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Population Bottlenecks in Quasispecies Dynamics

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Abstract The characteristics of natural populations result from different stochastic and deterministic processes that include reproduction with error, selection, and genetic drift. In particular, population fluctuations constitute a stochastic process that may play a very relevant role in shaping the structure of populations. For example, it is expected that small asexual populations will accumulate mutations at a higher rate than larger ones. As a consequence, in any population the fixation of mutations is accelerated when environmental conditions cause population bottlenecks. Bottlenecks have been relatively frequent in the history of life and it is generally accepted that they are highly relevant for speciation. Although population bottlenecks can occur in any species, their effects are more noticeable in organisms that form large and heterogeneous

populations, such as RNA viral quasispecies. Bottlenecks can also positively select and isolate particles that still keep the ability to infect cells from a disorganized population created by crossing the error threshold.

1 Introduction

The characteristics of natural populations result from different stochastic and deterministic processes that include reproduction with error, selection, and genetic drift. All these processes make possible the adaptation to changing environmental conditions. In particular, population fluctuations constitute a stochastic process that may play a very relevant role in shaping the structure of populations. For example, it is expected that small asexual populations will accumulate mutations at a higher rate than larger ones. As a consequence, in any population the fixation of mutations is accelerated when environmental conditions cause population bottlenecks. Then the size of the population, and thus its genetic diversity, is strongly reduced and selected through processes that do not depend on fitness. Any mutation present in this small number of individuals will be transmitted to most of their progeny. Bottlenecks have been relatively frequent in the history of life and it is generally accepted that they are highly relevant for speciation. Although population bottlenecks can occur in any species, their effects are more noticeable in organisms which form large and heterogeneous populations, such as RNA viral quasispecies.

The high mutation rate of RNA viruses has as a consequence that each new individual generated has 0.1 to 1 mutation relative to the consensus sequence (Batschelet et al. 1976; Drake and Holland 1999). Differences in the replicative ability of coexisting types, the continuous generation of new mutations, and the action of selective mechanisms, eventually define a complex population of interrelated individuals. If the environment is stable enough, the distribution of phenotypes attains a mutation-selection equilibrium characteristic of the environment where the population evolves. In terms of Wright's fitness landscapes, the quasispecies occupies a fraction of the genotype space where survival of the population as a whole is optimized. In addition to a number of different genomes representing the phenotype with maximal fitness, there are many other variants coexisting in the population. The very structure of viral quasispecies permits the presence of minority genomes which explore regions of the genotype space far from the optimum fitness peak.

The structure of the genotype space that is occupied by a quasispecies has not been studied in depth experimentally, since it would require sequencing a very large number of genomes. However, it is known that virus

isolates which have evolved under different adaptive pressures have different genotype structures and explore diverse regions of the fitness landscape, even in cases where they share the same consensus sequence. Given appropriate conditions, such as strong environmental changes or bottlenecks, genomes that are present at a low amount can be selected to generate a new quasispecies with suitable properties, and thus guarantee the persistence of the population. We have strong evidence that the formation of self-sustained and highly heterogeneous populations is an intrinsic feature of RNA viruses that strongly conditions their adaptability, robustness, and evolutionary dynamics.

This review is structured as follows. In Sects. 2 and 3, we discuss a number of theoretical approaches relevant to mutation and selection processes in heterogeneous populations. We start with classical predictions on the effect of Muller's ratchet and present more recent mathematical models which already take into explicit account the presence of bottlenecks. In Sect. 4, experiments and observations of bottlenecks in different organisms are reviewed, and Sect. 5 is entirely devoted to empirical results with RNA viruses. In that section, it is shown that periodic bottlenecks applied to optimized populations result in decreases in fitness. Changes in genomic sequences accompanying the fitness losses are discussed in Sect. 6. Section 7 presents a number of mechanisms through which viral fitness can be recovered. In particular, stationary states of fitness can be expected if compensatory mutations occur. The role of epistatic interactions in halting the progressive loss of fitness and experimental and theoretical observations on interactions between mutations are reviewed in Sect. 8. Finally, Sect. 9 discusses how a long history of in vitro bottlenecks can affect the adaptability of viral populations. We conclude with some final remarks in the last section.

2
Theoretical Approaches to Mutation and Selection Processes

In the early days of population genetics, little was known about the actual molecular mechanisms driving evolution and adaptation of populations. The first attempts to formalize the process of appearance of variants and the eventual fixation or disappearance of the mutation involved were carried out by Haldane, Fisher, and Wright, in a series of works of the highest relevance which settled the basis of theoretical population genetics (Haldane 1924; 1927; Fisher 1922, 1930; Wright 1931, 1939). One of the main concerns at the time regarded the fate of mutations in asexual populations. Since the appearance of change is an unavoidable effect of reproduction, mutations would necessarily accu-

mulate in the population if mechanisms such as recombination or sex, which could eliminate them, were absent. This process was studied, among others, by Muller (1964), who compared the unavoidable accumulation of mutations in asexual populations to the clicks of a ratchet mechanism. However, and contrary to some current interpretations, Muller did not equate accumulation of mutations to degradation of the population. In his own words:

> Under conditions where only stability of type is needed, a non-recombining population does not actually degenerate as a result of an excess of mutation over selection, after the usual equilibrium between these pressures is reached. However, a kind of irreversible ratchet mechanism exists in the non-recombining species (unlike the recombining ones) that prevents selection, even if intensified, from reducing the mutational loads below the lightest that were in existence when the intensified selection started, whereas, contrariwise, "drift" and what might be called "selective noise" must allow occasional slips of the lightest loads in the direction of increased weight.

In asexual populations, deleterious mutations can be fixed, particularly if the population size is small (Crow and Kimura 1970). Eventually, an asexual population of size N subjected to a mutation rate u and a selection coefficient s against deleterious mutations attains a deterministic mutation–selection equilibrium. At that point, there is a fixed amount of genomes n_m with m mutations. The equilibrium distribution of the population was calculated by Kimura and Maruyama (1966) and reads

$$n_m = N \frac{\exp(-u/s)}{m!} \left(\frac{u}{s}\right)^m . \tag{1}$$

As long as N is large enough, there will be a significant amount of wild-type (mutation free) genomes,

$$n_0 = N \exp(-u/s) . \tag{2}$$

However if N is small or the coefficient u/s is sufficiently large, the number of genomes in the mutation-free class can be small enough that population fluctuations cause the disappearance of all of the individuals in that class. If this happens, the ratchet has clicked once and the least loaded class corresponds now to individuals carrying one mutation. Analogously, the one-mutation class can disappear due to population fluctuations, and the ratchet clicks again. This model implicitly assumes that reversions (an exceedingly rare process implying in this framework a change from the class m to the class $m-1$) are the only mechanism able to produce an increase in fitness. If the

highest fitness class is identified with the mutation free class and is assigned value 1, the fitness $w(m)$ of individuals with m mutations will be

$$w(m) = (1 - s)^m \,. \tag{3}$$

This defines a multiplicative fitness landscape for the mutated genomes and implicitly assumes that each mutation affects fitness independently (epistatic effects are not included).

Nowadays, most interpretations of Muller's ratchet equate accumulation of mutations with fitness loss. The rationale behind this identification comes from presupposing that the mean genotypic value of the population is close to the optimum. Indeed, if one accepts that populations are well adapted to their natural environment, any further change, that is, any new mutation, should have a deleterious effect on their fitness with high probability. Comparatively, advantageous changes would be much rarer, to the point that they can be ignored. The first formal study of Muller's ratchet under the previous assumptions, and using the results of Kimura and Maruyama (Eq. (1)) was carried out by Haigh (1978) on a model proposed by Felsenstein (1974). He concluded that the speed of the ratchet (the time between two successive clicks or losses of the least-loaded class) depended chiefly on n_0 (see also Bell 1988a). However, more recent analyses (Stephan et al. 1993; Gordo and Charlesworth 2000) reveal that the speed of the ratchet depends not only on the number of individuals in the least mutated class, but also on the parameters u and s. Seen from a different viewpoint, this is equivalent to saying that it is the whole structure of the population that determines the fate of each and every class of mutants present.

Following the initial studies of Felsenstein and Haigh, other authors analysed and discussed different properties of Muller's ratchet under similar assumptions and proposed recombination as the most effective mechanism in order to arrest the ratchet and thus recover high-fitness (or mutation-free) genomes (Maynard Smith 1978; Kondrashov 1982). Epistatic effects among mutations where also considered as a possible mechanism capable of accelerating or slowing down the speed of the ratchet. Synergistic epistasis refers to the situation where the joint effect of two deleterious (or beneficial) mutations is larger in absolute value than the sum of the individual effects, and antagonistic epistasis occurs when two mutations in the same genome change fitness in an amount smaller than the sum of their individual effects.

Charlesworth et al. (1993) observed that under weak antagonistic epistasis, the time between successive clicks of the ratchet grows, though the rate of fitness decline is barely affected (as in previous models, it is assumed that all mutations have a negative effect on fitness). However, under stronger epistatic selection the ratchet can be effectively halted, such that, in practice,

a finite population can survive almost indefinitely (Kondrashov 1994). In contrast, Colato and Fontanari (2001) studied the speed of the ratchet in a population subjected to bottlenecks (with a time long enough between bottlenecks such that mutation–selection equilibrium could be attained), and introduced a fitness function of the form

$$w(m) = (1 - s)^{m^{\alpha}} . \tag{4}$$

The parameter α is the epistasis parameter: synergistic and antagonistic epistasis is described by $\alpha > 1$ and $\alpha < 1$, respectively, and Eq. (3) (absence of epistasis) is recovered for $\alpha = 1$. While, in all cases studied, antagonistic epistasis slows down the ratchet and synergistic epistasis accelerates it, other effects, such as a decrease of the population size facilitated by the steady accumulation of mutations, can notably accelerate the extinction of the population due to mutational meltdown (Lynch and Gabriel 1990).

3
Mathematical Models Including Compensatory Mutations

The studies reviewed up to now describe the theoretical understanding of the operation of Muller's ratchet in large populations of approximately constant size, with the exception of Colato and Fontanari's work. There were earlier attempts to include population bottlenecks in mathematical models. Those first studies were motivated by the form of transmission of mitochondrial genomes in mammals, which have no recombination and mostly undergo monoparental inheritance. With a simple model devised to represent such a situation, Bergstrom and Pritchard (1998) demonstrated that "rather than hastening genetic degradation, a bottleneck may be essential in maintaining mitochondrial genetic quality over evolutionary time." This result is in contrast with most previous theoretical expectations, which predicted that mutations would accumulate more easily in small populations, such that they would consequently suffer from faster degradation than larger ones.

However, the large amount of asexual, nonrecombining species subjected to strong population bottlenecks in their natural environments which, despite of them, seemed to keep a high average fitness, turned the attention to other forms in which the continued degeneration predicted by most models could be compensated. The occurrence of compensatory mutations (not reversions) which could have phenotypic effects and even outweigh the effect of the accumulated deleterious mutations has been considered (Wagner and Gabriel 1990; Bergstrom et al. 1999; Lázaro et al. 2002; Rouzine et al. 2003; Bachtrog and Gordo 2004). Wagner and Gabriel (1990) review the assumptions and

implications of previous models of Muller's ratchet and suggest that compensatory mutations can be as effective as recombination in halting the deleterious effect of the ratchet. Interestingly, they separate effects in the genotype from changes in the phenotype, a relevant difference not considered in most previous theoretical approaches. This distinction is especially relevant in the context of quasispecies, since the mutation–selection equilibrium is defined as the stationary distribution of fitness values selected in the given environment. This equilibrium does not fix the consensus sequence, which would surely be affected by (quasi-) neutral drift. Starting with a high-fitness variant, Wagner and Gabriel observed decreases in fitness for a number of generations due to the action of the ratchet. However, at a certain point compensatory mutations stop the effect of deleterious mutations and the average fitness of the population keeps an average value with small fluctuations around it. Hence, the evolution of the population displays a biphasic behavior, reaching a statistically stationary state after a transient state where fitness decreases, thus avoiding extinction. Actually, in all models where compensatory mutations are explicitly included, it is possible to recover higher fitness states in the population, and to escape extinction. As a side effect, biphasic evolution is observed whenever the initial fitness of the population is far from the average stationary fitness at the corresponding mutation–selection equilibrium. This is so even when bottlenecks, and thus strong fluctuations in the population size – leading to an enhanced mutation fixation rate – are periodically applied.

Aiming at understanding the relationship between the mode of transmission of a pathogen and its virulence, Bergstrom and co-workers (Bergstrom et al. 1999) proposed a simple model where the population was structured in several fitness classes. A down-mutation (occurring with probability p) moved the offspring of a genome from class w to class $w-1$, while an up-mutation increased fitness from w to $w+1$ (this happening with probability q). By means of numerical simulations, it was shown that vertically transmitted pathogens suffer decreases in fitness much stronger than when transmission is horizontal. A similar model was numerically (Lázaro et al. 2002) and analytically (Manrubia et al. 2003) studied in order to understand the appearance of biphasic behavior and large fluctuations in the viral yield observed in an in vitro system for viral evolution (Escarmís et al. 2002). An additional parameter of this model is the development time between bottlenecks, which determines the distance to the mutation–selection equilibrium (which would only be attained after very many generations), and also constrains the degree of heterogeneity of the population before the bottleneck.

This model has a number of differences when compared to classical descriptions of Muller's ratchet, where only deleterious mutations were taken into account. First, the fitness of a sequence with m mutations depends now

on its history, that is to say, in the number of compensatory and deleterious mutations it has suffered. On the average,

$$\langle w(m)\rangle = 1 - m(p-q)s\,, \tag{5}$$

since a fraction p of the accumulated mutations are deleterious and a fraction q are compensatory or beneficial, both modifying fitness in the same amount according to the model. However, the actual value of fitness of an individual carrying m mutations derived from an original sequence of fitness w_0 can take any value between $w_0 - ms$ and $w_0 + ms$ (with a minimum at $1/s$ and a maximum at 1), corresponding to all the mutations being deleterious or all being beneficial, respectively. By construction, the selection coefficient in this model is

$$s = \frac{1}{F}\,, \tag{6}$$

where F is the number of different fitness classes considered. If all mutations are deleterious, the fitness of the genome is

$$w(m) = 1 - ms\,, \tag{7}$$

which corresponds to a first-order expansion of Eq. (3). In this model, all mutations have the same effect on fitness regardless of the fitness state of the genome suffering the mutation. Though other models assume that mutations decrease or increase fitness in a fixed percent, and not in a fixed amount, it is possible, under certain conditions, to demonstrate that at least the changes in fitness due to beneficial mutations act in an additive way (Orr 2003).

Some models, including beneficial mutations, have tackled the problem of fixation of an advantageous mutant in this new context. Peck (1994) argued that mutations with a positive effect on fitness could appear in stable environments because no species is likely to be perfectly adapted. In changing environments, beneficial mutations should not be that rare at all. Still, he discussed that a beneficial mutation (a ruby) appearing in a background of deleterious mutations (the rubbish) would have little chance of being fixed in an asexual population. As in previous analysis, Peck's results supported the great advantage of sex and recombination in promoting adaptation, but, indirectly, also pointed to the relevance of the population structure (through the amount and distribution of deleterious mutations, in that case) in determining the fate of a mutant. The fixation of higher fitness variants in the framework of quasispecies has been explored further (Wilke 2003), with the conclusion that the chances for an advantageous mutant to be eventually fixed in the quasispecies can be only accurately estimated if the fitness of all potential members of the invading quasispecies is known.

The equilibrium distribution in Eq. (1) is attained after a long development time, for large enough populations, and only if all mutations have the same effect on fitness. Most populations, even in carefully controlled experiments, are expected to be out of the equilibrium described by Eq. (1). In particular, bottlenecks condition the structure of the population and its phenotypic characteristics in nontrivial ways. Following a bottleneck, both the frequency and variance of the population's genetic composition change (Zhang et al. 2004). If bottlenecks are regularly applied such that the development time of the population between bottlenecks is much shorter than the time required to achieve mutation–selection equilibrium, the (out-of-equilibrium) composition of the population in the presence of deleterious and beneficial mutations can be analytically calculated (Manrubia et al. 2003).

Several of the models reviewed have been designed in order to better understand the dynamics of in vitro experiments. These will be described in the forthcoming sections, where further results from the models discussed here will be presented.

4
Experimental Effects of Population Bottlenecks

A population is well adapted to a given environment when the processes of competition and selection have acted for a time long enough to reach an equilibrium close to the optimum. Nevertheless, mutations are inherent to replication and, in any well-adapted population, many of them will have a deleterious effect on fitness. In a situation where only a small number of individuals found a new population, the genetic diversity is reduced and mutations are transmitted to most of the descendants. Under these circumstances, the chances that the least mutated sequence is lost increase notably.

The process of mutation accumulation is particularly effective when the mutation rate is high, as in RNA viruses. Attending to theoretical predictions derived from models where compensatory mutations were not taken into account, it was expected that the repetition of serial bottlenecks could lead to the extinction of the population. Early theories on population evolution left open many questions that could be solved only experimentally, as for instance the actual role that sex and recombination play as mechanisms able to counteract the ratchet mechanism. Other debated questions concerned the way in which the progressive accumulation of mutations affect fitness: do mutations have a simple multiplicative effect? Are epistatic interactions important in the process? Is the effect of a mutation the same irrespective of other mutations accumulated in the genome? Can beneficial or compensatory mutations really

outweigh the effects of the ratchet? In the forthcoming sections, we will discuss the experimental studies carried out to answer these important questions.

The empirical implementation of Muller's ratchet in RNA viruses has been carried out through the classical protocol of plaque-to-plaque transfers of clonal populations (Fig. 1). Each transfer starts with a single viral genome. To this end, the starting population is appropriately diluted and plated under a semisolid agar layer. The virus contained in a single plaque of the progeny is isolated, diluted again, and plated a second time. A second plaque from the latter progeny is isolated and the process is serially repeated. The effect of Muller's ratchet following this procedure was experimentally observed for the first time by Lin Chao with the phage ϕ6 (Chao 1990). Since then, it has been studied in several microorganisms, such as the phage MS2 (de la Peña et al. 2000), several animal viruses, such as vesicular stomatitis virus (VSV) (Duarte et al. 1992), foot-and-mouth disease virus (FMDV) (Escarmís et al. 1996), and human immunodeficiency virus (HIV) (Yuste et al. 1999). All these viruses were subjected to plaque-to-plaque transfers, and it was documented that this transmission regime resulted in losses of viral fitness. Although in this review we will refer to RNA viruses, the effect of bottlenecks on evolution has also been studied in DNA viruses, such as phage T7 (Bull et al. 2003), where strikingly similar conclusions have been reached. In that case, the

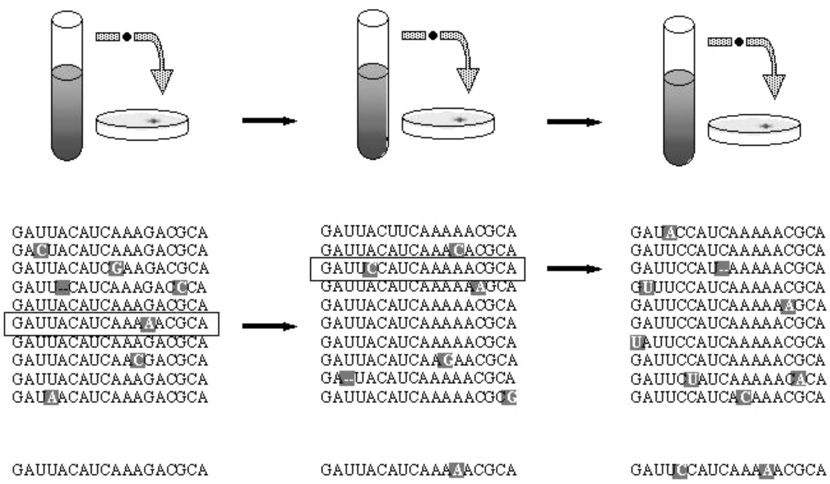

Fig. 1 Scheme of the plaque-to-plaque protocol. At each transfer a lytic plaque is generated from a single viral particle. The progeny present in the plaque is resuspended, diluted and plated to generate the next plaque (*top*). The consensus sequence of the population at each transfer carries the mutations from the parental genome (*bottom*)

authors increased the mutation rate of the DNA phage through the addition of a mutagen during growth. Evidence of the operation of Muller's ratchet has also been obtained in studies with protozoa (Bell 1988b), bacteria (Andersson and Hughes 1996; Kibota and Lynx 1996; Funchain et al. 2000), yeast (Zeyl et al. 2001), and multicellular organisms (Fry et al. 1999; Vassilieva et al. 2000). The issue of whether a bottleneck occurred among our human ancestors is a question that is strongly debated by geneticists and anthropologists (Ayala et al. 1994; Harpending and Rogers 2000).

5
Bottlenecks and Muller's Ratchet in RNA Viruses

The experiments carried out by Chao (1990) with the tripartite bacteriophage $\phi 6$ involved 40 consecutive plaque-to-plaque transfers, which led to a significant decrease in mean fitness. No extinction of infectivity was observed. The experimental design precluded reassortment of the three genome segments of the phage while allowing the comparison of the relative replication rates of wild-type phage vs repeatedly bottlenecked clones. In this way, sex (understood as encapsidation in the same particle of genomes from different viruses), as a mechanism able to repair genetic lesions was excluded. In subsequent studies carried out by the same group (Froissart et al. 2004), it was suggested that the mutational load could be purged faster in the absence of co-infection, thus hinting at the possibility that complementation diminishes the benefits of sex.

Duarte et al. (1992) studied the relative fitness of VSV, the prototype model for negative-stranded RNA viruses, when subjected to serial bottlenecks. VSV has a single nonsegmented genome and does not experience recombination at a detectable level. The assay to determine fitness was direct competition between the parental clone and genetically marked monoclonal antibody-resistant (MAR) clones. They found that after only 20 plaque-to-plaque transfers, the viral clones displayed wide variations in fitness. The overall trend was towards mean fitness reduction, in agreement with the classical interpretation of Muller's ratchet. Again, no extinctions of infectivity were observed. An additional study concerning the accumulation of mutations through bottlenecks in the same virus has been carried out by Elena and Moya (1999), who theoretically calculated a deleterious mutation rate of 1.2 mutations per genome and generation, with a mean fitness effect of −0.39% per generation. The average decrease in fitness per transfer may also change depending on the fitness of the viral clones at the beginning of the experiment, as Elena and co-workers have shown. High-fitness clones lose fitness faster than clones

with lower fitness (Elena et al. 1996): a well-adapted clone can accumulate deleterious mutations more easily than a clone that is less well adapted. The opposite is also true, since low-fitness clones are comparatively more prone to acquiring advantageous mutations and thus to improving fitness.

In the study conducted by de la Peña and collaborators (de la Peña et al. 2000), four MS2 clones were subjected to 20 serial bottlenecks. Although the number of transfers was rather low, they found a sharp decrease in fitness (about 96% at the end of the experiment). They observed that in three of the clones under study, fitness decreased at a constant rate as the number of transfers progressed. One clone lost fitness at a rate significantly higher during the first ten transfers than in the last ten transfers, thus showing biphasic behavior in its evolution. As discussed in the sections devoted to theoretical models, the decrease in fitness should stop after a period of variable length whenever compensatory mutations are included (Wagner and Gabriel 1990; Lázaro et al. 2002). However, in plaque-to-plaque transfers carried out with HIV-1 (Yuste et al. 1999), only four out of ten clones could produce viable progeny after 15 plaque-to-plaque transfers, and three of the four survivors showed important decreases in fitness. This represents a relevant difference with other viral systems, where extinctions were not observed.

The most extensive study on the effect of repeated bottlenecks on fitness evolution and mutation accumulation has been carried out by Escarmís and co-workers (Escarmís et al. 1996; 1999; 2002; Lázaro et al. 2002; 2003) using the animal pathogen FMDV, a positive-strand RNA virus of the *Picornaviridae* family that, in contrast to VSV, can undergo recombination. The main objective was to determine whether successive reductions in the population size could lead viral populations to extinction. The titer of virus in the plaques (PFU/plaque) at the successive transfers was taken as a measure of its fitness. If the titer of each plaque is represented as a function of the number of plaque transfers, biphasic dynamics are observed (Fig. 2). After a period where the plaque titer decreases exponentially, a stationary state with large fluctuations around a constant average fitness value is reached. A detailed analysis of the statistical properties of fluctuations in the viral yield was performed, with the conclusion that the expected yield at the stationary state follows a Weibull distribution (Lázaro et al. 2003). By means of a simple model (Lázaro et al. 2002; Manrubia et al. 2003), it was shown that this type of function is to be expected if an exponential growth of the population (affected by both deleterious and compensatory mutations) takes place between successive bottlenecks.

The results obtained with FMDV sharply contrast with the high number of extinctions observed when HIV-1 clones were subjected to successive bottlenecks. This can be ascribed to the number of replication rounds that takes place during the development of the HIV-1 plaques. This number is probably

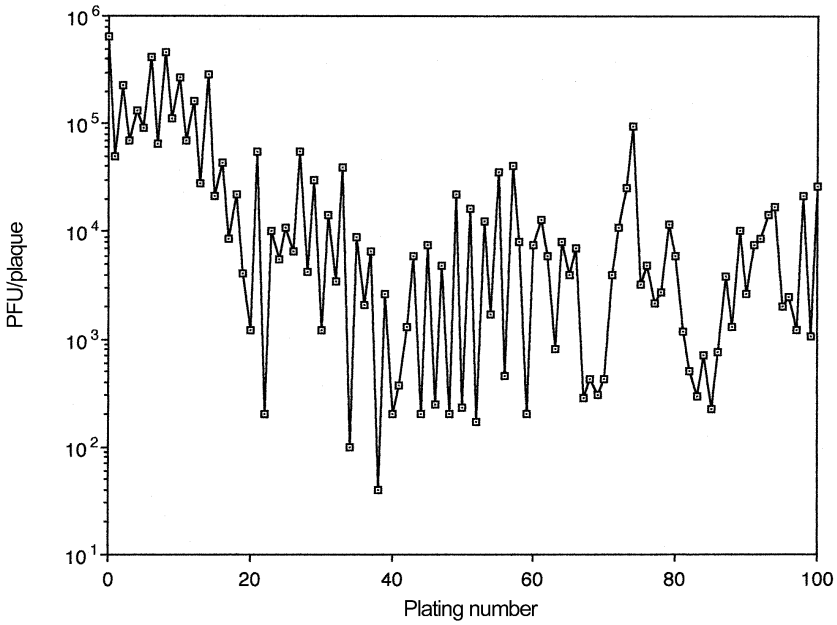

Fig. 2 Titer of successive plaques of a clone subjected to plaque transfers

lower than in other viruses and, therefore, the possibilities of competition and selection inside HIV-1 plaques are more limited than in the case of FMDV. Even a single plaque is formed by a heterogeneous genome population, and the degree of optimization of this population depends on the number of replication cycles undergone by the virus (Manrubia et al. 2005).

Mutations accumulated steadily in the consensus nucleotide sequence in the fitness decrease phase as well as in the constant average fitness phase (Escarmís et al. 2002). These results are unexpected in the light of classical Muller's ratchet interpretations. This might indicate that, although mutations always occur at the same rate, their nature and effects can vary depending on the number and type of mutations previously accumulated in the genome. Selection may act with different strengths depending on the average fitness of the population, since genomes with low fitness are less tolerant to the introduction of additional deleterious mutations.

The average rate of fitness decline (determined as the slope of the straight line obtained when the logarithm of fitness is represented as a function of the transfer number) in the study conducted with the phage MS2 is similar to that observed in the exponential phase of fitness losses in the case of FMDV (0.16 for MS2 and 0.12 for FMDV). The similarity between both results is striking, especially considering that, in the case of the phage MS2, fitness was deter-

mined by quantifying genotypes (using the Northern blot technique) instead of phenotypes (counting the number of plaque-forming units for FMDV).

It could be very interesting to study whether the documented resistance of bottlenecked FMDV clones to extinction (Escarmís et al. 2002, Lázaro et al. 2003) is a generic fact or a particular property of this virus. Testing this would require subjecting other viruses to a comparably large number of plaque-to-plaque transfers. There are many viral systems that have already experienced dramatic fitness losses. It could be checked if those clones become extinct after subjecting them to additional bottlenecks.

The characteristics of the state attained by the virus after many bottlenecks depend on the number of viral particles that generate each new population. The effect of different bottleneck sizes on fitness changes has been studied experimentally (Novella et al. 1995a). Novella and co-workers used clones of different fitness values as starting populations. They observed that the effective size of a genetic bottleneck causing fitness loss is larger when the fitness of the parental population increases. For example, for starting virus populations with low fitness, population transfers of five-clone-to-five-clone passages resulted in a fitness increase. However, when a parental population with high fitness was transferred, 30-clone-to-30-clone passages were required simply to maintain fitness values. This result can be explained by the probability of sampling variants of lower, equal or greater fitness than the fitness of the original population. As a consequence of this dependence between initial fitness and size of the bottleneck required to observe Muller's ratchet effect, Novella et al. (1999) suggested that bottleneck effects limited exponential fitness gains of RNA virus populations passaged at a high population concentration. In this regime, viral fitness reaches a plateau at which stochastic fitness variations were observed. In their view, transmission through a large number of particles may represent a bottleneck at this high fitness value and might limit further increases in fitness. Their results are in agreement with a simple model where it has been shown that, at the stationary state, the average fitness of the population is determined by the size of the population bottleneck, and does not depend on the initial fitness of the population (Lázaro et al. 2002).

6
Sequence Changes Accompanying Loss of Viral Fitness Due to Genetic Bottlenecks

Several genomes of viruses subjected to serial plaque transfers have been sequenced to investigate the genetic changes that accompany fitness losses. In general, in all the viruses analyzed, unusual mutations have been found. This

result indicates that, during the development of a plaque, negative selection has insufficient time to act, that mutants that would be otherwise eliminated by competition with fitter genomes remain in the population and have a chance to be selected for the next transfer.

In a study with HIV (Yuste et al. 1999, 2000), the analysis of the mutations present in several clones revealed a predominance of transitions over transversions. One of the clones analyzed displayed a remarkably high mutation frequency (28 mutations accumulated after 15 transfers) and showed a very high abundance of G → A transitions uniformly distributed along the genome. This had been previously observed in natural isolates of HIV-1. The mutations in the remaining clones were distributed in an unusual way. There was a statistically significant higher accumulation of mutations in *gag* and the first third of the genome, compared to *env*, which appeared to be the most conserved region in all the clones studied. This is in sharp contrast to the mutation distribution observed in natural HIV isolates and in large population passages of HIV-1 clones subjected to positive selection. In these populations, *gag* and *pol* are more conserved than *env*. The reduced action of purifying selection during plaque development can cause the differences observed. However, the low number of mutations in *env* remains puzzling. A possible explanation might be that mutation rates are not the same in different genomic regions, and that the replication of *gag* is more error prone.

In a study carried out with VSV (Novella and Ebendick-Corpus 2004) the mutations accumulated during the operation of Muller's ratchet were found in both coding and noncoding regions. Mutations accumulated at a rate of 1.92×10^{-5} per nucleotide and per transfer. Transitions were 2.1-fold more abundant than transversions, as observed in many viral systems. Nonsynonymous mutations were also more frequent than synonymous mutations. However, if the number of mutations of each type is corrected by the number of synonymous (ds) and nonsynonymous (dn) sites in the genome, a different result is obtained: dn/ds = 0.48. Several strains accumulated a high number of mutations in the N open reading frame, contrasting with the conservation of this region in populations under positive selection or in natural isolates of the virus. This indicates again that genomic regions that appear to be very conserved in different viruses can mutate at a rate comparable to the remainder of the genome, though they may experience stronger constraints. Negative selection eliminates most of the mutants in these conserved regions, and mutations are not observed unless negative selection is reduced, as happens when bottlenecks occur. That is to say, bottlenecks allow isolating and maintaining genomes that would be eliminated in other situations.

The changes observed in FMDV qualitatively differed from those fixed during large population passages (Escarmís et al. 1996; 2002). The dominance

of nonsynonymous mutations associated with large population passages diminished upon repeated plaque transfers. The percentage of nonsynonymous mutations ranged from 39% to 48% (Escarmís et al. 1996), although this number is not normalized with respect to the total number of synonymous and nonsynonymous sites in the genome. Of substituted residues in capsid proteins VP1, VP2 and VP3, 33% were internal (exposed neither in the outer nor in the inner surfaces of the protein shell, as determined by their accessibility to a probe of 0.3 nm radius; Lea et al. 1994). In contrast, only 4% of the substitutions found in viruses subjected to large population passages affected internal amino acid residues. Around 50% of the clones subjected to 30 serial plaque transfers acquired different extensions of five adenosine residues preceding the uridine of the second AUG initiation codon (see Fig. 3) (Escarmís et al. 1996, 2002). The phenotypic effect of this internal poly A elongation is not known, but its presence is deleterious for the virus. From data in Fig. 4, it can be deduced that there is an inverse linear correlation ($r = -0.77$) between the logarithm of plaque titer and the length of the poly A tract ($p < 0.001$, Student's t test) (Escarmís et al. 2002).

To predict possible phenotypic effects of the fixed mutations in the plaque-to-plaque transfers, each mutation was analyzed with regard to potential effects on RNA and protein structure and function, as well as the variability of the residues according to the FMDV sequences available. Mutations that affected conserved residues and therefore could account for the loss of fitness observed in the plaque transfers were disrupting a pseudoknot, changing the Y of VPg2 responsible for binding to the RNA rendering VPg2 inactive; a mutation in the S fragment that destabilizes its secondary structure; amino acid replacements in capsid proteins VP1 and VP4; and amino acid replacements in nonstructural proteins 2C, 3A and Vpg1 (for a detailed map of FMDV,

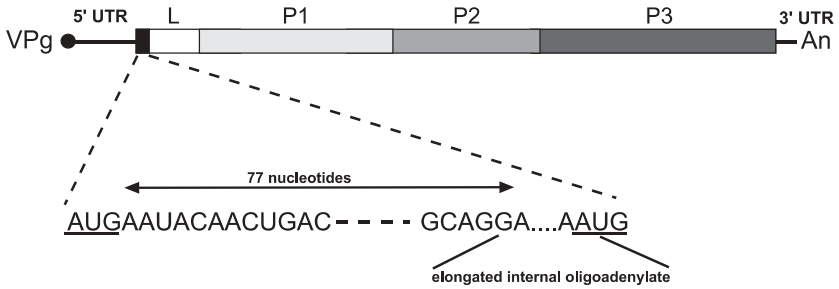

Fig. 3 Scheme of the genome of foot-and-mouth disease virus. Location of the elongated internal oligoadenylate preceding the second initiating AUG. The number of As preceding the AUG in the wt is four

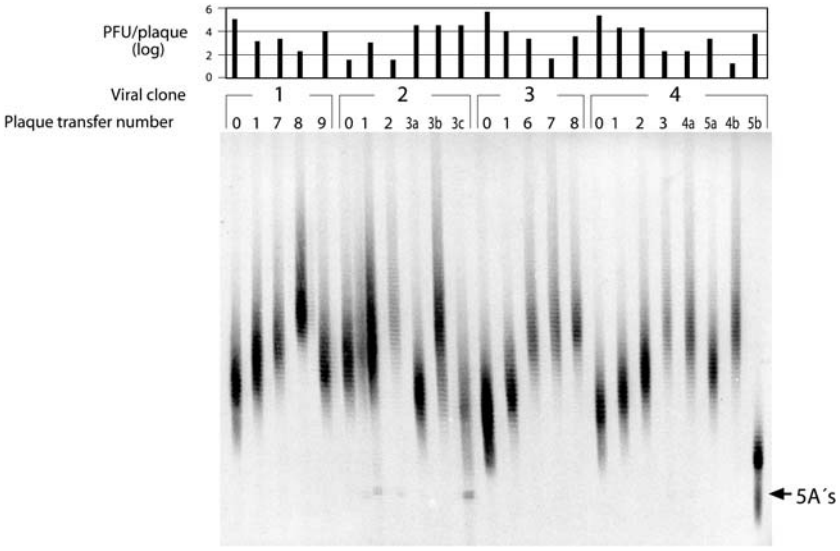

Fig. 4 Dependence of the titer of a plaque on the length of the internal elongated oligoadenylate present in the genome. The length of the oligoadenylate was determined by amplifying the RNA (RT-PCR) in the presence of ^{35}S-dATP with two primers flanking the oligoadenylate. The amplification products were electrophoresed through a 6% polyacrylamide sequencing gel. The heterogeneity of the oligoadenylate gives rise to a number of bands. The position in the gel of the product obtained from the wt genome (total of 5 A s) is marked with an *arrow* on the right. The determination was done for four clones. Clones 3a, 3b and 3c are three clones isolated in plaque transfer number 3 of clone 2.Clones 4a and 4b are two clones isolated in plaque transfer 4 of clone 4. These clones were transferred, giving rise to clones 5a and 5b

see Mason et al. 2003). However, confirmation of the adverse effects of these mutations on virus viability would require their separate inclusion in a cDNA clone, growth-competition experiments between viruses with and without each mutation and depending on the outcome, the monitoring of nucleotide sequences of the progeny virus. No back mutations (reversions) were observed in the plaque transfers.

Two FMDV clones were subjected to prolonged plaque-to-plaque transfers and the genomic sequences were determined at different plaque transfers. The comparison of the sequences shown in Fig. 5 revealed a new mechanism of clonal diversification by mutation clustering. It consists of the accumulation of mutations in some genomic regions, with localized mutation frequencies significantly larger than those observed in the whole genome. Mutation clustering was demonstrated by dividing the genome into 250 nucleotide-long fragments with a 200-base overlap and comparing the mutation frequency of

each fragment to that of the whole genome. In this way, three regions with a significantly enhanced mutation frequency were identified: the region of the L protein, the 2A2B region, and the 3A3B region. The mechanism by which this clustering is produced is unknown. Three possible mechanisms have been suggested (Escarmís et al. 2002): local variation of the copying fidelity of the viral replicase due to the presence of certain mutations; production of compensatory mutations close to the triggering ones; some mutations making a particular genomic region more tolerant to neighboring mutations. Weak evidence of mutation clustering has been obtained at some loci of FMDV genomes subjected to large population passages, but clustering is less obvious, presumably due to a more active negative selection.

In successive plaque transfers of FMDV, mutations accumulated at a rate of about 0.3 nucleotides per plaque transfer, even with constant average fitness. The mutation frequency attained after 130 plaque-to-plaque transfers was 4.8×10^{-3} changes per nucleotide. The rate of accumulation of mutations during large population passages of FMDV clones in liquid culture medium was 0.15 mutation per passage. It is difficult to compare the number of genome copying rounds during the development of a plaque from 1 to 10^2–10^4 PFU (the range observed for FMDV clones) with the number of rounds in one passage in liquid culture medium (generally a tenfold increase in PFU). Nevertheless, the important selective difference between the two regimes (plaque-to-plaque transfers and large population passages) appears to be the nature and location of the mutations rather than the rate of fixation (Escarmís et al. 2002).

7
Recovery of Viral Fitness

A large number of experimental studies have been devoted to understanding the mechanisms that permit recovery of fitness in bottlenecked viruses. Some experiments have analyzed the transfer regimes that cause fitness gains. More recent studies have also investigated the number and type of mutations appearing during the process.

As discussed, large population passages of RNA viruses usually result in an increase in relative fitness due to competition and selection of genomes within the quasispecies. (Novella et al. 1995b; Weaver et al. 1999). This transmission regime is also effective to recover fitness in viruses that have been debilitated by the accumulation of mutations through bottlenecks (Clarke et al. 1993; Escarmís et al. 1999; Novella and Ebendick-Corpus 2004).

One study (Duarte et al. 1993) showed that two consecutive large population passages intercalated between bottlenecks were not enough to avoid the

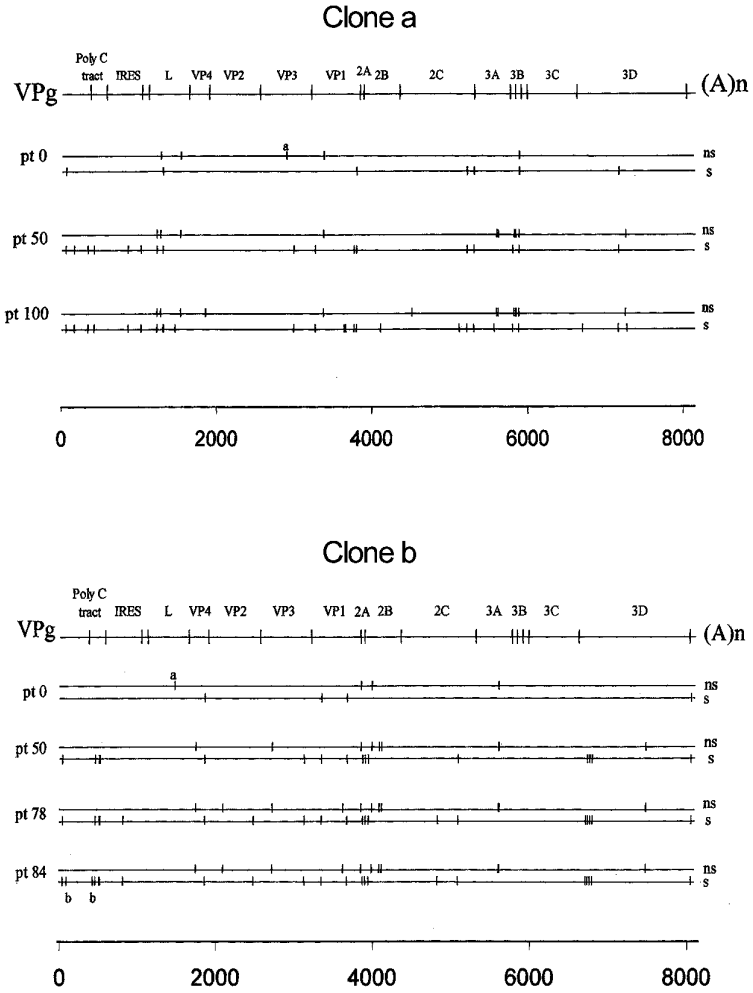

Fig. 5 Location of the mutations found in the genome of two FMDV clones subjected to serial plaque-to-plaque transfers. In each panel, the *top horizontal line* is a scheme of the FMDV genome, in which the main regulatory regions and encoded proteins are indicated. The *lines below the genome* indicate nonsynonymous (ns) and synonymous (s) mutations present in virus of plaque transfers 0, 50 and 100 for clone a, and 0, 50, 78 and 84 for clone b. The *bottom line* indicates the size of the FMDV genome. The letter *a* indicates the presence of a mixture of two nucleotides not seen in subsequent transfers. *b* Represents a mixture of two nucleotides, and the mutant residues became dominant upon multiplication of the virus in liquid culture medium

effect of the ratchet. These experiments were carried out with viruses of high to moderately low fitness. Other studies (Elena et al. 1998) have explored the evolution of VSV subclones derived from a very debilitated clone obtained after 20 successive bottlenecks. All the subclones analyzed gained fitness under different transmission regimes. As pointed out above, a possible explanation for this discrepancy is that, although mutation rates should be the same for all the clones, the fraction of beneficial mutations is larger in a clone with lower fitness. There is a higher chance of improving a debilitated virus than a well-adapted one. This reveals that the assumption that mutations have a multiplicative effect on fitness, as assumed in most theoretical models used to study the accumulation of mutations in genomic sequences, is probably too simplistic.

The recovery of the fitness of a debilitated FMDV clone through large population passages and the mutations accompanying this recovery were also studied (Escarmís et al. 1999). The debilitated clone included the internal polyadenylate extension and a number of additional mutations scattered throughout the genome. Three different pathways, regarding the internal polyadenylate tract, were followed by several subclones to recover fitness: (a) a true reversion to yield the wild-type sequence; (b) a shortening of the internal polyadenylate tract; and (c) a deletion of 69 residues spanning the site of the internal polyadenylate tract. These results indicate that an RNA virus can find multiple pathways to reach alternative high-fitness peaks on the fitness landscape. In large population passages, only a few reversions were observed (Escarmís et al. 1999). This low number of reversions is also true for bacteria. It has been documented that the deleterious effects of a resistance mutation in *Salmonella typhimurium* is compensated by a variety of new mutations, and only four out of 81 independent lineages contained true streptomycin-sensitive revertants (Maisnier-Patin et al. 2002).

Novella and Ebendick-Corpus (2004) also analyzed the genetic changes involved in the recovery of the negative effect of Muller's ratchet by large population passages. They found that fitness increases were associated with both reversions and compensatory mutations. A significant level of fitness increase was observed in several bottlenecked strains with no obvious changes in the consensus sequence. The structure of the ensemble of genomes composing the quasispecies must have been altered in some way, confirming that the consensus sequence represents only a fraction of the genomic information contained in the quasispecies. Three of these strains which did not alter the consensus sequence were subjected to additional large population passages. At passage 20, each population further increased its fitness and a single mutation was observed in each case. None of the recovered strains reverted to a wild-type sequence. This is consistent with the observation that bottlenecks

force a genome to constitute a new quasispecies at a different position of the genomic landscape, where the adaptive value of a given mutation might vary. Once more, this speaks of the enormous adaptive capacity of RNA viruses: a single viral clone is able, at each passage, to generate an optimized though different quasispecies under the action of mutation and selection.

Yuste et al. (2005) showed that a few mutations were sufficient to meditate fitness recovery of HIV clones that had been subjected to plaque-to-plaque transfers (Yuste et al. 1999; Yuste et al. 2000). Of all mutations observed in different clones, 25% were reversions, and 12 out of 20 mutations were located in the primer-binding-site loop for initiation of reverse transcription.

In addition to the fitness gains observed in very debilitated viruses when subjected to massive passages, other studies reveal the possibility of gaining fitness between bottlenecks. Elena et al. (1998) observed that several subclones could regain fitness after having been subjected to additional bottlenecks. The authors explained this result on the basis of the heterogeneity of the viral population inside a plaque. It had been generally accepted that transfers through small population sizes precluded the appearance of advantageous mutations, but this study demonstrated the possibility of the generation of beneficial mutants that could be selected for the next transfer due to its faster replication.

The possibilities of observing fitness rise after a bottleneck increase when the virus is highly debilitated. This is what happens in the stationary state reached by FMDV clones (Escarmís et al. 2002). In this case, the large number of bottlenecks experienced by the virus possibly leads to the minimal average fitness permitted under the transmission regime of plaque-to-plaque transfers. However, the virus still possesses an enormous capacity to recover from the negative effect of the serial bottlenecks, probably due to the selection of mutants with advantageous mutations. RNA viruses are robust enough to find solutions to outweigh the negative effect of the accumulated mutations. The transmission regime of plaque-to-plaque transfers introduces a positive bias, since it selects virus able to form plaques, and thus isolates genomes with a particular combination of mutations such that the virus is viable. It is likely that many genomes become extinct during the development of a plaque due to the accumulation of deleterious mutations, but epistatic interactions and compensatory mutations might permit the appearance of genomes which still maintain the ability to generate a progeny.

As shown in Fig. 4, debilitated FMDV clones (Escarmís et al. 2002) can recover fitness in a single plaque transfer. In most clones shown in Fig. 4, the recovery of viral fitness in consecutive transfers was accomplished by reduction of the length of the elongated poly A tract. In one clone, the recovery of plaque titer was caused by a change of adenosine to guanosine in the internal poly A tract. These results show the great plasticity of RNA genomes.

As a general conclusion, fitness in highly debilitated viruses can be recovered through massive passages, which permit selection processes to act efficiently. In addition to this generally accepted mechanism, fitness can also be recovered at consecutive bottlenecks. Our interpretation is that, when a virus has reached a low fitness state, the chances of selecting a virus with a compensatory mutation for the next transfer are enhanced. This permits sudden gains in fitness and leads to the fluctuating pattern in viral yield observed in FMDV (Lázaro et al. 2003, Manrubia et al. 2003).

8
Epistatic Interactions Among Mutations. The Role of Sex and Recombination

There have been few experimental studies devoted to the analysis of epistatic effects among mutations. In a recent study (Sanjuán et al. 2004), the effect of a number of mutations was compared when they were present as single mutations or in pairs in the same genome. Between two deleterious mutations, interactions were mainly antagonistic, meaning that their combined effect is significantly smaller than expected under a simple multiplicative model. This fact can partly account for the nonlinear fitness loss in the study carried out with FMDV clones, although this explains neither the arrest of the population around a mean fitness value (and thus its resistance to extinction) nor the strong fluctuations observed. In addition, it has to be considered that, under the plaque-to-plaque transmission regime, many mutations are accumulated, and the effect of their interactions on fitness is much more difficult to quantify than in a genome with only two mutations. In the same study of Sanjuán and co-workers, antagonistic epistasis between beneficial mutations was also found. These results imply that recombination and sex in RNA viruses would not necessarily result in an immediate adaptive benefit. Other experiments on epistatic interactions after mutation accumulation in RNA viruses have used bacteriophage $\phi 6$ (Burch and Chao 2004) and FMDV (Elena 1999). The former study found that viral genomes with low fitness were less sensitive to deleterious mutations, pointing to antagonistic epistasis. The latter study failed to find epistasis. It would be very interesting to continue the study of Sanjuán et al. (2004) with genomes containing both types of mutations, deleterious and advantageous.

We have reviewed some theoretical models which essentially demonstrate that antagonistic epistasis slows down Muller's ratchet, while synergistic epistasis accelerates it (Charlesworth 1993; Kondrashov 1994; Colato and Fontanari 2001). A more complete understanding of the role of mutations and their

interactions in RNA genomes requires the explicit introduction of (at least) the secondary structure of the molecules. Few studies have been devoted to this question to date, though the results obtained are extremely interesting. Analysis of the population structure in sequence space by explicitly considering the secondary structure of RNA sequences of 76 nucleotides reveals that the sequences represented in the population are clustered (that is to say, form groups differing in one or two point mutations) around a small number of variants (which differ in several to many point mutations), all of them folding in the same secondary structure (Huynen et al. 1996). The fraction of the sequence space explored by a finite population strongly conditions its adaptability to new environments (represented in the model by a different secondary structure). Algorithms for folding RNA into its secondary structure predict that the fitness landscape is extremely rugged and that there are extended connected networks of sequences with identical structure. When secondary structure is taken into account, a clear prevalence of antagonistic epistasis in RNA secondary structure folding is observed, together with the appearance of mutations at a long distance in the sequence with compensatory effects (Wilke et al. 2003).

9
Adaptability of Viral Populations with a Long Bottleneck History

It has been pointed several times throughout this review that systematic bottlenecks force an ensemble of viral clones to move through the genotypic (and phenotypic) landscape as the number of transfers increases. These viral populations continue accumulating mutations at a constant rate while eventually maintaining a constant average fitness value. However, an open question is whether the mutational load has consequences in the adaptability of these populations.

A VSV strain obtained after successive bottlenecks showing a fitness equal to that of the wild type, always lost in long-term competition experiments with the wild type (Quer et al. 1996; 2001). The accumulation of mutations, as a consequence of the repeated bottlenecks, has been thought to be the reason for this lower adaptability. In order to test this explanation, an additional experiment was carried out. Several populations were subjected to repeated bottlenecks and allowed to achieve a relative fitness equal to one through additional large population passages (Novella 2004). These populations were competed over 79 passages with the wild-type virus, and they were shown unable to out-compete the wild type, apparently reflecting a lower adaptive ability. A striking observation is that some clones experienced a period of

higher fitness than the wild type but, as the number of passages progressed, and in all the cases analyzed, the wild type performed eventually better than the bottlenecked virus. The authors of this study argue that mutant populations have a lower beneficial mutation rate than the wild type (Novella 2004). It is likely that a full analysis of the structure of the new quasispecies generated after the bottlenecks can give a more complete answer to this question. Maybe it is the structure of the quasispecies what confers higher or lower adaptability to a population, as theoretical studies suggest (Huynen et al. 1996; Wilke et al. 2001).

It would also be very interesting to compare the adaptive ability to a new environment of clones that, having the same fitness, differ in the number of accumulated mutations. These experiments are nowadays possible, since the research of Escarmís and co-workers has produced a number of viral clones with these characteristics. It might be that the bottlenecked clones display lower adaptability, as shown by Novella (2004), when forced to compete with the wild type in an environment where the latter has been optimized. However, this does not imply that a similar result would be found if both viruses compete in a new environment.

10 Final Remarks

Many of the results shown in this review indicate a great resistance of RNA viruses to extinction through the accumulation of mutations upon repeated bottlenecks. However, there are many experiments documenting extinction in viruses experiencing an enhanced replication error rate due to the action of mutagens (Sierra et al. 2000; Pariente et al. 2001; Crotty et al. 2001; Grande-Pérez et al. 2002; Severson et al. 2003). This result agrees with early molecular evolution theories (Eigen and Schuster 1979; Swetina and Schuster 1982; Eigen and Biebricher 1988, Eigen 2002) postulating that viral replication operates very close to the error threshold. When this limit is crossed, the population enters into “error catastrophe”, and the genetic information is lost. Near this threshold, RNA viruses can maintain a population structure organized in quasispecies, with maximal variability, and from which fittest viruses in a given environment can be rapidly selected. Nevertheless, when this limit is crossed the population disorganizes and forms an ensemble of random sequences unable to maintain the genetic information. Bottlenecks can positively select and isolate particles that still keep the ability to infect cells. Thanks to the bottleneck, these minority particles are separated from the unstructured group of mutants and allowed to generate a new popula-

tion without the interfering effect of a highly complex quasispecies (see also chapter by Domingo et al., this volume).

It is, however, difficult to assess the relevance that bottlenecks can have in vivo. In nature, population bottlenecks are very frequent during the transmission of many viruses and may affect the severity of disease outbreaks. In the case of vertically transmitted viruses, bottlenecks may be particularly severe, since only a small amount of viruses are able to cross the barriers necessary to infect the embryo. In addition, competition among genomes can only take place inside the infected host, so the action of selection is highly reduced. Bottlenecks may also be frequent in horizontally transmitted viruses and during the intra-organ transmissions inside an infected organism. Under horizontal transfer, each virus can infect every susceptible individual of the host population, and competition can also happen at the inter-host level (Wilson et al. 1992; Chao et al. 2000). In HIV, newly infected individuals typically contain a smaller diversity of sequences than long-term infected patients, suggesting that, initially, a low number of viral particles was transmitted (Nowak et al. 1991; Pang et al. 1992). Bottlenecks also occur in many diseases transmitted via respiratory droplets, because most droplets only contain one or a few virions (Artenstein et al. 1966; Gerone et al. 1966). Population bottlenecks have been suggested to be partly responsible for the observed evolution of poliovirus in an individual (Hovi et al. 2004) and have been invoked as an explanation for the extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period (Wittke et al. 2002).

Theoretically, it was demonstrated that while fitness weakly declines under horizontal transmission of viruses, vertically transmitted viruses are affected by dramatic decreases in fitness, since this form of transmission involves frequent bottlenecks (Bergstrom et al. 1999). This provides an alternative explanation to Ewald's theory (Ewald 1987), which claims that vertically transmitted viruses evolve to lower virulence because reproduction of the pathogen is limited by the reproductive success of the host. Population bottlenecks are one out of many factors determining the structure of a viral quasispecies and its characteristics. Further analysis, both theoretical and experimental, is required in order to disentangle the relative roles played by the different mechanisms involved in the evolution and adaptation of viral populations.

Mutation rates are probably not independent of the transmission mode used by viruses in nature. The optimum mutation rate for a certain virus may be selected according to the characteristics of the environment where the viral population evolves and adapts (Earl and Deem 2004). In particular, this optimum may depend on the frequency of transmission through bottlenecks and on the degree of intra- and inter-host optimization that takes place before the next bottleneck occurs. These parameters acting in close concert confer viral

populations their plasticity and robustness and prevent an easy extinction of viruses in nature.

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