represents the unmarked internal control for each competition passage series, but the slopes for each of the bottlenecked MAR mutant clones (A–H) must be compared with the slopes of control (non-bottlenecked) MAR mutants. Therefore, the relative fitness values given in the text have been scaled as described by Chao (7) to set the mean fitness of the marked starting clones to 1 versus wild-type virus (i.e., the slope of each bottlenecked MAR clone is divided by the slope of the control starting clone to determine the mean fitness change of each clonal population following 20 bottleneck transfers).

RESULTS

Fig. 1 shows the results of three separate experiments in which genetically marked MAR mutant viruses were tested for relative fitness before (controls) and after 20 consecutive plaque-to-plaque bottleneck transfers at 32°C. The results are

Fig. 1. Fitness vector diagrams (see ref. 9) of VSV clones following 20 consecutive plaque-to-plaque transfers. The marked virus (MAR mutant) was tested for competitive fitness following 20 consecutive plaque-to-plaque genetic bottleneck passages on the indicated cell line at 32°C. The vector lines depict the average rate of change of ratios of marked test virus (in each case, eight separate transfer series, labeled A–H) versus wild-type virus during replicative competition passages on the indicated cell line. Controls (large arrowhead) show the competitive fitness versus wild-type virus of the original marked clone (prior to genetic bottleneck transfers). Fitness values per competitive transfer can be obtained from the slope of each vector line. Zero slope (dotted horizontal line) denotes fitness equal to wild-type virus. Because control starting clones exhibit a positive fitness slope in each of the three sets of experiments, the relative fitness values given in the text have been scaled as described by Chao (7) to normalize the mean fitness of the marked starting clones to 1 versus wild-type virus [i.e., the slope of each test (bottlenecked) clone was divided by the slope of the control (con.) starting clone]. Competition passages were carried out at 37°C with 10^{-4} dilutions of the virus yield from each previous transfer of the competing virus mixture; cells were incubated until virus cytopathology was complete. The initial ratios of marked (test) virus to wild-type ranged from 0.08 to 2.3. Each fitness data point represents the mean of triplicate plaque titrations counting between 100 and 350 plaques in most titrations. Similar fitness slopes were usually observed during competition transfers at 32°C (data not shown). (*Top*) The marked starting virus (9) (mutant C) with fitness $W = 1.09$ relative to wild-type virus was tested for relative fitness following 20 plaque-to-plaque passages on BHK-21 cells (the cell line on which this virus strain has been replicated for decades). (*Middle*) BHK-21 cells were the host cells, but a very high-fitness variant of VSV (mutant X, selected following 61 consecutive low-multiplicity passages on BHK-21 cells; see ref. 8) was the marked starting virus ($W = 1.25$ relative to wild type). (*Bottom*) The marked starting virus clone was mutant C, but genetic bottleneck passages were done on a new cell line (HeLa cells). This VSV strain had been replicated exclusively on BHK-21 cells for the past two decades, yet the marked starting (control) virus exhibited higher relative fitness on HeLa cells than it did on BHK cells (W relative to wild type = 1.22).
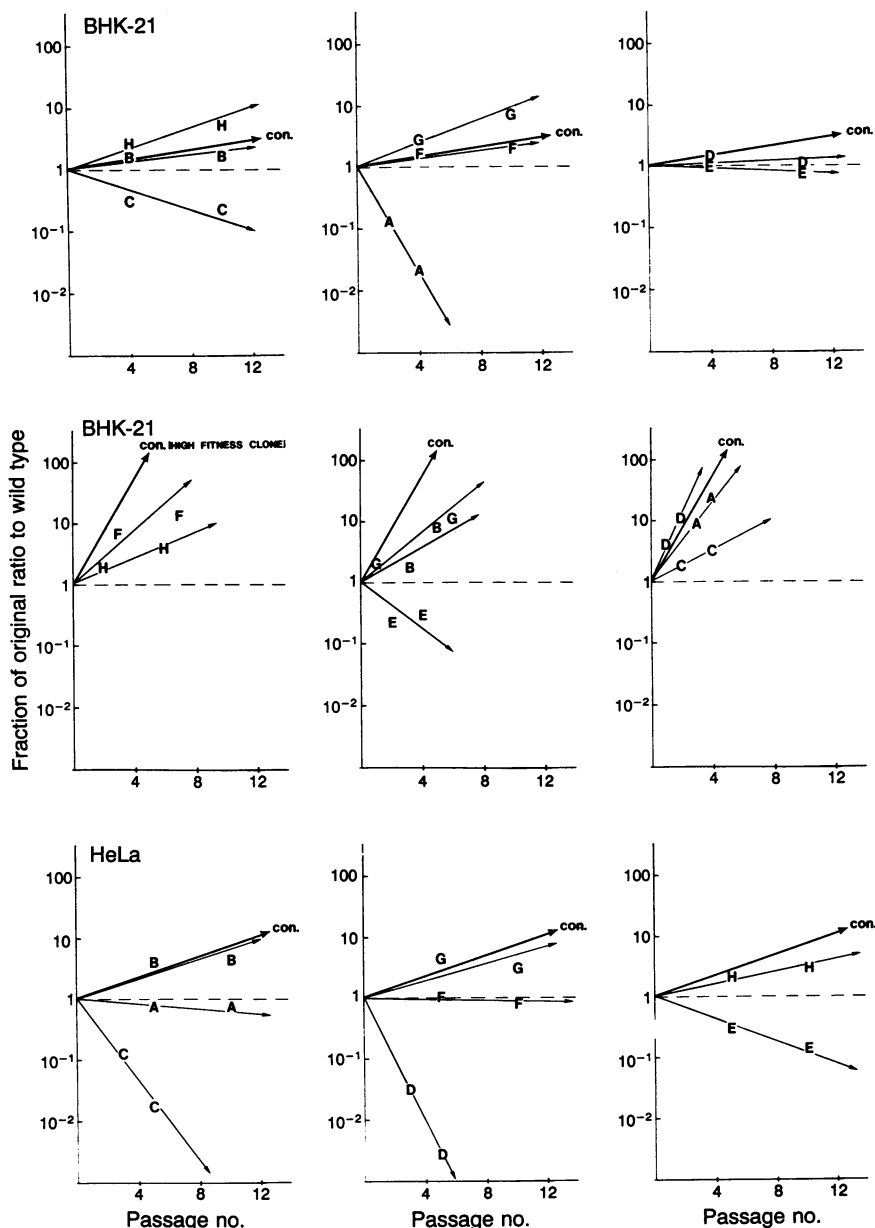
presented as fitness vector diagrams that graphically depict the exponential replicative advantage or disadvantage of marked virus compared with wild-type parental virus in direct replicative competition during low-multiplicity passages at 37°C on the indicated cell line. All of the fitness values (W) per passage given below were derived from the slope of each fitness vector line and were normalized by dividing the directly observed fitness values (versus wild-type parental virus) by the relative fitness of the starting marked virus clone (7).

Fig. 1 *Top* shows the results after 20 bottleneck passages of marked virus on BHK-21 cells (this VSV strain had been passaged for years exclusively on this cell line and is well adapted to it) (8). In this set of experiments, the starting marked clone was close to wild-type virus in fitness. Following 20 transfers, only one clone exhibited a great decrease in fitness compared with control (series A, $W = 0.63$). The series C and E clones also showed a significant fitness drop ($W = 0.78$ and 0.90, respectively) while all other clones were

not significantly different from the starting (control) clone. Two clones exhibited apparent increased fitness (series G, $W = 1.2$; series H, $W = 1.1$), but these were not reproducible (see below). Fig. 1 *Middle* shows that when a marked virus variant of very high fitness (relative to wild-type VSV) was used as the starting clone, clones from every plaque-to-plaque transfer series except D decreased significantly in fitness following 20 bottleneck passages. Values ranged from $W = 0.97$ for series A to $W = 0.53$ for series E. Finally, Fig. 1 *Bottom* shows the results obtained when the starting marked virus clone (which is well adapted to BHK-21 cells) was subjected to 20 bottleneck passages on HeLa cells, a "new" cell line. Surprisingly, clones from every transfer series exhibited either significantly decreased fitness (series A, C, D, E, and F) or no significant fitness change (series B, G, and H) relative to the starting marked VSV clone. Severe fitness decreases were observed for series D ($W = 0.25$), series C ($W = 0.36$), and series E ($W = 0.68$). Sharp decreases in mean fitness were also observed following 20 plaque-to-

plaque transfers on another "new" host cell line, the MDCK (Madin–Darby canine kidney) cell line (data not shown).

These rapid, sharp drops in mean fitness on HeLa cells were unexpected because we recently observed that BHK-adapted VSV gained significantly *increased* fitness after only 10 adaptive transfers on HeLa cells where large virus populations (>10⁴) were transferred at each passage (8). Obviously, genetic bottleneck transfers often led to rapid, severe fitness losses whereas our previous transfers of a large clonal population had led to rapid adaptive fitness gains. In all of the above experiments, care was taken to pick plaques randomly, but of representative size, since control experiments (data not shown) indicated that deliberate and repeated selection of extremely small or extremely large plaques can sometimes lead to biased fitness results. It should also be noted that the marked fitness changes we observed after 20 plaque transfers were not observed after the first plaque transfer (data not shown), so most fitness losses were probably cumulative as expected (1–5).

Because it was important to determine the reproducibility of these relative fitness assays, we repeated a number of the replicative competitions. All the data points in Fig. 2 represent averages from triplicate plaque assays in the presence and in the absence of mAb. Fig. 2 *Upper* presents the results obtained when the relative fitness competition assays of Fig. 1 *Top* were repeated in independent experiments. It can be seen that similar results were obtained. The only notable differences are that clone A exhibits an even steeper negative fitness slope than in the original fitness assay and that clones H and G exhibit neutral slopes compared with the control (rather than the slightly increased fitness slopes observed originally).

Fig. 2 *Lower* presents results obtained when the relative fitness competition assays of Fig. 1 *Bottom* were repeated. In this case, three independent competition passages were done for clones A, B, C, and D on HeLa cells (Fig. 2 *Lower*).

Similar results were observed for all four fitness assays for all four clones (compare Fig. 1 *Bottom* and all three panels of Fig. 2 *Lower*). The relative fitness assays were quite reproducible although slight variability can be observed, indicating that minor differences in fitness slopes are not significant (see below).

For statistical analysis, the slopes of mutant and control lines were compared by means of covariance analysis (23) with the program ANCOVA of the BIOM package (24). A total of 36 covariance analyses were carried out. The covariance analyses utilized individual ratio data values rather than the mean ratio values employed for the fitness vector plots. In Fig. 2, eight analyses were for the BHK-21 series (*Upper*) and twelve were for the HeLa series (*Lower*). All of the slope comparisons for Fig. 2 were statistically highly significant. For Fig. 1, eight were for the high-fitness clone/BHK-21 series (*Middle*) and all slope comparisons were highly significant, except for bottleneck mutant D, for which the very slightly increased apparent fitness is not statistically significant. Eight were for the HeLa series and all of these slope comparisons were highly significant except for bottleneck mutants B, G, and H (Fig. 1 *Bottom*). Note, however, that when mutant B was retested three more times independently with more data points in each comparison, its slope comparisons were statistically highly significant (Fig. 2 *Lower*). We conclude that following only 20 clone-to-clone bottleneck transfers, some clones have undergone significant fitness losses due to Muller's ratchet effects, while others exhibit no significant fitness alterations. In no case did we observe a significant or reproducible fitness increase.

DISCUSSION

The most remarkable findings in Fig. 1 are the wide fitness variations observed following only 20 plaque-to-plaque genetic bottleneck transfers. It should be emphasized that in all three experimental groups, the starting (control) marked
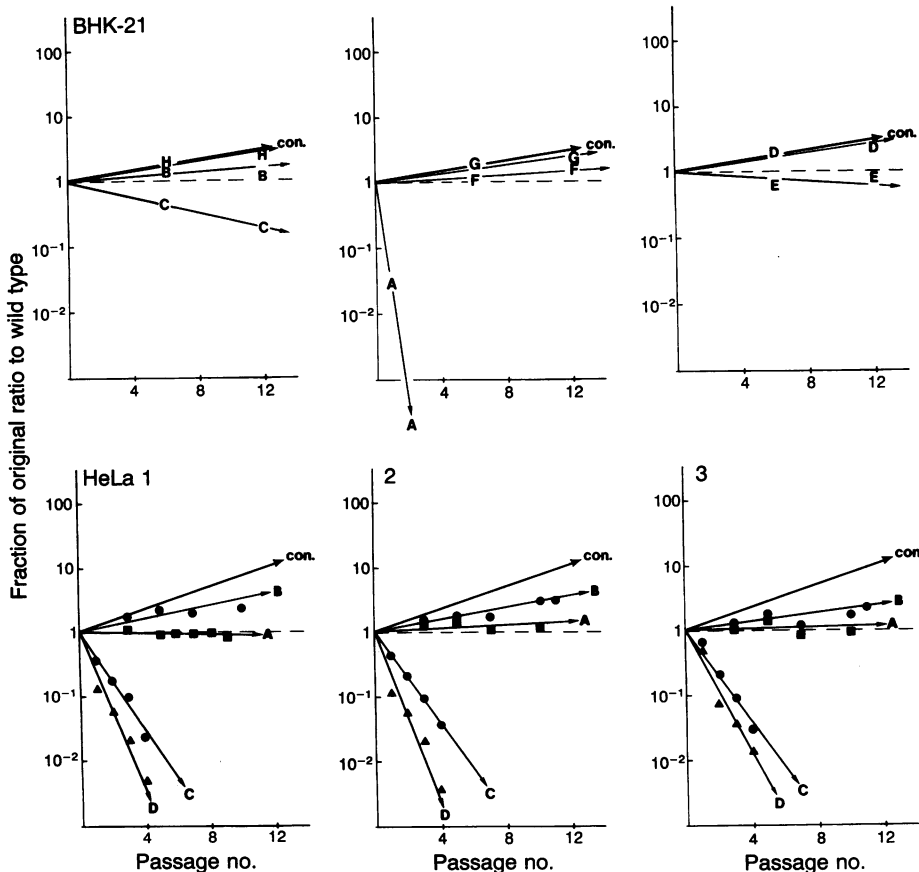

FIG. 2. Reproducibility of relative fitness competition assays. (*Upper*) Fitness vectors obtained when the clones of Fig. 1 *Top* were retested in independent relative fitness assays. (*Lower*) Fitness vectors obtained when four of the clones from Fig. 1 *Bottom* were retested in three additional independent competition assays. Experiments utilized the same (frozen) mixtures of wild-type and bottleneck-passaged marked virus that were employed for Fig. 1. All experimental conditions and procedures were as described for Fig. 1, and each data point represents average values from triplicate plaque assays.

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virus was itself a clone. These results verify the great genetic and biological variability (i.e., quasispecies, or genetic swarm nature) of clones of animal RNA viruses (10–17). Although we have not tested the possibility, the extreme variability in replicative fitness observed here suggests that repeated genetic bottleneck replication might also produce variability in disease pathogenesis and virulence *in vivo*. Despite the observed variability in fitness among bottleneck-transferred clones, the overall trend is toward mean reduction in fitness, in confirmation of the Muller's ratchet effect reported for RNA phage φ6 by Chao (7).

These observations of Muller's ratchet effects in an RNA animal virus may have important implications for the pathogenesis and natural history of viruses transmitted in nature. Bottleneck transmission by single infectious particles must occur frequently in the case of viruses transmitted by respiratory droplets. The small size of most human respiratory droplets (<1 to 10 μm), and generally low virus concentrations in these droplets dictate that average droplets must contain either no virus particles or only one or several infectious particles (25, 26). The high mutation frequencies of RNA viruses have focused attention on their great variability and adaptability (reviewed in refs. 10–17). However, Muller's ratchet may confer on highly mutable human and animal RNA viruses the ability to ratchet-down fitness (and virulence) during occasional bottleneck transmissions. This might ensure that even the most virulent ribovirus or retrovirus could immunize (rather than kill or debilitate) at least a fraction of its host population.

Again, it should be noted that we carried out only 20 bottleneck passages for each clone, and a much larger number would be likely to provide a greater opportunity for Muller's ratchet to operate.

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