

Quantitation of Relative Fitness and Great Adaptability of Clonal Populations of RNA Viruses

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We describe a sensitive, internally controlled method for comparing the genetic adaptability and relative fitness of virus populations in constant or changing host environments. Certain monoclonal antibody-resistant mutants of vesicular stomatitis virus can compete equally during serial passages in mixtures with the parental wild-type clone from which they were derived. These genetically marked “surrogate wild-type” neutral mutants, when mixed with wild-type virus, allow reliable measurement of changes in virus fitness and of virus adaptation to different host environments. Quantitative fitness vector plots demonstrate graphically that even clones of an RNA virus are composed of complex variant populations (quasispecies). Variants of greater fitness (competitive replication ability) were selected within very few passages of virus clones in new host cells or animals. Even clones which were well adapted to BHK21 cells gained further fitness during repeated passages in BHK21 cells.

There is considerable evidence that the replicases of most RNA viruses are generally quite error-prone during genome replication (3, 4, 12, 27, 56, 59–62), and this leads to a generally high mutation frequency. Some studies have suggested much lower mutation frequencies at certain sites (11, 14, 23, 46, 54, 56, 62), but high rates of virus evolution and mutation frequencies on the order of 10^{-4} to 10^{-5} base substitutions per single base site have been reported (1–13, 17–22, 24–31, 36, 37, 41–44, 48–52, 55–62, 69). For RNA virus genomes averaging approximately 10 kb in size, such replicase infidelity could produce one (or more) mutations during a high percentage of genome replications. Thus, even a recently cloned virus population might consist of a complex mixture of related virus genomes differing from each other at one or more sites. Such heterogeneous populations have been referred to as quasispecies (15). The mutation frequencies at several single base sites in vesicular stomatitis virus (VSV) and poliovirus approximate 10^{-4} and are so high that chemical mutagenesis could not increase these frequencies more than 1.1- to 2.8-fold (25). High mutation frequencies would seem to be very energy inefficient, since a considerable fraction of all mutations should be either lethal or deleterious. However, the great genetic diversity of quasispecies populations should provide considerable biological adaptability even within a clone. We demonstrate below that clones of wild-type VSV do exhibit great adaptability and a striking capacity to increase relative population fitness very rapidly (see below for definition of fitness). Martinez et al. (36) have observed similar rapid adaptability of foot-and-mouth disease virus clones. RNA viruses have long been known to have considerable adaptability (3, 4, 12, 27–31, 36–38, 51, 62), and this adaptability has been used for many decades to produce attenuated live-virus vaccines (16, 53, 63, 64). The present study and similar results of Martinez et al. (36) quantitate this adaptability and verify the quasispecies nature of RNA virus clones.

The approach we have used here involved mixing various clones of monoclonal antibody-resistant (MAR) mutants with wild-type virus; the two virus populations were then allowed to replicate in competition with each other during a series of low-multiplicity (dilute) passages in cell culture. MAR mutant/wild-type ratios were determined for the original mixture and at intervals during subsequent passages. This allowed an internally controlled method for determining the relative competitive ability (relative fitness) of each MAR mutant clone vis-à-vis wild-type virus during simultaneous replication in any cell culture or animal host. The relevant monoclonal antibody (MAB) was not present during competition passages but was used only to determine MAR mutant/wild-type genome ratios at intervals during the competition passage series. To avoid problems with phenotypic mixing and masking, the MAB was incorporated into the overlay medium added following virus attachment and penetration into plaque assay monolayers (24, 65). It is shown below that MAR mutant clones with certain amino acid substitutions in the antibody-binding epitope are less fit than wild-type virus, but MAR mutant clones with certain other amino acid substitutions are of approximately equal relative fitness. The latter mutants can be used as genetically marked “surrogate wild-type” clones for study of virus adaptability, virus population behavior, etc. They are used here to demonstrate how relative fitness (and changes in relative fitness) can be compared readily by using simple fitness vector plots. We also used vector plots to quantitate the great biological adaptability of VSV. Since all of the virus adaptability quantitated below was observed in virus clones, this is evidence that even clones of an RNA virus must consist of complex populations of related variants, in agreement with the quasispecies concepts of Eigen and Biebricher (15).

Some standard terms of population biology employed below are fitness, relative fitness, founder effect, and genetic bottleneck. For our studies of virus populations, we consider fitness to be determined by the frequency with which the (complex quasispecies) progeny of a genetically marked virus clone appear following many replicative generations (passages) in a given host environment. Relative fitness is quantitated below by the changing MAR mutant progeny/

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wild-type progeny ratios after two defined clones are mixed and allowed to compete directly during many replicative generations of a particular host environment. Founder effects refers to the founding of a clonal (but quasispecies) population from a single parental virus particle. Clonal populations by definition have passed through a genetic bottleneck during cloning (or by natural transmission of a single infectious particle).

MATERIALS AND METHODS

Cells and virus. All cells used here were grown as monolayer cell cultures in Eagle's minimal essential medium (MEM) containing heat-inactivated (60°C, 30 min) calf serum, which was tested to ensure the absence of antibody to VSV or other virus inhibitors. Calf serum was added to a final concentration of 7% for growth of all cell lines except the IC-21 mouse macrophage cell line, for which it was added to 25%. The IC-21 mouse macrophage cell line, the BHK21 hamster kidney cell line, the MDCK canine kidney cell line, and the CVI monkey kidney cell line were all originally obtained from the American Type Culture Collection. The HeLa human cervical carcinoma cell line was originally obtained from Bert Semler. BHK21 cell monolayers were used for all virus plaque assays. The wild-type Mudd-Summers strain of VSV Indiana serotype was used for all studies below. It was originally obtained from Donald Summers (39), and it has been replicated for two decades in this laboratory exclusively on BHK21 cells with either low-multiplicity passages or plaque-to-plaque clonal propagation to minimize interference by defective interfering (DI) particles. The clonal population of wild-type virus used here was a large clonal pool stored in aliquots at -70°C. This clonal pool was prepared by growth of VSV at a low multiplicity of infection (MOI) following 17 consecutive plaque-to-plaque cloning procedures on monolayers of BHK21 cells.

Mouse hybridoma cells and MAbs. The mouse hybridoma cell line and the corresponding I1 MAb used here were produced and characterized by Lefrancois and Lyles (33-35) and VandePol et al. (66). Leo Lefrancois of the Upjohn Co. kindly provided the I1 antibody-producing hybridoma cells. These cells were propagated in MEM with 20% CaS as loosely adherent or nonadherent cells in 32-oz (ca. 950 ml) culture flasks. During the replication phase, these hybridoma cells grew rapidly, with frequent expansion to new culture vessels. Finally the expanded cell population was allowed to reach maximum numbers in each flask and to secrete MAb for 2 to 3 days at 37°C under these conditions of maximal cell density and restricted replication. A pool containing many liters of this I1 MAb was prepared, characterized, and frozen in multiple aliquots for use in this study.

Assays of wild-type and MAR mutant VSV. In the virus competition studies and virus fitness vector assays described below, the plaque assays were performed exactly as described previously except that the I1 MAb and I1 MAR mutants of VSV were used instead of I3 (24, 66). As described previously, plaque assays for relative numbers of MAR mutant genomes must be carried out under conditions which neutralize wild-type virus genomes but avoid neutralization of MAR genomes that have acquired complete or partial glycoprotein coats from wild-type virus (phenotypic masking and mixing) during mixed infections. Following the competition passages described below, mixtures of wild-type and MAR mutant virus were diluted, and appropriate dilutions were plaque-assayed on BHK21 cell monolayers

without antibody to determine total virus yields. The same dilutions were plated under agarose overlay medium containing maximum neutralizing levels of I1 MAb to allow normal development of I1 MAR mutant virus plaques while suppressing the formation of visible plaques by wild-type virus (24, 65). Accurate quantitation of wild-type virus therefore requires that MAR mutant virus be at least severalfold less abundant than wild-type virus during competition passages (see below).

The wild type-plus-MAR mutant assays (24) involve adsorption of 0.1 ml of the diluted virus mixture to confluent monolayers of BHK21 cells for 15 min at room temperature, followed by 35 to 40 min of continued adsorption and penetration at 37°C (to allow phenotypically mixed or masked MAR genomes to escape extracellular neutralizing antibody). Then, 7 ml of 0.4% agarose-MEM overlay medium was added to control flasks (for total virus assays), and the same overlay medium containing maximum neutralizing levels of I1 antibody was added to matched flasks at the same dilution for MAR mutant genotype assays (24, 65). Because MAR mutants arise spontaneously from wild-type virus clones at a frequency of about 10^{-4} to 10^{-5} (24, 49, 65), it is necessary to start competition experiments at MAR mutant/wild-type ratios higher than about 10^{-4} . Finally, as will be demonstrated below, the more abundant of the two virus clones in a mixture has a higher probability of increasing its relative fitness by selection of fitter mutants arising during repeated passages. Therefore, the starting fitness of two viruses competing during passages of a mixture of each is best determined when the starting ratio is not extremely high and the number of passages during competition is kept to a minimum.

Mutational changes in the I1 MAb-resistant MAR mutants. The I1 MAb-resistant MAR mutants used here were derived by methods already described (66) following exposure of wild-type clonal pool virus to the I1 MAb (33-35, 66). The following single-amino-acid substitutions in the I1 major antigenic epitope of the VSV Indiana surface glycoprotein were inferred by RNA genome sequencing (66): MAR mutant A, Asp-257 to Gly; MAR mutant B, Asp-259 to Asn; MAR mutant C, Asp-259 to Ala; MAR mutant X, Asp-257 to Val; MAR mutant Y, Asp-257 to Tyr; MAR mutant Z, Ala-263 to Glu. Clonal pools of each of these MAR mutants were prepared by a single low-multiplicity passage on BHK21 cells of MAR mutant virus recovered directly from a well-isolated plaque of each. These first-passage clonal pools were mixed with a first-passage clonal pool of wild-type virus and allowed to compete during multiple low-MOI passages *in vitro* or *in vivo* as described in the figure legends. The starting ratios of wild-type and MAR mutant viruses in the original mixture and after serial passages of each mixture were determined as described above. The changes in ratio (fractions of original ratio) at selected passage numbers were used to plot relative fitness vectors, as shown in Fig. 1.

RESULTS

Identification of MAR mutants with fitness equal to wild-type virus for use as wild-type surrogates. We show below that certain MAR mutants of a virus can compete equally in BHK21 cells with the wild-type clone from which they were derived. This provides a genetically marked surrogate wild-type fitness clone for use in relative fitness studies. Either the wild-type or the equally fit MAR mutant can be used as a control fitness standard when the other is tested for adaptation to new host environments. Such sensitive inter-

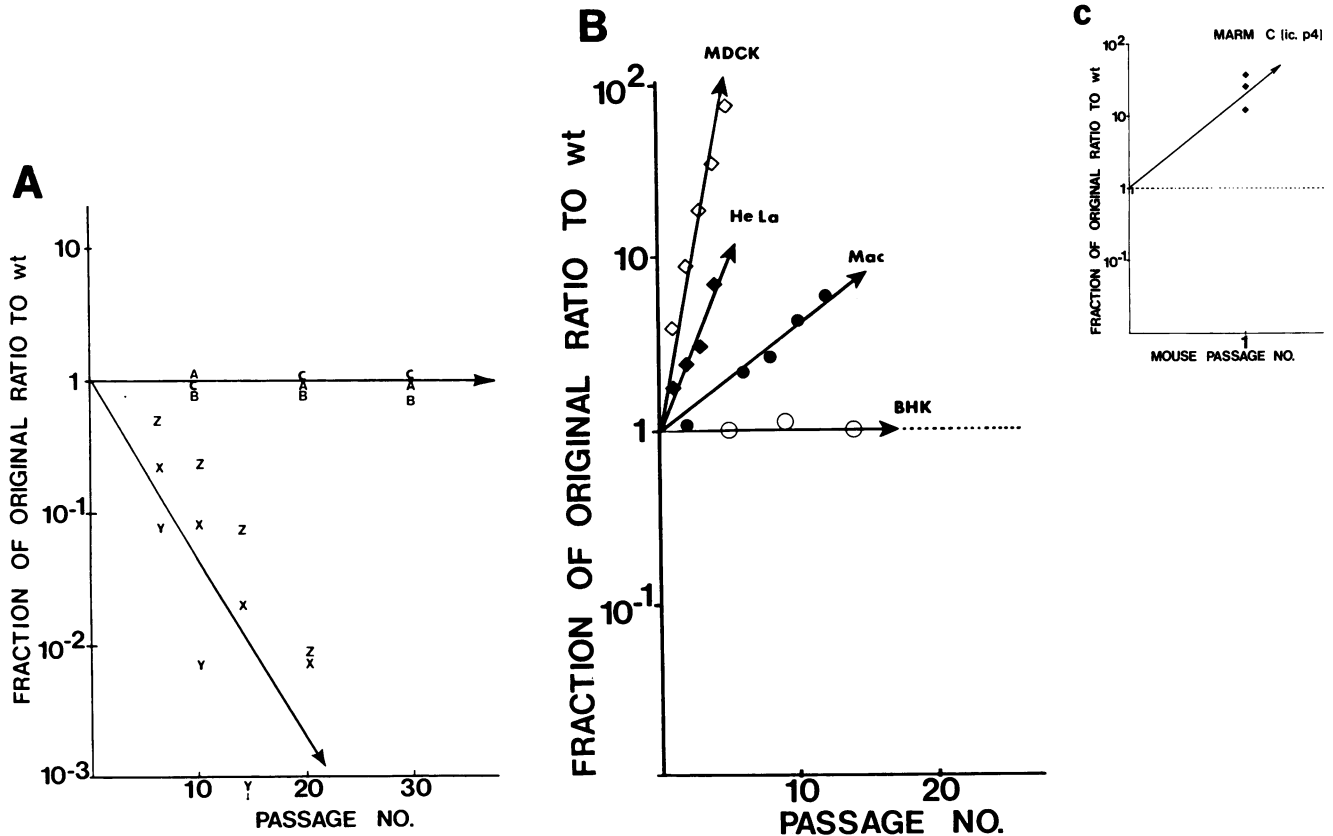


FIG. 1. Relative fitness vector plots of competition between wild-type (wt) virus and MAR mutants (MARM) during passages of mixtures of each. Vector arrows show losses, gains, or zero slope (neutral) maintenance of MAR mutant/wild-type ratios during passages in cell culture or in mice. (A) Preliminary characterization of relative fitness of six MAR mutants of VSV exhibiting single-amino-acid substitutions in the major (I1) antigenic epitope (4). See Materials and Methods for a list of the amino acid substitutions in each MAR mutant. MAR mutant A was mixed with wild-type VSV at a starting wild-type/MAR mutant ratio of 8:1; mutant B at a starting ratio of 6:1; C at 10:1; X at 7:1; Y at 5:1; and Z at 3.8:1. Each wild-type-MAR mutant mixture was then passaged in BHK21 cells, with 10⁴-fold dilution between passages. Starting ratios and subsequent ratios of wild-type and MAR mutant viruses were carefully quantitated by plaque assays with and without addition of I1 MAb to the plaque assay overlay medium (6). The fractions of subsequent ratios to starting ratios are indicated by letters at each passage level tested for each MAR mutant-wild-type virus mixture. Total virus yields at all passages averaged nearly 10⁴ PFU per cell. (B) Adaptation of the MAR mutant C clone following 10 adaptive passages in new host cell lines. The MAR C mutant (which exhibited a neutral [zero slope] vector on BHK21 cells [panel A above]) was passaged by itself 10 times on either MDCK cells, HeLa cells, a mouse macrophage (Mac) cell line (IC-21), or BHK21 cells. Following this adaptation to each cell line, each adapted MAR C mutant was mixed with wild-type virus, and relative fitness was determined as for panel A above during further competition passages in the cognate cell line. Starting wild-type/MAR C mutant ratios were 23:1 on MDCK cells, 9:1 on HeLa cells, 7:1 on the IC-21 cells, and 40:1 on BHK21 cells. (C) Relative fitness of the MAR C mutant in vivo after four intracerebral (ic) passages for neural adaptation. A 40:1 mixture of wild-type VSV and MAR C mutant clone was injected intracerebrally into three halothane-anesthetized Swiss Webster mice. After the mice died 2 days later with neural symptoms of VSV infection, the wild-type/MAR C mutant ratio in each mouse brain was determined as for panel A above (see Table 1) after homogenizing each brain in medium. Total virus yields per brain averaged nearly 10⁹ PFU, as shown in Table 1.

nal controls allow unequivocal demonstration and precise quantitation of rapid genetic adaptability of clones of VSV to new hosts. Martinez et al. have shown similar rapid adaptability of foot-and-mouth disease virus clones (36).

The I1 antigenic epitope on the surface glycoprotein of VSV Indiana constitutes a major antigenic segment at which any one of a number of different single-amino-acid substitutions can confer resistance to neutralization by the I1 MAb (66). The six MAR mutants used here were derived from a wild-type VSV clone which had been passaged and cloned for decades only on BHK21 cells in this laboratory. The single-amino-acid substitutions in the major epitope of each of these six MAR mutants are listed in Materials and Methods.

Each MAR mutant clone was tested for relative fitness by

mixing it with approximately four- to fivefold greater amounts of wild-type virus. Each mixture was then passaged repeatedly at 10,000-fold dilutions in BHK21 cells to allow competition. At intervals, the wild-type/MAR mutant ratios were compared to the original ratios by plaque assays with and without I1 MAb in the overlay medium (24). This method quantitates total virus and MAR mutant virus without inaccuracy due to phenotype mixing. Figure 1A shows the results plotted as vector diagrams for the relative fitness of MAR mutants versus the wild-type. Three mutants (MAR mutants X, Y, and Z) could not compete effectively in mixtures with wild-type virus, exhibiting negative relative fitness slopes during passages. However, three other MAR mutants (A, B, and C) maintained their approximate wild-type/MAR starting ratio for 40 passages (only 30 passages

TABLE 1. Relative fitness data for neural virus replication in three mice^a

Mouse no.	Original MAR C/wt ratio in inoculum	Virus yield (PFU)/brain at death on day 2		Yield ratio, MAR C/wt	MAR C fraction of original ratio to wt ^b
		No MAb (wt + MAR C)	I1 MAb ^c in overlay (MAR C)		
1	0.0245	1.5×10^9	5.9×10^8	0.64	26
2	0.0245	1.03×10^9	7.6×10^8	>1.0	40 ^d
3	0.0245	1.05×10^9	2.3×10^8	0.28	11

^a Raw data from Fig. 1C. Each young adult mouse was injected intracerebrally with about 0.02 ml of a mixture containing 9.8×10^4 PFU of wild-type (wt) virus and 2.4×10^3 PFU of the MAR C mutant clone which had previously been adapted to neural growth in vivo by four intracerebral passages in mice. The exact MAR C/wt ratio in the inoculum was determined by plaque assays in the presence and absence of I1 MAb (24).

^b This fraction represents the change in the MAR mutant/wt ratio during replicative competition.

^c I1 MAb was incorporated into plaque assay agarose overlay medium after virus attachment and penetration at 37°C as described before (24) to avoid phenotypic mixing and masking.

^d A MAR C fraction of 1.0 is used for calculations when MAR C/wt yield ratios exceed 1. This prevents overestimation of MAR C/wt ratios due to statistically insignificant differences in plaque counts.

are shown), exhibiting a slope of 0 in Fig. 1A. Thus, MAR mutants A, B, and C provide a genetically marked wild-type fitness standard or surrogate wild-type on BHK21 cells. Undiluted passages of wild-type and MAR mutant mixtures showed similar fitness results (not shown).

Increases in virus relative fitness on new host cell types. We next determined whether MAR mutant C could gain increased relative fitness in several "new" host cell types to which it had not previously been adapted. Figure 1B shows that MAR mutant C clonal populations very quickly gained fitness during only 10 adaptive passages in new cell types. After 10 passages by itself on MDCK cells, MAR mutant C showed a remarkable gain in relative fitness when it was mixed with larger amounts of wild-type virus and the two were allowed to compete directly during multiple passages on MDCK cells (Fig. 1B). Similar but lower-level fitness gains were also observed on the homologous cell line after 10 adaptive passages of MAR mutant C in HeLa cells or in a mouse macrophage cell line, but no fitness gain was observed after 10 passages on BHK21 cells or on CVI monkey kidney cells (data not shown). Adaptation to new host cell types was rather specific. Virus adapted to MDCK cells showed increased relative fitness only on MDCK cells and not on BHK21 or HeLa cells (data not shown). Virus adapted to HeLa cells showed no increased fitness on BHK21 cells, but did show a slight increase in relative fitness on MDCK cells (not shown). Figure 1C and the cognate data in Table 1 depict similar results in vivo. MAR C adapted to neural replication after only four intracerebral passages by itself in adult Swiss mice quickly outcompeted larger numbers of wild-type virions during a single intracerebral competition passage of a mixture of the two in three different mice. Since both wild-type virus and the MAR variants were clonal pools prepared directly from plaques, it is clear that even clones of RNA viruses quickly generate diverse populations of great genetic adaptability in changing host environments.

Analysis of founder effects and gains in fitness of mutant clones that were originally poorly fit. We next used MAR mutants X, Y, and Z, which are less fit than the wild type, to examine founder effects and virus population dynamics. We

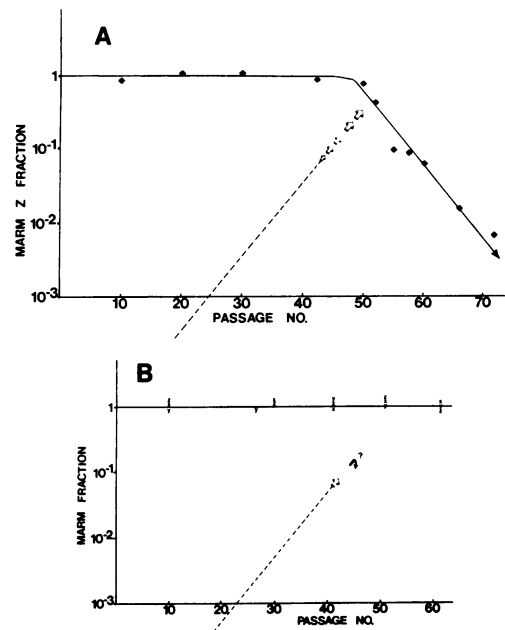


FIG. 2. Population behavior of low-fitness MAR mutant (MARM) clones during many passages in BHK21 cells. (A) Passages of the MAR mutant Z clone alone in BHK21 cells at 10^{-4} dilutions between passages. The MAR (I1 MAb-resistant) fraction of total virus remained near unity until about passage 50, when wild-type revertants began to displace the less-fit MAR Z clone. The dotted line outlines the inverse slope of the MAR Z mutant decline slope (i.e., the apparent rate of ascendancy of wild-type revertants after passage 50). (B) Passages of MAR mutant clones X, Y, and Z in BHK21 cells at 10^{-4} dilutions between passages. Wild-type virus was mixed into each MAR mutant clone at very low levels (approximately 1×10^{-4} to 2×10^{-4} fraction) before starting a passage series of each mixture. I1 MAb-resistant MAR mutants dominated the yields at every passage level tested for 85 passages (only the first 61 passages are shown). As indicated by the dotted line, the added wild-type viruses presumably increased their proportion of total virus for an unknown number of passages but never achieved dominance (due to increasing fitness of the MAR mutants during repeated passages; see Fig. 3).

passed MAR mutant Z alone on BHK21 cells (10,000-fold dilutions between passages). Figure 2A shows that spontaneously arising wild-type revertants (which should appear at a frequency of nearly 10^{-4}) did not begin to displace the MAR Z population until after approximately 50 passages. This emphasizes how founder effects (genetic bottlenecks) can allow virus clones of poor relative fitness to propagate extensively (up to $10,000^{50}$ in this example) before displacement by fitter wild-type virus. The dotted line in Fig. 2A shows the inverse slope of the MAR Z mutant displacement by revertant virus (i.e., the approximate rate of ascendancy of the revertant). This was somewhat slower than expected for an average mutation frequency of about 10^{-4} to 10^{-5} (24, 49, 65). Therefore, we repeated this experiment with independent passage series of MAR mutants X, Y, and Z in BHK21 cells, but in this experiment wild-type virus was seeded into the starting MAR mutant clones at a level of 10^{-4} to ensure its presence at at least that level during the initial passage in the series.

Figure 2B shows a rather surprising result; the added fitter wild-type virus did not replace the originally less fit MAR mutants even after 85 passages. Apparently the more popu-

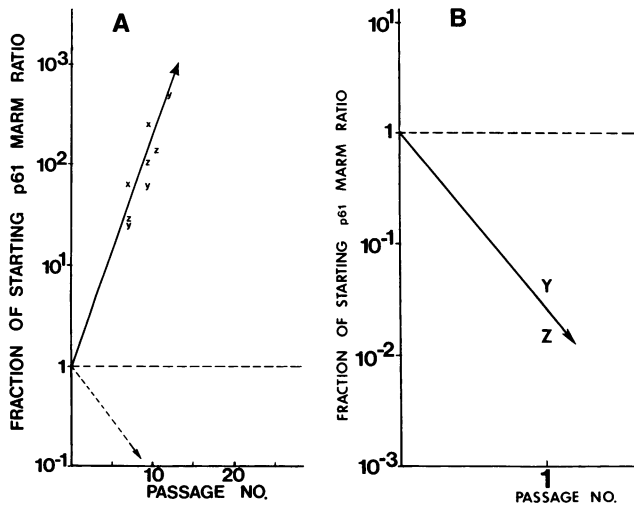


FIG. 3. Greatly increased fitness of MAR X, Y, and Z mutants on BHK21 cells but not in mice following 61 passages on BHK21 cells. (A) Clones of MAR mutants X, Y, and Z were plaque-purified from the 61st BHK21 passage of virus (see Fig. 2B). Each clone was then mixed with a larger amount of wild-type virus, and each mixture was separately passaged in BHK21 cells to determine relative fitness vectors (as for Fig. 1). Virus yields were diluted 10⁴-fold between passages. Starting wild-type/passage 61 (p61) MAR mutant ratios were 250:1 for MAR X, 500:1 for MAR Y, and 143:1 for MAR Z. The descending dotted line shows the average fitness vector of the original MAR mutants X, Y, and Z (from Fig. 1A). (B) Plaque-purified MAR mutant clones Y and Z from passage 61 in BHK21 cells (Fig. 2B) were mixed with smaller amounts of wild-type virus, and each mixture was injected intracerebrally into mice anesthetized with halothane. After the mice died with VSV neural symptoms, the wild-type/MAR mutant ratio was determined (as for Fig. 1) and compared with the starting ratio. The starting wild-type/MAR mutant Y ratio injected was 2.5:1, and the starting wild-type/MAR mutant Z ratio was 24:1. Approximately 1.5×10^2 PFU of total virus was injected into each mouse.

lous MAR mutant virus had, during all three passage series, significantly increased its relative fitness and suppressed the added wild-type virus indefinitely. As suggested by the (hypothetical) dashed-line vector arrow, the fitter wild-type virus presumably increased as a fraction of the total population for an undetermined number of passages until increased fitness of the MAR mutants prevented further increase. Figure 3A confirms that by passage 61, all three MAR mutants had greatly increased their relative fitness versus wild-type virus.

When clones of the X, Y, and Z mutants obtained from BHK21 passage 61 were mixed with larger amounts of wild-type virus, they exhibited remarkable competitive increases. Instead of the original negative fitness slope (Fig. 1A and dotted line in Fig. 3A), these passage 61 MAR mutant clones now exhibited strongly positive slopes in competition with wild-type virus (solid vector arrow, Fig. 3A). Obviously a very large number of passages on BHK21 cells selected for significant increases in fitness. It should be noted that such increases in fitness were not apparent in Fig. 1B after 14 passages on BHK21 cells or in Fig. 2A by 50 passages (at which point wild-type revertants displaced MAR Z). Virus which was already well adapted to BHK21 cells clearly could, after numerous passages in BHK21 cells, exhibit significantly increased fitness, but this did not occur quickly or reproducibly. This result, observed three times (Fig. 2B),

is apparently more probable than is displacement by very small numbers of wild-type virus, which we observed once (Fig. 2A). Increased fitness in one host cell environment does not necessarily extend to another. Figure 3B shows that this increased relative fitness of passage 61 MAR mutants Y and Z on BHK21 cells does not necessarily apply to a different host environment. Wild-type virus quickly outcompeted the two passage 61 MAR mutants during a single intracerebral passage in adult Swiss mice. The positive relative fitness of MAR mutants Y and Z on BHK21 cells (Fig. 3A) changed markedly to negative slopes in the neural selective environment (Fig. 3B).

DISCUSSION

The methods used here can allow reproducible quantitation of very small (or quite large) differences in the relative fitness of virus populations. To facilitate comparisons, all data in Fig. 1 to 3 were plotted on the same scale, and there is obviously a very wide range of relative fitness changes possible. We have not yet tried to determine whether large fitness changes most often evolve by successive incremental mutations, as seems likely. Martinez et al. (36) similarly observed rapid alterations in fitness (and antigenic changes) of foot-and-mouth disease virus. Their virus clones were recently isolated from diseased pigs and had not been well adapted to *in vitro* cell culture. The VSV clones used here had been previously well adapted to BHK21 cells, so fitness alterations in BHK21 cells were much slower (less probable) than for foot-and-mouth disease virus in its BHK21 host cell (or for VSV in new host cell types, as shown in Fig. 1). We have not yet sequenced any of the viruses exhibiting fitness alterations, because mutations in any region of the genome might affect fitness.

It is well established that RNA viruses (and many DNA viruses) are extremely adaptable. For decades vaccine developers have regularly adapted virulent viruses to "foreign" host cells to select attenuated live-virus vaccine strains (15, 53, 63, 64). It is now known that during these multiple passages for vaccine attenuation, a number of genome mutations accumulate, not all of which are involved in attenuation (6, 29, 38, 43, 45, 47, 68). Presumably, some but not all of these mutations were involved with adaptation to the host cells used during the attenuation passages. The methods used above should facilitate quantitation of such adaptive (and deadaptive) changes during attenuation of live-virus vaccine strains. They should be generally applicable for quantitating the genetic adaptability of any virus (RNA virus, DNA virus, or retrovirus) during environmental changes such as replication in different host animals, insect vectors, different host organs and tissues, acquisition of drug resistance during antiviral drug therapy, etc.

It is obvious from the data presented here that the relative fitness vector plots in Fig. 1 to 3 might have been altered by changing the passage history of the virus clones. For example, in Fig. 2, the relative fitness vector plot of the HeLa cell-adapted MAR mutant is approximately linear, as is the plot for the MAR mutants adapted to MDCK cells and mouse macrophages. Presumably, this occurred because the previous 10 adaptive passages of each MAR mutant on the cognate "new" cell line provided a strong (and rather constant) relative fitness advantage at each passage during later competition experiments with wild-type virus. This advantage could then be rather constant even though the wild-type virus was added to the original mixture at a 7:1, 9:1, or 23:1 ratio and therefore had greater numerical oppor-

tunity for adaptation to the HeLa, MDCK, or macrophage cell lines during the early passages of the competition experiments. This probably would not have been observed if, for example, the adaptive passages of MAR mutant C on HeLa cells had been carried out for only four or five passages prior to competition passages with wild-type virus. Likewise, it might not have been observed if we had started the competition experiments with a mixture of wild-type and HeLa-adapted MAR mutant at a ratio of 10,000 to 1. In that case, the much more abundant wild-type virus would itself have adapted well to HeLa cells very early during the competition experiments. Obviously, therefore, use of the relative-fitness assays described here requires attention to these parameters. We are presently starting to determine whether adaptation of VSV to new animal (and insect) host cells involves multiple stepwise adaptive mutations during an increasing number of adaptive passages. It must be expected that early adaptive changes will occur probabilistically (since they are based upon mutation probabilities) but rather dependably for RNA viruses, since their mutation frequency is high and even clones appear to be quasispecies populations.

As for any stochastic process, apparent or real changes in relative fitness must be expected to occur occasionally even when equally fit virus clones are competing in a consistent host environment. Apparent changes can occur when statistical sampling errors during dilute passages cause genetic drift. Real changes in relative fitness will appear when significant changes in relative population fitness accrue due to probabilistic distributions of mutations (as observed in Fig. 2B). Therefore, the neutral surrogate wild-type behavior of MAR mutant clones A, B, and C in competition with wild-type virus observed in Fig. 1A should not be anticipated to continue for an indefinite number of passages or to be completely reproducible in a large number of relative-fitness trials. Drift due to sampling error is minimized during dilute (10^{-4} dilution) passages of VSV in BHK21 cells (Fig. 1A) because infectious VSV yields approximately 10^{10} PFU/ml. However, even rather improbable leaps in fitness might occur at any time due to stochastic mutation events.

It should be recognized that the methods used here to quantitate relative fitness in any host environment measure total fitness differences regardless of the many cellular or molecular reasons for the fitness differences observed. For example, we carried out adaptation passages and competition passages after high dilution (i.e., at a low input MOI) to minimize DI particle interference and other direct viral interference effects. Nevertheless, DI particle interference or direct virus interference could have occurred late in each passage when cells became infected by multiple input viruses, and interference would therefore be one component affecting the overall fitness values observed. In fact, this approach should be useful for accurate quantitation of relative virus and DI particle interference effects, interferon effects, and acquisition of genetic resistance to interference in various host environments.

Finally, the availability of methods for quantitating relative fitness changes of rapidly evolving RNA viruses should allow quantitative evaluation of some of the theories of population biology regarding constant or changing fitness landscapes (32, 40, 70).

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