

Reproducible Nonlinear Population Dynamics and Critical Points During Replicative Competitions of RNA Virus Quasispecies

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RNA virus evolution is generally considered to be highly unpredictable, but tests of determinism in the evolution of competing populations during viral infections have not been performed. Here we study the fate of two closely related evolving quasispecies of vesicular stomatitis virus, by determining the relative concentration of a wild-type clone and a surrogate marked virus subclone (MARM-C) upon extensive competitive replication in a constant cell culture environment. A highly predictable nonlinear behaviour of the two competing populations was found. In addition, the presence of critical points, which are defined as points from which viral competitions may follow different trajectories, has been documented. Critical points were reached after nearly constant periods of time. The dynamics of relative fitness values for both competing populations were calculated during the replication passages. Concomitant with expected fitness gain of both competing viral populations (which follow the Red Queen hypothesis) a tendency for the MARM-C to gain less fitness than the wild-type was observed. Although fitness variations were noisy, this tendency was seen in all evolutionary replicas. Thus, despite the stochastic process of mutation that leads to a continuous generation of mutant genomes during RNA virus replication, a nonlinear, nearly deterministic evolutionary behaviour has been observed. It is proposed that such a behaviour is mediated by a low-pass filter (averaging of mutational noise signals) due to competitive selection among variants.

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Introduction

Mutation rates for RNA viruses are usually between 10^{-4} to 10^{-5} mutations per base incorporated per round of replication (Domingo & Holland, 1994). As a consequence of these high mutation rates, RNA viral populations are complex mixtures of different but closely related variants which replicate while being subjected to continuous mutation and competitive selection. Such extremely variable populations are known as quasispecies

(Eigen, 1971; Eigen & Schuster, 1977, 1978a,b; Eigen *et al.*, 1987, 1988). A viral quasispecies can be characterized by a consensus or average sequence that may coincide with the most represented sequence called the master sequence. For any given environment each mutant from the quasispecies has a relative fitness value (replicative value) directly dependent of its genetic composition and of the mutant spectrum surrounding it (de la Torre & Holland, 1990; Eigen *et al.*, 1988). The first experimental demonstrations of the quasispecies nature of RNA virus populations were reported for the bacteriophage Q β (Domingo *et al.*, 1978) and with mini-variants derived from Q β RNA (Biebricher, 1983). Since then, many RNA viruses have been shown to have a quasispecies structure (reviewed by Domingo & Holland, 1988, 1994; Holland *et al.*, 1992).

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Abbreviations used: VSV, vesicular stomatitis virus; wt, wild-type; MARM, monoclonal antibody-resistant mutant; p.f.u. plaque forming units.

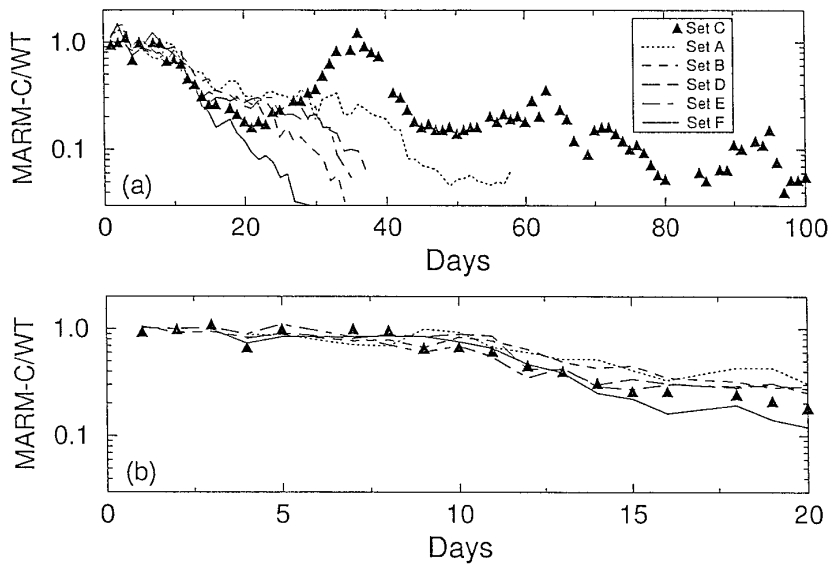


Figure 1. Change of ratio MARM-C:wt during competitions in BHK₂₁ cells. A mixture of MARM-C and wt at a 1:1 ratio (2×10^5 p.f.u.) was used initially to infect six BHK₂₁ monolayers (2×10^6 cells). After complete cytopathic effect (about 24 hours) ratios MARM C:wt were determined, and again around 2×10^5 p.f.u. (out of the total 10^{10} viral yield) were used for the second passage (see Materials and Methods). Competitions were followed during 30 to 100 days. (a) Shows the results of each of the six independent Sets (A to F) during long term competitions. (b) Shows the first 20 days of competition in detail.

RNA viruses follow many of the basic principles of classical evolutionary biology (Chao, 1990; Clarke *et al.*, 1994; Domingo *et al.*, 1996; Duarte *et al.*, 1992). Work with vesicular stomatitis virus (VSV) has shown that when two populations are allowed to replicate in long-term competitions, eventually one or the other excludes the competitor (Clarke *et al.*, 1994), supporting the competitive exclusion principle of population genetics (Gause, 1971). Both the winners and the losers in these competitions gain fitness (Clarke *et al.*, 1994), as predicted by the Red Queen hypothesis (Van Valen, 1973). Newly arising, higher fitness mutants often outcompete lower fitness mutants, and thus quasispecies can adapt rapidly to a changing environment.

Despite the underlying stochastic process of mutation, this report documents a nonlinear, highly predictable population dynamics of RNA viruses, as previously included in the framework of the quasispecies theory of molecular evolution (Eigen, 1971, 1992; Eigen & Schuster, 1977, 1978a,b; Eigen *et al.*, 1987, 1988). Eigen and co-workers proposed a rather deterministic model for RNA viral populations in which, despite the stochastic nature of mutation, mutants with higher adaptive (or relative fitness) value determine the evolutionary route, and whole quasispecies rather than individual genomes, are the units of selection. Viral quasispecies provide a framework to examine possible deterministic evolutionary features in a quantitative way.

Here we describe the evolution of a VSV virus population named wild-type (wt), in competition with a genetically marked mutant population resistant to a monoclonal antibody (MARM-C) which was derived from wt virus. MARM-C/wt ratios were determined as a function of passage number. A highly predictable, nonlinear behaviour of these two competing populations as well as the

presence of critical points in the outcome of the evolutionary process are described. The results suggest that selection within the competing quasispecies may act as a low-pass filter to generate a highly reproducible behaviour. Biological implications in terms of population fitness gains and mutational load are discussed.

Results

Outcome of repeated competition passages between viruses

Wild-type VSV (Mudd-Summers strain, Indiana serotype; Mudd & Summers, 1970) (assigned a fitness value, $W = 1$) was mixed with a MARM-C mutant resistant to I1 monoclonal antibody (neutral variant, relative fitness value of 1 compared to wt, described in Materials and Methods). With this initial mixture, six parallel passage Sets were carried out, and the proportion of MARM-C and wt populations was determined at each passage (day; Figure 1). In all six Sets during a period of 8 to 11 passages the ratio of MARM-C to wt virus remained very similar to the starting mixture. Then, the proportion of MARM-C gradually decreased until it became undetectable by passage 60 in five out of six passage series (Figure 1(a)). The rate of MARM-C decline in Sets A, B, D, E and F was different in each case. In Set C, MARM-C was also eventually outcompeted by wt, but in this case the competition followed a remarkable nonlinear fluctuating dynamics which was followed during 100 consecutive days (Figure 1).

Critical points in RNA virus evolution

Passage Set C was studied in more detail by following the proportion of MARM-C and wt in several replicas of the same starting viral mixtures

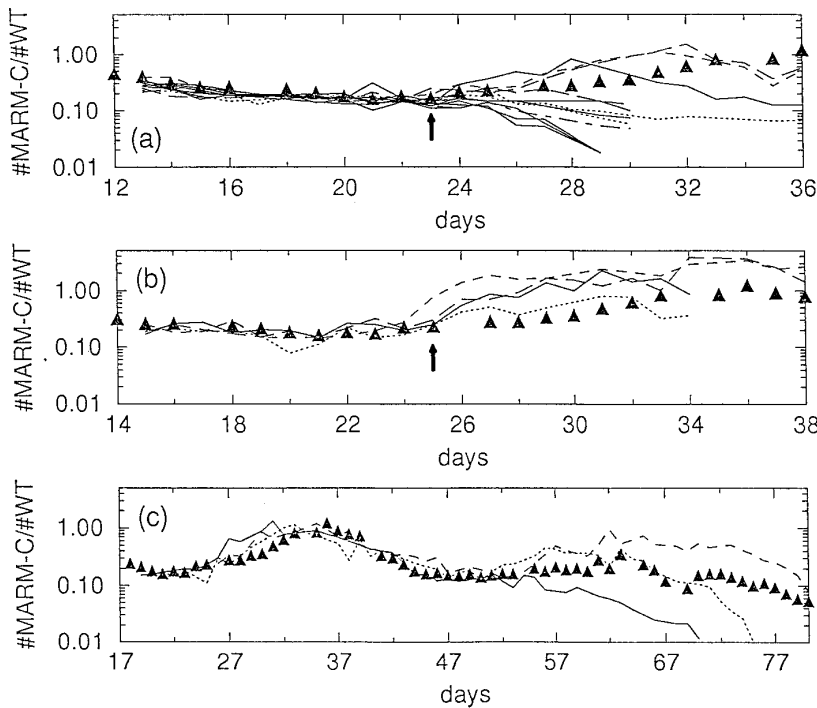


Figure 2. Changes of MARM-C:wt ratio in independent replicas of competition Set C (depicted as in filled triangles, Figure 1(a)). In each panel the MARM-C:wt ratio of the original competition Set C (Figure 1) is shown in filled triangles. (a) Eighteen replicas restarted from passage 12. (b) Six replicas restarted from passage 14. (c) One replica from each of passages 18 (continuous line), 20 (dotted line), 25 (broken line). Critical points are indicated by arrows.

(Figure 2). The results showed two remarkable features. First, the behaviour of both competing viral populations was highly predictable and reproducible before apparently reaching a critical

point; and, second, critical points were reached after a nearly constant number of passages (arrows in Figures 2 and 3). A critical point is defined as the time point at which variance among replicas

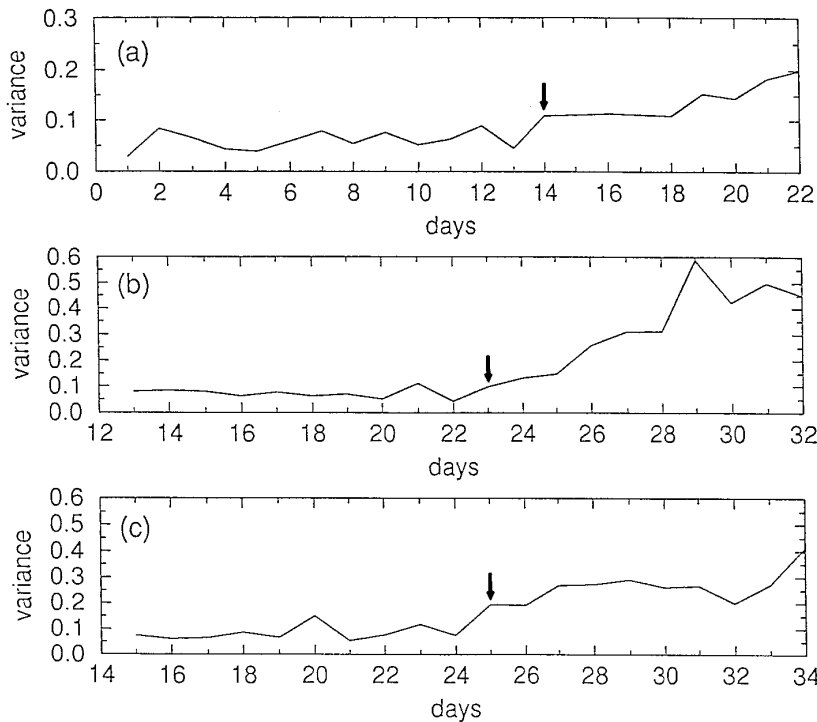


Figure 3. Analysis of variance of MARM-C:wt ratios from several competition Sets. (a) Variance of competition Sets A to F depicted in Figure 1(a). (b) Variance of the eighteen replicas starting from passage 12 of Set C, and depicted in Figure 2(a). (c) Variance of the six replicas starting from passage 14 of Set C, and depicted in Figure 2(b). Critical points indicated by arrows were observed when variances were higher than the experimental error of 0.08. From these critical points trajectories diverged rapidly. Variances were calculated as:

$$\sigma(t) = \sqrt{\frac{1}{N} \sum_{i=1}^N (\text{Mean}(t, i) - \log_{10} C(t, i))^2}$$

where

$$\text{Mean}(t) = \frac{1}{N} \sum_{i=1}^N \log_{10} C(t, i)$$

and

$$C(t) = (\text{MARM} - C/\text{wt})(t)$$

(where N is the number of replicas and t is the time in days). Assuming that the variance of the fitness ratio distribution also increases exponentially in time $\sigma = \sigma_0 \exp(\alpha t)$, the critical point might be associated with time (t_{cr}) when the variance first exceeds the measurement error level $t_{cr} = \alpha^{-1} \ln 0.08/\sigma_0$.

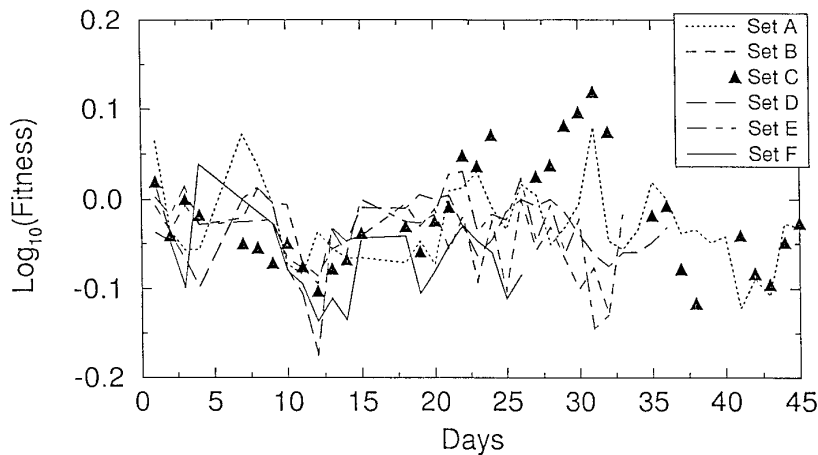


Figure 4. Kinetics of relative fitness (W) values during daily passages from Sets A to F (Figure 1). Experimental error of fitness determinations was reduced by filtering the data with a moving average of three days using the formula $w(t) = C(t+1)/C(t)$. This transformation was possible because of the normal distribution of independent measurements, and allowed us to reject noise due to experimental error as the cause of the observed variation.

cannot be explained by the experimental error in the measurement of MARM-C/wt ratios. The experimental error was calculated as the variance of the distribution of the repeated measurements of the $\log_{10} \text{MARM-C/wt}$, and was 0.08. To find the time of trajectory divergence (critical point), the variance at each point was compared with the variance due to sampling (Figure 3). The time point of divergence is the point at which the variance could not be accounted for by experimental error (arrows in Figure 3). The critical points occurred within a narrow time frame.

A filter imposed by selection

As expected from earlier studies on the Red Queen hypothesis as applied to RNA virus evolution (Clarke *et al.*, 1994; reviewed by Domingo *et al.*, 1996) both populations acquired an average increase of fitness during replicative competition (not shown). Although the relative fitness values in the time Sets were noisy (Figure 4), MARM-C fitness values generally tended to be lower than wt fitness at each passage. In biological terms, the noise in fitness values is expected from the continuous and stochastic generation of mutant variants, which may alter relative fitness values. However, due to a very large number of mutants simultaneously present in the quasispecies, this noise may be effectively averaged out in the selection process (Clarke *et al.*, 1994; Novella *et al.*, 1995a). Using terminology of signal processing, this selection constitutes a low-pass filter between the multiple stochastic mutations and the overall fitness of the populations. This gives rise to a rather smooth deterministic evolution with relatively small high-frequency oscillations of average fitness. This filter constrains the possible outcomes of competition, thus inducing a highly reproducible evolutionary pattern. In contrast, a more erratic and unpredictable behaviour was seen when quasispecies populations were reduced to relatively few infectious particles (genetic bottleneck) at one or more passages (Duarte *et al.*, 1992, 1994).

Discussion

The standard tools of nonlinear dynamics for defining deterministic behaviour require an amount of data which far exceeds that generally amenable to experimental determinations in biological systems. The deterministic nature of the evolution of a quasispecies as was originally defined by Eigen and colleagues (Eigen, 1971, 1992; Eigen & Schuster, 1977, 1978a,b; Eigen *et al.*, 1987, 1988) can only be approached, albeit in an approximate fashion, with experimental systems that permit the analysis of the behaviour of many replicas of the same biological entities. Here we have reported that different runs of competition started from the same initial mixture of two virus populations, reproduced the same dynamics for a long time within the experimental error inherent to the measurements. Therefore, the viral competitions showed indications of determinism. Nevertheless, after some period of reproducible behaviour (about 12 days) trajectories corresponding to different tests, diverged rather quickly. We call these moments of divergence critical points of evolution. From the point of view of nonlinear dynamics, these critical points bear resemblances to glitches which interrupt deterministic trajectories of chaotic systems in the presence of noise (see Grebogi *et al.*, 1990). Random mutations (equivalent to noise in dynamical systems) brings a system to a different deterministic trajectory with its particular glitch. The biological nature of the critical points is not yet understood. Careful measurements of the fitness distribution within quasispecies accompanied by sequencing before and after critical points may shed some light on their origin. It was previously established that a virus population with lower average fitness value has a greater probability to undergo advantageous mutations than does a more highly fit population (Novella *et al.*, 1995a,b). This may help explain why MARM-C virus populations on occasions (Set C in Figure 1) can recover even when they may be on the way to be outcompeted by wt virus. However, fitness gains

and their effect on virus evolution may be punctuated and irregular (Clarke *et al.*, 1994; Nichol *et al.*, 1993; Novella *et al.*, 1996; Pelletier *et al.*, 1995). It is interesting that after restarting serial infections from passages 18, 20 and 25 (Figure 2(c)), trajectories were very similar to those of the original reference competition, and followed the same general fluctuation pattern. This suggests that the advantageous mutations which caused the fluctuations, were either already present in the quasispecies or occurred later with high probability. Both, the virus population history and mutational events probably contribute to quasispecies evolution during regular competition dynamics. Despite the same initial average fitness of wt and MARM-C, the latter may have a compromised constellation of mutations which affects its movement through the fitness landscape, limiting the extent of its fitness gain relative to the wt. The possibility that MARM-C may be inherently limited in adaptability relative to its parental wt virus is now under study. Also, possible genetic changes leading to the occurrence of critical points in which the competition trajectories diverge remain to be identified in future research.

The experimental results reported here are in excellent agreement with the concepts of quasispecies populations proposed by Eigen and co-workers and reinforce the concept that whole quasispecies rather than individual genomes are the target of selection (Eigen, 1992; Eigen & Schuster, 1977, 1978a,b; Eigen *et al.*, 1987, 1988). Despite the stochastic generation of mutations, regular RNA viral competitions can follow a highly reproducible behaviour. This may also be at the basis of previous observations of viral fluctuations of foot-and-mouth disease virus serotypes in tissue culture (Woodbury *et al.*, 1995), and may also contribute to the long-term invariance of consensus sequences in spite of the continuous appearance of new mutant genomes (Domingo *et al.*, 1978; Nichol *et al.*, 1993; Steinhauer *et al.*, 1989). A dynamical theory to explain biphasic fitness increases during large population passages of VSV (Novella *et al.*, 1995a) has been recently presented (Tsimring *et al.*, 1996). Work on a specific model to try to simulate viral competitions is now in progress.

It is noteworthy that both wt and MARM-C populations, as well as any other RNA viral population, when replicating in infected cells become complex quasispecies. Thus, the nearly deterministic effects documented here may probably reflect the behaviour of other evolving quasispecies, not just a peculiarity of this particular mixed population system. It must be recognized, however, that the reproducible behaviour documented was probably favored by the close genetic relatedness of wt and MARM-C, and by the constancy of the cell culture environment in which viral replication took place. It will be difficult to provide evidence for deterministic behaviour of viruses in nature, because of the number of variables which affect such complex quasispecies at

any time. However, in natural evolution, some viruses exhibit periods of replicative evolutionary stasis, also called of population equilibrium (Domingo & Holland, 1988) in spite of continuous viral replication. Such evolutionary stasis can at times be followed by periods of rapid evolution, usually when the selective environment changes (Nichol *et al.*, 1993; Webster *et al.*, 1992). Recently, it has been shown that artificially reproducing the first step in the origin of a recombinant species of sunflower (*Helianthus anomalus*), the resulting hybrid lines converged to a nearly identical gene combination despite the different crossing schemes used. Moreover, the gene combinations evolving in the experimental lines were similar to those seen in the naturally occurring *H. anomalus* (Rieseberg *et al.*, 1996). This is a clear example of highly reproducible complex evolutionary steps leading to speciation.

Experiments are now in progress to analyze effects of various factors (changes of temperature, presence of mutagens, interference by defective particles, etc.) on the evolutionary dynamics of competing viruses. It may also be possible to mix virus from different passages with different trajectories at different ratios to observe variation in fitness dynamics, and to determine possible points of the trajectory associated to evolutionary instability.

Materials and Methods

Cells and viruses

BHK₂₁ cells were grown as cell monolayers in Eagle's minimum essential medium containing heat-inactivated (60°C, 30 minutes) bovine calf serum. A wild-type population of vesicular stomatitis virus (VSV) Mudd-Summers strain, Indiana serotype (Mudd & Summers, 1970) and a genetically-marked monoclonal antibody-resistant mutant (MARM-C) were used. The competing wild-type (wt) quasispecies population has been replicated in our laboratory exclusively on BKH₂₁ cells. It was stored frozen at -85°C. This stock is called the wild-type (wt), and it was assigned a fitness value (*W*) of 1.0, to be used as a fitness internal reference point (Holland *et al.*, 1991). MARM-C is a subclone of wt obtained after 20 bottlenecks (plaque-to-plaque) passages of a VSV clone resistant to I1 monoclonal antibody. MARM-C has an Asp259 → Ala substitution in the surface glycoprotein. This clonal MARM-C population, in short-term competitions (up to eight competition passages), exhibits a fitness value of 1 (neutral fitness) with respect to the competing parental wt population.

Virus passages and competitions

Transfer of large virus populations and competition assays were carried out as previously described (Duarte *et al.*, 1994; Holland *et al.*, 1991). Briefly, samples of wt and MARM-C populations were mixed at a starting proportion of 1:1. From the same initial mixture, six independent competition Sets were followed (A to F). In each competition series, a new monolayer of BHK₂₁ cells was infected with 10⁵ to 10⁶ plaque-forming units (p.f.u.) from the previous competition passage and incubated

until cytopathic effect was complete. Each passage involved one day needed for complete cytopathic effect. Thus, in this report time (in days) and passage number are used indistinctly. The average multiplicity of infection at each competition passage was adjusted to about 0.1 p.f.u./cell, in order to avoid interference by defective interfering particles (Holland, 1990). Ratios of MARM-C:wt were calculated daily by triplicate plaque assay in the presence and absence of I1-monoclonal antibody as described (Duarte *et al.*, 1994; Holland *et al.*, 1991). Samples of each viral competition were frozen at -85°C , to allow to rerun each virus population history from any time point, providing a very powerful tool to study evolution and population dynamics in replicate series of experiments.

Statistical analysis of data

Multiple time series of the proportion of MARM-C and wt VSV were analyzed using standard statistical tools (Lee, 1982). The variance was calculated at each passage, and its dynamics was observed in the original run starting from the initial competition mixture, and also in replicas starting from intermediate passages. Calculations are detailed in the relevant figure legends. This procedure allowed the identification of critical points in the competitions as those passages (days) at which variance exceeded a noise level.

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