Evolution of Fitness in Experimental Populations of Vesicular Stomatitis Virus

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ABSTRACT

The evolution of fitness in experimental clonal populations of vesicular stomatitis virus (VSV) has been compared under different genetic (fitness of initial clone) and demographic (population dynamics) regimes. In spite of the high genetic heterogeneity among replicates within experiments, there is a clear effect of population dynamics on the evolution of fitness. Those populations that went through strong periodic bottlenecks showed a decreased fitness in competition experiments with wild type. Conversely, mutant populations that were transferred under the dynamics of continuous population expansions increased their fitness when compared with the same wild type. The magnitude of the observed effect depended on the fitness of the original viral clone. Thus, high fitness clones showed a larger reduction in fitness than low fitness clones under dynamics with included periodic bottleneck. In contrast, the gain in fitness was larger the lower the initial fitness of the viral clone. The quantitative genetic analysis of the trait "fitness" in the resulting populations shows that genetic variation for the trait is positively correlated with the magnitude of the change in the same trait. The results are interpreted in terms of the operation of MULLER's ratchet and genetic drift as opposed to the appearance of beneficial mutations.

RNA viruses are receiving increasing attention from population and evolutionary geneticists as experimental systems for studies on molecular evolution (CHAO 1990; GOJOBORI *et al.* 1990; BUONAUGURIO *et al.* 1991; FITCH *et al.* 1991; GILLESPIE 1993; MOYA *et al.* 1993). Likewise, virologists are increasingly using concepts of evolutionary genetics theory to understand the dynamics of viral populations (HOLLAND *et al.* 1982; MARICH *et al.* 1992; MARTÍNEZ *et al.* 1992; NICHOL *et al.* 1993; CLARKE *et al.* 1994).

A hallmark of the biology of RNA viruses is their error-prone replication, with mutation rates estimated at 10^{-3} to 10^{-5} substitutions per nucleotide and round of copying (for review, see STEINHAUER and HOLLAND 1987; DRAKE 1993). In addition, many RNA viruses display high frequencies of both homologous and heterologous recombination. Consequently, replicating populations of RNA viruses, even clonal populations, rapidly evolve to become complex mixtures of variants termed viral quasispecies (DOMINGO *et al.* 1978; EIGEN and SCHUSTER 1979; DOMINGO *et al.* 1985; EIGEN and BIEBRICHER 1988). Quasispecies is not only a description of the extreme heterogeneity of RNA populations but also of the complex dynamics of competition between extant and newly arising variants. Upon further diversification in different infected hosts, RNA populations may exhibit extensive genetic polymorphism (differences among average or consensus sequences of independently evolved quasispecies). The remarkable result of high mutation rates is the coexistence of multiple mutant swarms during disease outbreaks or within infected individuals (HOLLAND *et al.* 1982, 1992; DELASSUS *et al.* 1991; COFFIN 1992; WAIN-HOBSON 1992, 1994; DOMINGO and HOLLAND 1994; SCOTT *et al.* 1994).

Vesicular stomatitis virus (VSV) has been extensively analyzed at the population level regarding both its natural evolution in the field (NICHOL *et al.* 1993) and its response to environmental changes in cell culture (HOLLAND *et al.* 1982). Early work involved the recognition of rapid VSV evolution in response to defective interfering particles generated at high rates during persistent infections or virus passages at high multiplicity of infection (the number of infectious particles per cell). Biochemical and genetic methods have documented mutation frequencies of 10^{-3} to 10^{-5} substitutions per site for VSV (STEINHAUER and HOLLAND 1986, 1987; WHITE and MCGEOCH 1987; STEINHAUER *et al.* 1989; HOLLAND *et al.* 1989). VSV is suitable for studies on evolution since it infects a wide range of cells in culture and it produces large numbers of progeny in a short time (each cell may yield over 10^4 infectious particles within 7–8 hr of infection).

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A critical parameter for interpreting the dynamics of viral evolution is fitness, understood as the ability of a viral quasispecies to complete an infectious cycle and produce progeny in a defined environment. Viral fitness may be altered during massive multiplication in cells or by bottleneck transmissions involving one or very few particles. CHAO (1990) reported remarkable fitness losses upon repeated plaque-to-plaque transfers of the tripartite RNA bacteriophage $\phi 6$. This was interpreted as the result of accumulation of deleterious mutations in populations of finite effective population size, an effect first proposed by MULLER (1964). Such a steady accumulation of mutations in replicating asexual organisms, leading to a gradual loss of fitness, is known as MULLER's ratchet (HAIGH 1978; MAYNARD SMITH 1989). Stochastic fitness losses have also been documented upon plaque-to-plaque transfers of VSV (DUARTE *et al.* 1992, 1993). In contrast, repeated massive infection of cells with VSV resulted in remarkable gain of fitness (CLARKE *et al.* 1993). Bottleneck transmissions occur in nature whenever one or very few viral particles colonize a new susceptible individual or are transported to a new site within an infected organism. Variations in fitness due to such single particle transmissions may affect viral pathogenesis by influencing the spread of variants with particular properties into new organs or hosts. Thus, model systems to study the effects of environmental, demographic or genetic factors on viral fitness are needed to provide new insights into the dynamics of viral populations during disease progression. This is an area of research in which population genetics meets viral pathogenesis and it has received very limited attention. In the experimental system described in the present report, fitness changes in VSV populations were quantitated by growth competition experiments between a reference wild-type virus and each genetically marked virus population to be tested (HOLLAND *et al.* 1991). This procedure yields a fitness value for each population relative to the reference virus and allows a quantitation of fitness loss or gain by mutant viruses subjected either to massive infections or to bottleneck transmissions. Here we report a statistical treatment to evaluate the effects of fluctuations in population size on the decrease of fitness, as well as to describe the demographic conditions that would counteract the negative effect of MULLER's ratchet and/or genetic drift acting in these populations. By considering fitness as a quantitative genetic trait, we have estimated genetic parameters such as broad sense heritability and mean fitness change per generation. This allows an interpretation of the effect of population dynamics on fitness change in genetic terms.

MATERIALS AND METHODS

Biological materials: The biological materials and experimental protocols used to obtain the data analyzed in the present study have been previously described (DUARTE *et al.* 1992, 1993, 1994; CLARKE *et al.* 1993). Briefly, BHK_{21} and HeLa cells were grown as monolayers under Eagle's minimum essential medium (MEM) containing 5% bovine calf serum. Cell monolayers were infected with VSV (the Mudd-Summers strain, Indiana serotype) at the multiplicity of infection indicated for each experiment. Virus was quantitated by plaque assays using confluent BHK_{21} cell monolayers under MEM solidified with 0.4% agarose. Differential quantitation of genetically marked MARM (monoclonal antibody-resistant mutant) clones compared with total virus was done by parallel platings of the virus with and without monoclonal antibody in the agarose overlay, respectively; usually triplicate platings were carried out for each virus plaque number determination.

The mouse monoclonal antibody employed was the I_1 (I_1 Mab) characterized by VANDEPOL *et al.* (1986). MARM viruses were derived from parental wild-type VSV by plaque selection under I_1 Mab-containing agarose. Three genetically marked viruses have been used. MARM clone C is an approximately neutral variant (with fitness 1.02 ± 0.03 relative to surrogate wild type) that contains an Asp → Ala substitution at position 259 of the VSV surface glycoprotein (G); this amino acid substitution permits replication of this mutant under I_1 antibody levels that completely neutralize the Mudd-Summers wild type. The second marked virus, R, was a MULLER's ratchet-derived clone of C, obtained after several plaque-to-plaque transfers; it showed a lower fitness than the parental virus (0.872 ± 0.005 relative to surrogate wild type). The third virus studied was MARM clone X, which showed a much higher relative fitness (3.0 ± 0.2) than the wild type. In this case the I_1 antibody-resistance phenotype is conferred by an Asp → Val substitution at position 257 of the G glycoprotein, and the high relative fitness was acquired due to 61 consecutive transfers of large virus populations on BHK_{21} cells.

Experimental population dynamics: The experiments were divided into three different population dynamics that were termed *A*, *B* and *C* (Figure 1). For dynamics *A*, the MARM clone to be tested was diluted and plated on a monolayer of either BHK_{21} or HeLa cells; then eight independent passage series were started with virus from eight well isolated plaques. For each series, viruses from a plaque were picked and transferred daily plaque-to-plaque for a total of 20 passages. Dynamics *B* consisted of 20 consecutive cycles each involving reduction to a single infectious particle, growth of a clone and infections of $2 \cdot 10^6$ cells with $\sim 10^5$ plaque-forming units (p.f.u.) of virus (massive infection), followed again by dilution, plating on a new monolayer and isolation of virus from a single plaque, etc. (compare *A* and *B* in Figure 1). For dynamics *C*, $2 \cdot 10^6$ cells were infected with $\sim 10^5$ p.f.u. of virus, the virus yield was diluted and the process was repeated a total of 20 times. Infections in all dynamics were carried out at 37°. In all cases, I_1 Mab was added at passages 10 and 19 to neutralize possible wild-type revertants. For each experiment, both the fitness of the initial and passaged viruses was estimated as described below.

Relative fitness assays: The MARM virus populations were mixed with wild-type virus and the mixture was used to infect either BHK_{21} or HeLa cells (HOLLAND *et al.* 1991; DUARTE *et al.* 1992, 1993, 1994; CLARKE *et al.* 1993). Competition experiments were initiated with a 10:1 wild type to MARM clone proportion with $\sim 10^5$ p.f.u. used to infect $2 \cdot 10^6$ cells. Progeny were collected after 24 hr, diluted to 10^5 p.f.u. and used to infect a fresh cell monolayer. Parallel competitions were performed with the corresponding marked virus populations before and after being subjected to either dynamics *A*, *B* or *C*. Competitions were carried out during a sufficient number of passages to obtain good estimates of relative fitness.

TABLE 1

Summary of the covariance analysis when grouping the experiments according to experimental dynamics and initial mutant clone

Dynamics	Viral clone	Source of variation	SS	d.f.	MS	F	P	m	m_o	$\bar{w}$
A	X	Between replicates	134.55	7	19.22	19.68	<0.0001	−0.1370 ± .156	1.1098 ± .0809	0.2874 ± .0681
		Within + residual	14.65	15	0.98					
	C	Between replicates	131.74	7	18.82	126.94	<0.0001	−0.1805 ± .018	0.0046 ± .0098	0.8310 ± .0231
		Within + residual	28.32	191	0.15					
	R	Between replicates	262.93	8	32.87	490.41	<0.0001	−0.3196 ± .019	−0.1367 ± .0055	0.8329 ± .0204
		Within + residual	21.91	327	0.07					
B	X	Between replicates	105.29	4	26.32	86.54	<0.0001	0.0203 ± .042	1.1287 ± .0666	0.3301 ± .0358
		Within + residual	51.71	170	0.30					
	C	Between replicates	52.44	4	13.11	184.20	<0.0001	−0.1040 ± .017	−0.0152 ± .0102	0.9150 ± .0249
		Within + residual	11.46	161	0.07					
	R	Between replicates	37.38	4	9.34	128.75	<0.0001	−0.1072 ± .015	−0.0694 ± .0081	0.9629 ± .0222
		Within + residual	11.90	164	0.07					
C	X	Between replicates	1.73	4	0.43	8.89	<0.0001	1.9075 ± .032	1.1157 ± .0112	2.2074 ± .0954
		Within + residual	10.49	215	0.05					
	C	Between replicates	8.16	2	4.08	44.43	<0.0001	1.2499 ± .035	0.2398 ± .0280	2.7459 ± .1730
		Within + residual	9.36	102	0.09					
	R	Between replicates	36.61	7	5.23	17.91	<0.0001	0.5892 ± .027	−0.1367 ± .005	2.0666 ± .0672
		Within + residual	84.65	290	0.29					

SS, sum of squares; d.f., degrees of freedom; MS, mean square; *P*, probability for the F value; m, m_o, common slopes of the passaged and parental clones, respectively; $\bar{w}$, average relative fitness. See text for more details.

RESULTS

Statistical analysis: Table 1 shows a summary of the relevant parameters and the results of nine serial passage experiments with VSV (each involving several replicates) carried out under dynamics *A*, *B* or *C* (Figure 1). Part of the competition experiment data was originally reported in DUARTE *et al.* (1993) and CLARKE *et al.* (1993); the remaining experiments concerning mutants C and X under dynamics *C* have been carried out for this work to complete the factorial design.

To test the effect of population dynamics on the outcome of the competition experiments between control wild type and each experimental virus, analyses of covariance, using the natural logarithm of the proportion MARM to wild type as dependent variable and the competition passage number as covariate, were performed. The F test indicates that there are differences among the replicates of the same starting virus subjected to the same infection dynamics. It should be pointed out that the different viral clones are not truly replicated, so in the purest statistical sense differences between them should be taken as random differences. Despite their high heterogeneity, a common pattern can be observed in the different "replicates" of each experiment with respect to the corresponding controls. For a given virus and given experimental dynamics, the different replicates showed a similar trend in fitness change. Hence, a common slope for the different replicates of each experiment was obtained from the regression of the logarithm of MARM to wild type ratio on competition passage number. These slopes (m and m_o, for derived and original clones, respectively) are shown in Table 1 and were used as estimates of fitness ($\bar{v}_i = e^{m_i}$) relative to control wild type (assumed equal to 1) (HARTL and CLARK 1989, pp. 182–184). Then, the mean relative fitness of a passaged clone with respect to its corresponding parental is defined as $\bar{w}_i = \bar{v}_i/\bar{v}_o = e^{m_i - m_0}$. This definition of fitness differs from the one previously used by DUARTE *et al.* (1992, 1993) and CLARKE *et al.* (1993). Our current definition does not involve the approximation for the logarithm of a small figure (such as the selection coefficient) and it has a wider range of applicability. A net increase in the average relative fitness

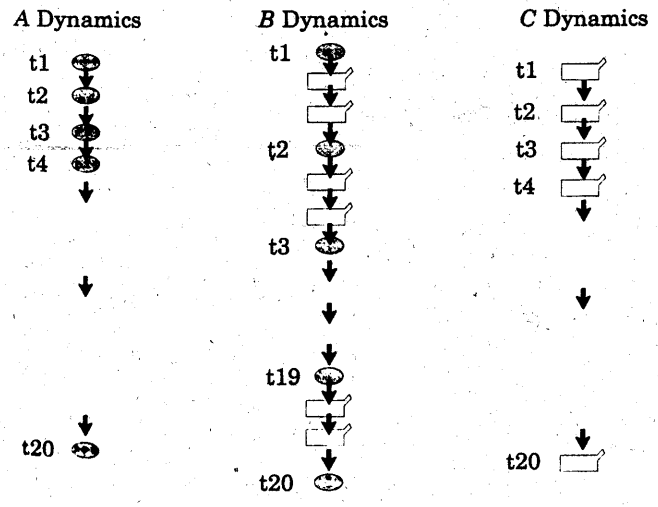

FIGURE 1.—Scheme of the three different experimental dynamics. The dark circles represent plaque passages, and bottles represent bottle passages. For further details, see MATERIALS AND METHODS.

(with respect to the control, unpassaged virus) is clearly seen in populations subjected to dynamics *C*, but not in those subjected to dynamics *A* or *B*. The results show that the type *C* dynamics, which allows a continuous competition among a large number of transferred viral genomes, can permit populations to reach fitness values higher than those attained when subjected to repeated or periodic bottleneck events.

The decrease in fitness under dynamics *A* and *B* is more evident with the initially high fitness clone X than with the neutral clone C or the low fitness clone R. X loses almost 72% of the initial relative fitness under dynamics *A* and ~67% under dynamics *B*. Also, under dynamics *C* the fitness gain of X is lower than the gain observed for the initially less fit clone C. Clones C and R did not show any significant decrease in fitness under dynamics involving bottlenecks but showed fitness gain under dynamics *C*. These results indicate that type *C* dynamics may permit populations to reach fitness values higher than those attained by populations subjected to repeated or periodic bottleneck events.

Expected distribution of deleterious mutations: We have applied HAIGH's (1978) model for the expected distribution of deleterious mutations in a haploid asexual population to the evolution of VSV under dynamics *A*, *B* and *C*. During replication, mutations are assumed to occur on the viral genome following a Poisson distribution with parameter μL. For VSV, $\mu = 2.5 \cdot 10^{-4}$, and $L = 11{,}200$, in which μ is the average mutation rate per nucleotide and L is the genome length (DRAKE 1993); thus, the mean number of mutations per genome will be $\mu L = 2.8$. The rate of accumulation of deleterious mutations will be given by $\theta = \mu L/s$, where s is the average selection coefficient of the generated mutants. The least mutated genome class (considered to be the one with the highest fitness in the mutant distribution) has an equilibrium population size of $\hat{n}_0 = N \cdot e^{-\theta}$, where N is the population size. When $\hat{n}_0 \gg 1$, deleterious mutations will accumulate very slowly and MULLER's ratchet will not operate (HAIGH 1978). HAIGH's model assumes constant population sizes. Hence, as it is customary in population genetics when fluctuating population sizes have to be considered as a single figure, the corresponding population size N was calculated from

$$\frac{1}{N} = 1 - \left[\prod_{i=0}^{t-1}\left(1 - \frac{1}{N_i}\right)\right]^{1/t} \quad \text{(WRIGHT 1938)},$$

where t is the number of generations (here estimated as the net number of genome doublings) needed to amplify the population from the initial number of genomes to the total number at the end of the passage series, and N_i is the population size at replication round i (Table 2).

Another parameter derived from HAIGH's model is the equilibrium mean fitness, given by $e^{-\mu L}$, which in the case of VSV has an expected value of 0.061. The experimental results indicate that none of the VSV populations studied are near such equilibrium (Table 1, last column). Away from equilibrium, the operation of MULLER's ratchet can be equated with loss of the less mutated genome class. Then, the second less mutated class becomes the fittest in the population and the ratchet has "clicked round one notch" (MULLER 1964). The process is then repeated concomitantly with an increase in mutational load and decrease in fitness. The number of generations needed to complete one such step in the operation of MULLER's ratchet was calculated by STEPHEN *et al.* (1993) using a diffusion model. This number is given by

$$T = \frac{1}{s} \ln \frac{\theta}{1.6} + 3.2Ne^{-\theta}.$$

In these experiments, we have estimated that clones under dynamics *A* and *B* have undergone ~480 and 1140 genomic doublings, respectively. As seen in Table 2, for dynamics *A* and *B*, T is lower than these values. This implies that all clones under dynamics *A* and *B* have had a large chance of losing the less mutated class, some of them (especially X) even several times. The values of s obtained for all clones under dynamics *C* imply that MULLER's ratchet is not operating under this kind of dynamics. Consequently, no inferences can be made either on the size of the less mutated class or on the number of generations needed to lose it.

Quantitative genetics: The expected mean square between replicates σ_T^2 estimated in the analysis of covariance (Table 1) can be partitioned in two components, σ_d^2 and $k_0\sigma_G^2$, where σ_d^2 is estimated by the error term (within + residual), k_0 is the average number of points used in the regressions (SOKAL and ROHLF 1981) and σ_G^2 is the variance of fitness. Accordingly, broad sense heritability, $H^2 := \sigma_G^2/(\sigma_G^2 + \sigma_d^2)$, and rate of fitness change per passage, $\delta\bar{w} = \Delta\bar{w}/\Delta t \cdot \bar{w}$, have been estimated and are shown in Table 3.

For all clones, estimates of genetic variation, both as σ_G^2 and H^2, decrease as the number of massive infections in the corresponding dynamics increases. This reflects the higher probability of appearance of deleterious mutations and their wider scope. In parallel, there is a lower chance of appearance of favorable mutations under dynamics *C*, as shown by the higher homogeneity of the replicates. It is also apparent that the initially high fitness clone X accumulates a wider spectrum of deleterious mutations than the neutral and low fitness clones, and that it can benefit from a rather small number of favorable mutations.

The rate of evolution of average fitness also supports the same pattern. The rate of change per generation is higher when MULLER's ratchet and strong genetic drift are at work: more deleterious mutations accumulate and less mutated classes are being lost. However, the

TABLE 2

Theoretical predictions following STEPHAN *et al.* (1993) model

Dynamic	Viral clone	N	s	θ	$\hat{n}_0$	T
A	X	7.5	0.7126	3.9293	0.1474	1.7
	C		0.1690	16.5680	0	13.8
	R		0.2671	10.4830	0.0002	7.0
B	X	22.0	0.6699	4.1797	0.3362	2.5
	C		0.0850	32.9412	0	35.6
	R		0.0371	75.4717	0	103.9
C	X	750044.8	−1.2074	−2.3190	—	—
	C		−1.7459	−1.6038	—	—
	R		−.7484	−3.7413	—	—

N is the population size, s is the coefficient of selection (defined as $1 - \bar{w}$), θ is $\mu L/s$ (see RESULTS), $\hat{n}_0$ is the size at equilibrium of the less mutated class and T is the mean time (in generations) required to eliminate the less mutated class from the population.

rate of change under favorable conditions is much lower, as only occasionally a single more favorable mutation will appear in the population, where it will have a chance of increasing in frequency under the nonsize-restricted regime of dynamics *C*. Also, clone X loses fitness at a higher rate than either C or R, but its gain under dynamics *C* is one order of magnitude lower than the gain experienced by clones C or R.

DISCUSSION

CHARLESWORTH *et al.* (1993) proposed that to understand the decline in mean fitness in finite populations it is important to separate two contributing processes. The first one is the fixation by drift of deleterious alleles at individual loci subject to irreversible mutation. The second is the operation of MULLER's ratchet *per se, i.e.*, the steady increase in the frequencies of genomes carrying large numbers of mutant alleles as a result of the stochastic loss of the least-loaded class of genomes. This can in theory occur without any noticeable concomitant fixation of deleterious alleles at individual loci (HAIGH 1978). However, FELSENSTEIN (1974) stressed the similarity between the fixation and ratchet processes, emphasizing that both depend on linkage disequilibrium and that the mutational load and the rate of complete fixation of deleterious mutations should increase for the same reason in the absence of recombination. In addition, CHARLESWORTH *et al.* (1993) noticed that high mutation rates (as in the present case) cause the ratchet to be the dominant process, at least in situations where it can operate at all.

MAYNARD SMITH and NEE (1991) proposed that genetic drift acting on a finite population has two distinct effects: chance elimination of the fittest individuals (with an important component due to MULLER's ratchet) and loss of the optimal genotype at equilibrium, *i.e.*, meltdown of sequence information by violation of the error threshold and entry in error catastrophe (EIGEN and SCHUSTER 1979). We have found evidence for the action of MULLER's ratchet, but we can make no assertion on the error threshold of these populations because any viral particle beyond the error threshold will not be able to form a visible plaque and hence will not be detected under our experimental conditions. The evidence for the lack of equilibrium according to HAIGH's model can be found in the larger average fitness of these populations than predicted under this model. MELZER and KOESLAG (1991) provided a set of demographic conditions under which equilibrium could be reached. Only under assumptions of populations experiencing large fluctuations in size (their model 4) does the average fitness of the population remain larger than the one predicted for equilibrium. They suggested that this could be due to the disguising effect that a few slightly beneficial mutations can have on the general background of deleterious and slightly deleterious mutations that accumulate under MULLER's ratchet.

TABLE 3

Quantitative genetic parameters estimated for each experimental line

Dynamics	Viral clone	k_0	σ^2_G	H^2	$\delta\bar{w}$
A	X	3.7	4.9600	0.8350	−0.1239
	C	25.9	0.7219	0.8280	−0.0102
	R	38.3	0.8566	0.9245	−0.0100
B	X	36	0.7228	0.7067	−0.1015
	C	34.1	0.3749	0.8427	−0.0046
	R	34.8	0.2421	0.7757	−0.0019
C	X	36	0.0086	0.1462	0.0274
	C	36	0.1108	0.5519	0.0318
	R	38.1	0.1293	0.3084	0.0258

k_0 represents the average number of points used in the estimation of fitness for each clone, σ^2_G represents the genetic component of variation, H^2 is the broad sense heritability of fitness, and $\delta\bar{w}$ represents the per generation rate of change in fitness.

In the experiments with VSV, both MULLER's ratchet and accumulation of mutations due to genetic drift act jointly, and their relative contributions to the decline in fitness depend on the specific population dynamics. In all cases, the mutation rate is very high and, in consequence, MULLER's ratchet is expected to be an important contribution, although the extreme reduction in population size during plaque-to-plaque passages implies an important role of drift. According to MAYNARD SMITH and NEE (1991), MULLER's ratchet *per se* is expected to act only in massive infection dynamics. However, no such effect has been observed in these experiments, hence providing evidence that MULLER's ratchet will operate only in sufficiently small populations. When plaque passages are included in the population dynamics, the ratchet effect is enhanced by genetic drift, which will render more likely the loss of the least mutated class. This will be accompanied by a concomitant reduction in fitness.

On the contrary, under dynamics *C* in which populations did not undergo bottlenecking, there is ample opportunity for beneficial mutations to appear. Once one such mutation has appeared, it will increase its frequency thus rising the average fitness of the population and counteracting MULLER's ratchet effects. As mentioned above, different initial fitness clones have different degrees of accumulation of beneficial and deleterious mutations, which explains the differences observed among clones X, C and R. Nevertheless, it is necessary to acknowledge that, given our experimental design, the use of the same clones under three different demographic regimes does not represent true replicates in the strict statistical sense. In consequence, differences among clones under the same demographic conditions cannot be attributed directly and exclusively to differences in prior fitness, as they might well be due to random differences among the clones. However, in our discussion, the relevant conclusions are based first on the differences among the results obtained for each clone under the different dynamics, and second on the consistency of our interpretations with the theoretical and empirical results obtained, for which the information on the prior history of each clone is actually taken into account.

The genetic variability of viral fitness, as measured from its broad sense heritability in an experimental design with different population dynamics, is not independent of the initial fitness of the population nor of the population dynamics. An initially high fitness clone, X in these experiments, loses fitness more rapidly and in a higher proportion than both clones with low or neutral fitness, R and C, respectively, when the experimental dynamics involves extreme population bottlenecks. This genetic drift phase enhances MULLER's ratchet, the effects of which are more pronounced on the high fitness clone. This is understandable, as this clone can accumulate more and more easily deleterious and slightly deleterious variants than the originally lower fitness ones. Not only does average fitness of X drop proportionally more than for clones C and R, but also its genetic variation is larger than for the two other clones under dynamics with intense bottlenecks. For the other dynamics, without an intense drift phase, the opposite is true. Few advantageous mutations can improve an already high fitness clone, but large genetically diverse populations derived initially from low fitness clones can quickly improve fitness, because a large proportion of mutations can confer a selective advantage to the virus.

In an adaptive landscape setting, clone X will initially be in a high fitness peak, whereas C and R would occupy lower peaks or may even be in adaptive valleys. If the population dynamics allowed the downward movement in the adaptive landscape, as when genetic drift (and hence MULLER's ratchet) is operating in bottlenecked dynamics, clones C and R decrease proportionally less in their fitness than clone X. If population dynamics does not involve genetic drift, MULLER's ratchet cannot be turned on, and the main evolutionary regime is governed by selection. In this scenario, only upward movements in the adaptive landscape are allowed. The chances for improvement of an already fit clone are lower than for C and R. This is reflected both in the lower relative gain in fitness of clone X, and in the lower genetic variation associated with it.

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LITERATURE CITED

BUONAUGURIO, D. A., S. NAKADA, J. O. PARVIN, M. KRYSTAL, P. PALESE *et al.*, 1986 Evolution of human influenza A virus over 50 years: rapid, uniform rate of change in NS gene. Science **232:** 980–982.

CHAO, L., 1990 Fitness of RNA virus decreased by Muller's ratchet. Nature **348:** 454–455.

CHARLESWORTH, D., M. T. MORGAN and B. CHARLESWORTH, 1993 Mutation accumulation in finite inbreeding and outbreeding populations. Genet. Res. **61:** 39–56.

CLARKE, D. K., E. A. DUARTE, S. F. ELENA, A. MOYA, E. DOMINGO *et al.*, 1993 Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses. J. Virol. **67:** 222–228.

CLARKE, D. K., E. A. DUARTE, S. F. ELENA, A. MOYA, E. DOMINGO *et al.*, 1994 The red queen reigns in the kingdom of RNA viruses. Proc. Natl. Acad. Sci. USA **91:** 4821–4824.

COFFIN, J. H., 1992 Genetic diversity and evolution of retrovirus. Curr. Topics Microb. Immunol. **176:** 143–164.

DELASSUS, S., R. CHEYNIER and S. WAIN-HOBSON, 1991 Evolution of human immunodeficiency virus type 1 nef and long repeat

terminal sequences over 4 years *in vivo* and *in vitro*. J. Virol. **65:** 225–231.

DOMINGO, E., and J. J. HOLLAND, 1994 Mutation rates and rapid evolution of RNA viruses, pp. 161–184 in *The Evolutionary Biology of Viruses*, edited by S. S. MORSE. Raven Press, New York.

DOMINGO, E., D. SABO, T. TANIGUCHI and C. WEISSMANN, 1978 Nucleotide sequence heterogeneity of an RNA phage population. Cell **13:** 735–744.

DOMINGO, E., E. MARTÍNEZ-SALAS, F. SOBRINO, J. C. DE LA TORRE, A. PORTELA *et al.*, 1985 The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance—a review. Gene **40:** 1–8.

DRAKE, J. W., 1993 Rates of spontaneous mutation among RNA viruses. Proc. Natl. Acad. Sci. USA **90:** 4171–4175.

DUARTE, E. A., D. K. CLARKE, A. MOYA, E. DOMINGO and J. J. HOLLAND, 1992 Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. Proc. Natl. Acad. Sci. USA **89:** 6015–6019.

DUARTE, E. A., D. K. CLARKE, S. F. ELENA, A. MOYA, E. DOMINGO *et al.*, 1993 Many-trillionfold amplification of single RNA virus particles fails to overcome the Muller's ratchet effect. J. Virol. **67:** 3620–3623.

DUARTE, E., I. S. NOVELLA, S. LEDESMA, D. K. CLARKE, A. MOYA *et al.*, 1994 Subclonal components of consensus fitness in an RNA virus clone. J. Virol. **68:** 1325–1330.

EIGEN, M., and C. BIEBRICHER, 1988 Sequence space and quasispecies distribution, pp. 211–245 in *RNA Genetics*, edited by E. DOMINGO, J. J. HOLLAND and P. AHLQUIST. CRC Press, Boca Raton, FL.

EIGEN, M., and P. SCHUSTER, 1979 *The Hypercycle. A Principle of Natural Self-organization*. Springer-Verlag, Berlin.

FELSENSTEIN, J., 1974 The evolutionary advantage of recombination. Genetics **78:** 737–756.

FITCH, W. M., J. M. E. LEITER, X.-Q. LI and P. PALESE, 1991 Positive Darwinian evolution in human influenza A viruses. Proc. Natl. Acad. Sci. USA **88:** 4270–4274.

GILLESPIE, J. H., 1993 Episodic evolution of RNA viruses. Proc. Natl. Acad. Sci. USA **90:** 10411–10412.

GOJOBORI, E., E. N. MARUYAMA and M. KIMURA, 1990 Molecular clock of viral evolution and the neutral theory. Proc. Natl. Acad. Sci. USA **87:** 10015–10018.

HAIGH, J., 1978 The accumulation of deleterious genes in a population: Muller's ratchet. Theor. Pop. Biol. **14:** 251–267.

HARTL, D. L., and A. G. CLARK, 1988 *Principles of Population Genetics*, Ed. 2. Sinauer Associates, Sunderland, MA.

HOLLAND, J. J., K. SPINDLER, F. HORODYSKI, E. GRABAU, S. NICHOL *et al.*, 1982 Rapid evolution of RNA genomes. Science **215:** 1577–1585.

HOLLAND, J. J., J. C. DE LA TORRE, D. A. STEINHAUER, D. CLARKE, E. DUARTE *et al.*, 1989 Virus mutation frequencies can be greatly underestimated by monoclonal antibody neutralization of virions. J. Virol. **63:** 5030–5036.

HOLLAND, J. J., J. C. DE LA TORRE, D. K. CLARKE and E. A. DUARTE, 1991 Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. J. Virol. **65:** 2960–2967.

HOLLAND, J. J., J. C. DE LA TORRE and D. A. STEINHAUER, 1992 RNA virus populations as quasispecies. Curr. Top. Microbiol. Immunol. **176:** 1–20.

MARICH, J. E., A. T. POTSLER, S. M. RICE, K. A. MCGRAW and T. W. DUBENSKY, 1992 The phylogenetic relationship and complete nucleotide sequence of human papilloma virus type 35. Virology **186:** 770–776.

MARTÍNEZ, M. A., J. DOPAZO, J. HERNÁNDEZ, M. G. MATEU, F. SOBRINO *et al.*, 1992 Evolution of the capsid protein genes of foot-and-mouth disease virus: antigenic variation without accumulation of aminoacid substitutions over six decades. J. Virol. **66:** 3557–3565.

MAYNARD SMITH, J., 1989 *Evolutionary Genetics*. Oxford University Press, New York.

MAYNARD SMITH, J., and S. NEE, 1991 Clicking into decline? Nature **348:** 391–392.

MELZER, A. L., and J. H. KOESLAG, 1991 Mutations do not accumulate in asexual isolates capable of growth and extinction—Muller's ratchet reexamined. Evolution **45:** 649–655.

MOYA, A., E. RODRÍGUEZ-CEREZO and F. GARCÍA-ARENAL, 1993 Genetic structure of natural populations of the plant RNA virus tobacco mild green mosaic virus. Mol. Biol. Evol. **10:** 449–456.

MULLER, H. J., 1964 The relation of recombination to mutational advance. Mutant. Res. **1:** 2–9.

NICHOL, S. T., J. E. ROWE and W. M. FITCH, 1993 Punctuated equilibrium and positive Darwinian evolution in vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA **90:** 10424–10428.

SCOTT, T. W., S. C. WEAVER and V. L. MALLAMPALLI, 1994 Evolution of mosquito-borne viruses, pp. 293–324 in *The Evolutionary Biology of Viruses*, edited by S. S. MORSE. Raven Press, New York.

SOKAL, R. R., and F. J. ROHLF, 1981 *Biometry*, Ed. 2. W. H. Freeman and Company, New York.

STEINHAUER, D. S., and J. J. HOLLAND, 1986 Direct method for quantification of extreme polymerase error frequencies at selected single base sites in viral RNA. J. Virol. **57:** 219–228.

STEINHAUER, D. S., and J. J. HOLLAND, 1987 Rapid evolution of RNA viruses. Ann. Rev. Microbiol. **41:** 409–433.

STEINHAUER, D. S., J. C. DE LA TORRE and J. J. HOLLAND, 1989 High nucleotide substitution error frequencies in clonal pools of vesicular stomatitis virus. J. Virol. **63:** 2063–2071.

STEPHAN, W., L. CHAO and J. G. SMALE, 1993 The advance of Muller's ratchet in a haploid asexual population: approximate solutions based on diffusion theory. Genet. Res. **61:** 225–231.

VANDEPOL, S. B., L. LEFRANCOIS and J. HOLLAND, 1986 Sequences of the major antibody binding epitopes of the Indiana serotype of vesicular stomatitis virus. Virology **148:** 312–325.

WAIN-HOBSON, S., 1992 Human immunodeficiency virus type 1 quasispecies *in vivo* and *ex vivo*. Curr. Top. Microbiol. Immunol. **176:** 181–194.

WAIN-HOBSON, S,, 1994 Is antigenic variation of HIV important for AIDS and what migh be expected in the future?, pp. 185–209 in *The Evolutionary Biology of Viruses*, edited by S. S. MORSE. Raven Press, New York.

WHITE, B. T., and D. J. MCGEOCH, 1987 Isolation and characterization of conditional lethal amber nonsense mutants of vesicular stomatitis virus. J. Gen. Virol. **68:** 3033–3044.

WRIGHT, S., 1938 Size of populations and breeding structure in relation to evolution. Science **36:** 1059–1066.

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