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Quasispecies dynamics and RNA virus extinction

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Abstract

The extinction of foot-and-mouth disease virus (FMDV) is strongly influenced by mutation rates, types of mutations, relative viral fitness and virus population regimens during infection. Here we review experimental results and theoretical models that describe a contrast between the effective extinction of FMDV subjected to increased mutagenesis, and the remarkable resistance to extinction of the same and related FMDV clones subjected to serial bottleneck events. The results suggest procedures to master key parameters to develop effective antiviral strategies based on virus entry into error catastrophe.

Keywords: Foot-and-mouth disease virus; 5-Fluorouracil; 5-Azacytidine; Mutation

1. Introduction

Critical for a therapeutic application of error catastrophe as an antiviral strategy is to understand the main factors (intrinsic to the virus as well as those related to population dynamics) that may contribute to loss of infectivity. This has proven a complex issue and the experiments carried out so far have raised more questions than provided answers. Here we review studies carried out over the last decade with the important animal pathogen foot-and-mouth disease virus (FMDV) aimed at understanding how high mutation rates and quasispecies dynamics (as opposed to low mutation rates and a non-quasispecies dynamics) affected the accumulation of mutations, fitness variations, and virus survival. The main point of this article is to compare the remarkable capacity to drive FMDV into error catastrophe when the effects of three critical parameters (mutation rates, viral fitness, and viral load) are understood and controlled, with the resistance to extinction despite accumulation of mutations upon subjecting FMDV to repeated bottleneck events (experimentally realized as plaque-to-plaque transfers). These very different responses regarding survival may shed light on strategies whose goal is the elimination of virus during infectious processes in vivo.

As recent introductions to FMDV the reader is referred to the volumes by Rowlands (2003) and Sobrino and Domingo (2004). For clarity, Table 1 includes a glossary of concepts and terms used in this article and in the literature on quasispecies and error catastrophe.

2. Extinction of FMDV by enhanced mutagenesis

Following pioneer work on the adverse effects of chemical mutagenesis on the infectivity of poliovirus and vesicular stomatitis virus (VSV) by Holland et al. (1990) and Lee et al. (1997), our group set to study the effect of the mutagenic base analog 5-fluorouracil (FU) and the nucleoside analog 5-azacytidine (AZT) on the infectivity and mutant spectrum complexity of FMDV (Pariente et al., 2001, 2003; Sierra et al., 2000). These experiments, and the effect of ribavirin on persistent FMDV infections (Airaksinen et al., 2003; de la Torre et al., 1987), are reviewed in detail by (Pariente et al., 2005). Here we extract the main conclusions only to serve

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Table 1
Glossary of some terms frequently used in the literature of quasispecies and error catastrophe

Complexity of the mutant spectrum	A measure of the nucleotide sequence differences among components of a mutant spectrum: it is generally given by the **mutation frequency** and **Shannon entropy**. Complexity has other meanings in science, including size of genomes, used also in the text
Consensus (or average) sequence	The sequence resulting from taking for each position the most frequent residue (nucleotide or amino acid) found at the corresponding position in the homologous set of aligned sequences: the consensus sequence may not exist physically in the mutant spectrum
Error threshold	A critical error rate above which the information encoded by a genetic system cannot be maintained: violation of the error threshold results in the system entering **error catastrophe**. The error threshold relationship is given by $\nu_{max} < \ln \sigma_o/(1-\bar{q})$ in which ν_{max} is the maximum length of the sequence (genetic complexity) that can be maintained during replication, σ_o is the superiority of the master sequence relative to the mutant spectrum, and $\bar{q}$ is the average copying fidelity (the average error rate is $(1-\bar{q})$)
Fitness	A parameter that quantifies the adaptation of an organism or a virus to a given environment: it is necessarily a relative value. For a virus, relative fitness measures its ability to produce infectious progeny relative to a reference viral clone, in a defined environment. **Epidemiological fitness** describes in semi-quantitative ways (sampling of definitory genomic sequences versus those of competitors) the relative capacity of a virus to become dominant in the field during (or as a result of) epidemic outbreaks
Master sequence	The dominant genomic nucleotide sequence in a quasispecies: it generally depicts a selective advantage over the other components of the quasispecies. It may or may not coincide with the consensus sequence. The master sequence may change as the environment is modified
Mutant spectrum	The ensemble of mutant genomes that compose a quasispecies
Mutation frequency	The proportion of mutants in a population of genomes: it may be calculated for an entire sequence of for a specific site of a genome (such as in the frequency of monoclonal antibody-escape mutants)
Mutation rate	The frequency of occurrence of a mutational event during genome replication: in the literature of population genetics, mutation rate is often used to mean the rate of fixation (or accumulation) of mutations, or rate of evolution
Population number	The number of individuals in a population: for viruses, the term refers to the number of infectious genomes present in a cell, tissue, organ or organism that at any given time are either replicating or can potentially replicate. The number of genomes quantified in an infected host is also termed the viral load. Not all viral genomes are infectious (see **Specific infectivity**)
Preextinction viral population	A mutagenized viral population that precedes the one from which no infectivity can be rescued: **Preextinction RNA** is the RNA extracted form a preextinction population
Quasispecies	A weighted distribution of mutants centered around one master sequence: in its initial mathematical formulation, a quasispecies was a steady state distribution of infinite size in equilibrium. Mathematical extensions to finite population under non-equilibrium conditions have been developed. Virologists use an extended definition of quasispecies meaning "dynamic distributions of non-identical but closely related mutant and recombinant viral genomes subjected to a continuous process of genetic variation, competition and selection, and which act as a unit of selection"
Rate of fixation (or accumulation) of mutations	The frequency of mutations that become dominant in a genome per unit time: for a virus it may be calculated for sequential genomes in an infected host of for viral genomes sampled at different times from different infected hosts. For viruses this rate is generally not constant. The assumption of a "molecular clock" is not realistic for RNA viruses
Sequence space	A theoretical representation of all possible variants of a genomic sequence: for a single stranded RNA virus of 10,000 residues (using four types of nucleotides) the total sequence space is $4^{10,000}$! Viral genomes occupy tiny portions of their theoretical sequence space
Shannon entropy	The proportion of different nucleotide sequences in a mutant spectrum (a value of 1 means each sequence is unique in the distribution; a value of 0 means that all sequences are identical)
Specific infectivity	The proportion of infectious particles (or infectious viral nucleic acid) in a viral (or viral genome) population: the transition into error catastrophe is generally preceded by decreases in specific infectivity
Viral load	The number of infectious (actively replicating or potentially replicating) particles in a viral population

Based on Domingo (1999, 2003) and Eigen (1992).

as the basis to compare extinction mutagenesis with survival despite accumulation of mutations associated with bottleneck events.

During cytolytic infections in cell culture, low viral load and low relative fitness favored extinction of FMDV by FU and AZC. Mutagenized populations, including preextinction RNA, did not show mutations in the consensus sequences analyzed, but displayed an increase in the complexity of mutant spectra. The maximum increases in complexity occurred in the polymerase (3D) gene, which is very conserved in FMDV. Several amino acid replacements found in the mutant spectrum of 3D in mutagenized populations have never been observed in natural or laboratory populations of the virus (Sierra et al., 2000; Airaksinen et al., unpublished results; review in Pariente et al., 2005), suggesting that occurrence of highly deleterious mutations is associated with proximity to the error threshold (Eigen, 2002).

The same mutagenic agent can have very different effects on the extinction of different viruses, for reasons that are not well understood. This is illustrated by comparing the results with FMDV with those obtained with the prototype arenavirus lymphocytic choriomeningitis virus (LCMV) (see

also accompanying article by de la Torre, 2005). LCMV is far more sensitive to FU mutagenesis than FMDV since in serial passages carried out at comparable m.o.i. and viral load, LCMV was extinguished after 2–3 passages in the presence of 50–200 μg/ml of FU while extinction of FMDV was stochastic and required around 16 passages in the presence of 200 μg/ml of FU in the culture medium (compare Sierra et al., 2000 with Grande-Pérez et al., 2002 and Ruíz-Jarabo et al., 2003). Possible reasons for such a different susceptibility to a mutagen include that (i) the polymerases of the two viruses show different affinity for fluourouracil-triphosphate (FUTP) as an abnormal nucleotide substrate, or different capacity to incorporate FUMP in the growing RNA chain; (ii) FU exerts stronger inhibitory activity on LCMV than on FMDV, despite a comparable intrinsinc mutagenic activity; this would result in a mutagenesis-independent reduction of the viral load, thereby favoring viral extinction (Pariente et al., 2001); (iii) the essential L (polymerase) gene of LCMV is 4.7-fold larger in complexity (size in nucleotides) and represents a larger proportion of the viral genetic information than the polymerase (3D) gene of FMDV (62% versus 17%); this may favor a higher frequency of deleterious mutations in the LCMV genome; (iv) the types of mutations induced by FU (mainly A → G and U → C) may affect more codons related to critical viral functions in LCMV than in FMDV. Whether this may be a significant influence will only be known when a dissection of the biological functions of individual amino acids (and sets of amino acids) together with their tolerances to accept substitutions are known. These are just four of many imaginable possibilities (and their combinations) that may underlie a different response of two viruses to the same mutagens. It seems quite obvious that many issues will have to be approached to control processes of lethal mutagenesis.

The point we want to address next is the resistance to extinction of the same FMDV clones vulnerable to mutagenesis, despite accumulation of mutations as a result of plaque-to-plaque transfers.

3. Accumulation of mutations in FMDV clones subjected to serial bottleneck events

Mutation rates during RNA virus replication are in the range of 10^{-3} to 10^{-5} substitutions per nucleotide (Batschelet et al., 1976; Drake and Holland, 1999). This means that an average in the neighbourhood of 0.5–1 mutation occurs each time a viral genome is copied into a complementary strand. Unavoidably, since many mutations are compatible with genome replication and viral multiplication, viral populations rapidly evolve into complex distributions of non-identical but closely related genomes, termed viral quasispecies (reviews in Domingo et al., 2001; Eigen, 1996; Eigen and Biebricher, 1988). Thus, RNA viruses exist as mutant spectra, meaning that, at any given time, individual genomes differ in one or several position from the consensus sequence (terms in Table 1). A mutation has a higher probability of being deleterious than advantageous. (This is because for any complex genetic organization, evolved over long time periods, there is an intricate interconnection of functions that is easier to perturb than to improve; viruses are not an exception.) As a consequence, repeated bottleneck events acting on mutant spectra will result in a progressive deviation from the initial consensus sequence, and a concomitant decrease of viral fitness. Experimentally, serial bottleneck events are accomplished by serial plaque-to-plaque transfers (Fig. 1). Average fitness losses accompanying plaque-to-plaque transfers constitute experimental evidence of the operation of Muller's (1964) ratchet, first proposed as a theoretical concept. It predicted that asexual populations of organisms should tend to accumulate deleterious mutations unless sex or recombination intervene as compensatory mechanisms (Maynard-Smith, 1976; Muller, 1964). The first experimental evidence of the operation of Muller's ratchet was obtained by Chao (1990) working with bacteriophage ϕ6, and then the evidence was extended to a number of animal viruses (Duarte et al., 1992; Elena et al., 1996; Yuste et al., 1999, 2000), including FMDV (Escarmís et al., 1996, 1999).

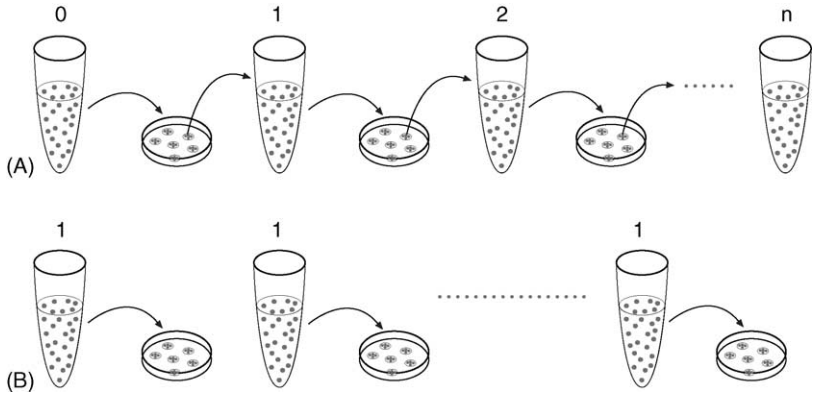

Fig. 1. Scheme of plaque-to-plaque transfers of a virus. (A) A viral population (0) is diluted and plated. Virus from a single plaque is diluted (1) and plated again. The process is repeated *n* times. Fitness evolution and accumulation of mutations can be studied by analyzing part of the same viral suspension used for a transfer, as discussed in the text. (B) As a control for possible fitness variations due to environmental factors and not to the serial plaque-to-plaque transfers, the same virus population (1) is plated in parallel, as discussed in the text.

The studies with FMDV were designed to explore: (i) the variation of relative fitness of FMDV clones subjected to serial plaque-to-plaque transfers; (ii) the number and types of mutations that accumulate in the genome as a consequence of the repeated bottleneck events; (iii) the use of numerical simulations to identify those parameters that can most influence the behavior observed. As a result of the theoretical investigations related to (iii), information has been obtained on mechanisms that can prevent (or delay) FMDV transition into error catastrophe. First, we examine the main conclusions derived from the experiments.

3.1. Biphasic evolution of fitness values and remarkable resistance to extinction of FMDV clones

Several FMDV clones, derived from the same biological clone C-S8c1 which is the standard FMDV used in our laboratory (Sobrino et al., 1983), have been subjected to plaque-to-plaque transfers (Fig. 1), with very similar conclusions. In these experiments the number of plaque-forming units titrated in a viral plaque was taken as a measure of relative fitness value. Usually, the time allowed for plaque development on monolayers of BHK-21 cells under our standard plating conditions (Escarmís et al., 1996) was 24 h. For some clones, this time was prolongued to 48 h to allow potential slow-growing plaques to become visible. During the first 20–40 transfers, relative fitness fluctuated around an average value that decreased in a nearly exponential fashion. Then, in the following transfers (in some cases transfer 280 has been reached), the amplitude of the fluctuations increased but the average fitness values remained constant (Escarmís et al., 2002; Lázaro et al., 2002) (Fig. 2). The main biological consequence of this behavior is a remarkable resistance to extinction since, when a clone reached a fitness valley, some mutational event appeared to trigger a sudden fitness gain (see Section 3.2). Some extinction events, however, have been documented: when the mutation frequency reached 6.5×10^{-3} mutations/nucleotide (after 190 serial transfers) in clones that had acquired an unusual genetic lesion consisting of an internal oligoadenylate (described in Section 3.2), the virus was presumably unable to acquire a compensatory mutation that could trigger a fitness gain and the virus became non-infectious in the next transfer (Escarmís, unpublished results).

The amplitude of the fitness oscillations was dependent on both the virus and the host cells, and it was larger the lower was the relative fitness of the virus subjected to plaque transfers. A statistical analysis of fitness values of the evolving clones indicated that they were best described by a Weibull distribution. In contrast, the fluctuations in control, non-evolving populations (the same population plated each time (as depicted in Fig. 1B)) followed a log-normal distribution (Lazaro et al., 2003). The presence of fluctuations in physical and biological systems is often indicative of the involvement of several interacting components simultaneously in the system. Log-normal distributions usually characterize multiplicative processes in which small differences are amplified considerably giving rise to fluctuations in the parameters measured at the end of the process (in this case a virus titer). Probably, the lognormal distribution of the titers

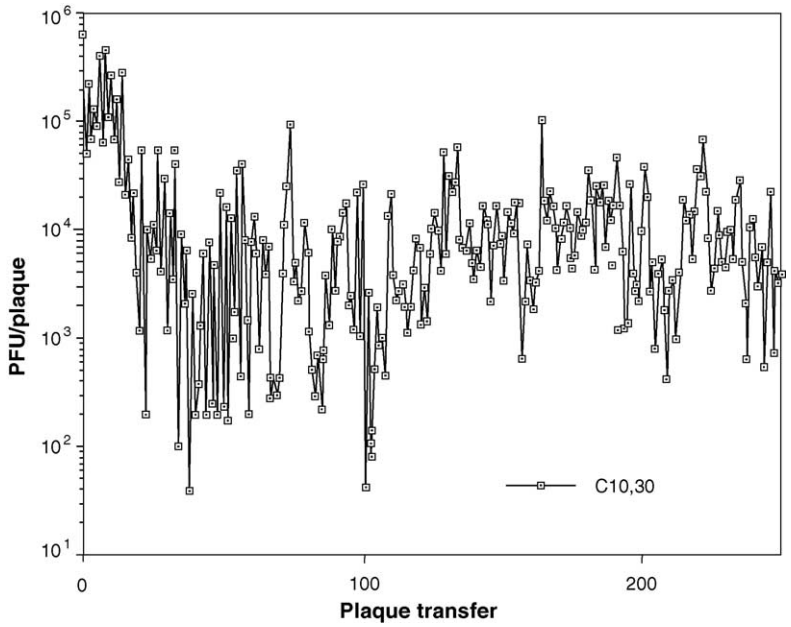

Fig. 2. Typical pattern of fitness evolution of a FMDV clone subjected to plaque-to-plaque transfers. The pfus per plaque (ordinate) are taken as a relative fitness value; the plaque transfer number (*n* in Fig. 1) is shown in abscissa. Each point corresponds to one transfer. A decrease of relative fitness value can be observed up to about transfer 40, and this is followed by a fluctuating pattern with a constant average fitness. This behavior has important implications for the understanding of quasispecies dynamics, as discussed in the text.

of the non-evolving, control viruses has its origin in a complex dependence of replication on the cellular state, since the latter influences several steps in the virus life cycle that must be independently completed for virion maturation to occur. In the evolving populations, repeated bottlenecks with new mutations preceding each founder event allow for larger variations in the initial state and larger fluctuations in fitness values which distribute according to a Weibull function (Lazaro et al., 2003; Manrubia et al., 2003). Put in even more general terms, the Weibull distribution reflects both a high mutation rate during each plaque development, and the dependence of the virus on the host cell. The genetic information in RNA viruses in general, and in picornavirus in particular, is very compact. Each genomic region and viral protein are involved in multiple functions in interdependence with cellular functions (as support of this concept for picornaviruses, see the different chapters of Semler and Wimmer (2002)). As a consequence, most mutations occurring in genomes that initiate plaque formation may trigger a cascade of alterations in the host–virus relationship, reflected in a modification of virus yield. In this view, many mutations should have a non-trivial effect on virtually each process of plaque development. It is now important that we examine experimental results on mutations in virus from different plaques and in viral genomes found within plaques.

3.2. Some mutational correlates of fitness variation

Fifty percent of the FMDV clones subjected to plaque-to-plaque transfers showed an unusual genetic lesion, consisting of an elongation of the number of adenylate residues that precede the second functional AUG translation initiation codon (Fig. 3A). The length of this internal oligoadenylate tract tends to increase with the number of plaque transfers and it correlates negatively with the relative fitness of the FMDV clone (Escarmís et al., 1996, 1999) (Fig. 3B). The

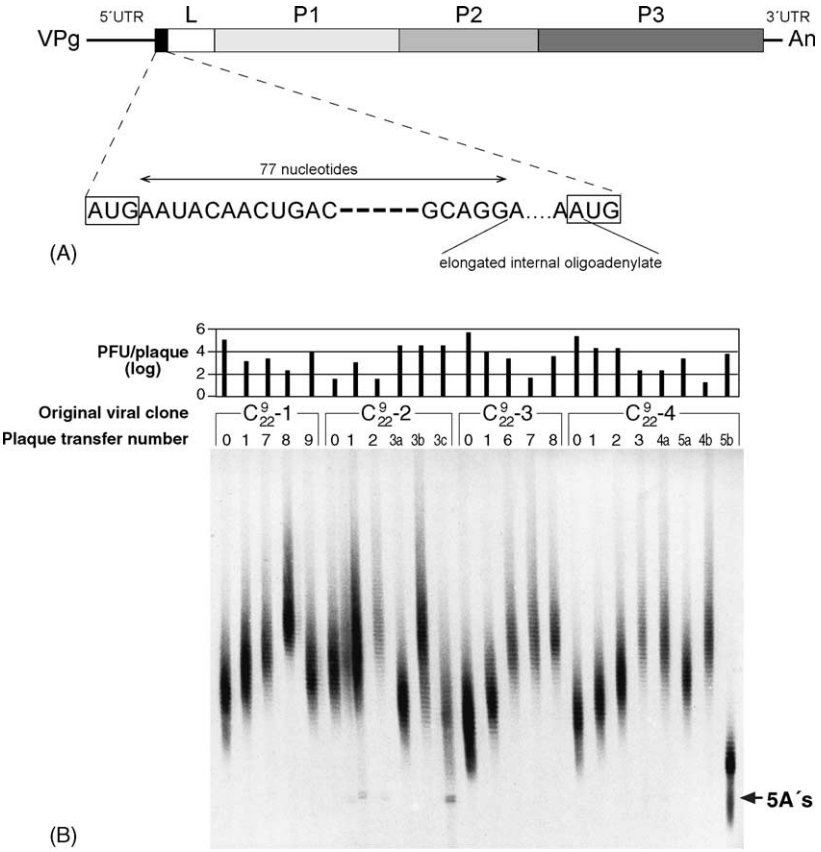

Fig. 3. Internal oligoadenylate tract generated upon serial plaque-to-plaque transfers of FMDV clones. (A) Scheme of the FMDV genome and the location of the elongated internal oligoadenylate. Lines depict regulatory 5′- and 3′-untranslated regions (UTR). VPg is the protein linked to the 5′-end of the RNA and An is the 3′-terminal polyadenylate. The open reading frame is depicted by horizontal boxes: L is the leader protease, P1 is the capsid-coding region and P2, P3 are the non-structural protein-coding region (for review see different chapters of Rowlands, 2003; Sobrino and Domingo, 2004). At least two forms of L, termed Lab and Lb are synthesized in FMDV-infected cells from two in-frame, functional AUG initiation codons. The coding region expressed only in Lab is indicated in black, and part of the nucleotide sequence between the two functional AUGs is given. The internal oligoadenylate is formed by elongation of four adenylate residues that precede the second AUG (Escarmís et al., 1996). Probably elongation occurs through slippage mutagenesis (Arias et al., 2001). (B) Correlation between the amount of infectious virus (pfu) per plaque and the size of the oligoadenylate tract. The measurements involved four subclones (1–4) of clone C^9_{22}, and in C^9_{22}-2 and C^9_{22}-4 also analysis of several subclones (a–c) as the progeny of the same parental clones. The range of lengths of the heterogeneous oligoadenylate was determined in a nucleotide sequencing gel (bottom part of the figure; the 5 adenylate size marker is indicated). The results show that in general a longer oligoadenylate tract corresponds to a lower virus titer per plaque (based in Escarmís et al., 2002).

deleterious effect of the tract was occasionally overcome during plaque development, either by a decrease in the number of adenylate residues or by a single A to G transition within the tract (Escarmís et al., 1999). In one of the clonal lineages, the sequence of the internal oligoadenylate tract was determined at each successive plaque transfer from number 23–50 (Escarmís et al., 2002). The oligoadenylate showed a different sequence at each transfer. In the progeny produced after nine of the transfers analyzed, the population was dominated by genomes that included interspersed G or U residues within the oligoadenylate (Table 2). These sequencing results illustrate once more the adaptive potential conferred by quasispecies dynamics: to overcome a fitness-decreasing lesion, FMDV finds alternative solutions at the molecular level. Such alternatives are manifested at different population size scales: large population passages (higher than 10^6 pfu to initiate an infection) or plaque development (1 pfu to initiate an infection) (Escarmís et al., 1999, 2002).

In the clones where, despite extensive plaque-to-plaque transfers, an internal oligoadenylate tract was not produced, fitness variations occurred and, therefore, other mutations must be involved, but their effect has not been quantified. The cases of extinction observed (described in Section 3.1) affected only clones with an internal oligoadenylate.

3.3. Within plaque genetic heterogeneity and steady accumulation of mutations

The continuous genetic variation in successive plaque transfers (Table 2), together with the oligoadenylate tract length heterogeneity of virus within a single plaque (Escarmís et al., 1996), constitute an additional demonstration that FMDV replicates as a spectrum of mutants, even in the process of plaque formation. Given the similar range of mutation rates for all RNA viruses analyzed to date (Drake and Holland, 1999) it is not too speculative to propose similar within-plaque heterogeneities for other RNA viruses, in view of the elegant studies by Yin and colleagues on mutant selection during plaque development of bacteriophage T7 (Lee and Yin, 1996; Yin, 1993).

Table 2
Evolution of the internal poly A of virus from lineage H^7_{30}

Plaque transfer[a]	Nucleotide sequence[b]							
2	AG	G	–	–	–	A_4	–	AUG
4	AG	G	–	–	–	A_4	–	AUG
6–10, 18–21	AG	A	–	–	–	A_4	–	AUG
22, 23	AG	A	–	–	–	A_5	–	AUG
24	AG	A	–	–	–	$A_{13–16}$	–	AUG
25	AG	A	–	–	–	$A_{17–20}$	–	AUG
26	AG	A	–	–	–	A_5	–	AUG
27	AG	A	–	–	–	$A_{6–7}$	–	AUG
28	AG	A	–	–	–	$A_{8–11}$	–	AUG
29	AG	A	–	–	–	$A_{24–27}$	–	AUG
30	AG	A	–	$A_{11–14}$	G	$A_{13–17}$	–	AUG
31	AG	A	–	–	–	$A_{15–17}$	–	AUG
32	AG	A	–	–	–	$A_{33–36}$	–	AUG
33	AG	A	–	–	–	$A_{20–22}$	–	AUG
34	AG	A	–	–	–	$A_{17–22}$	–	AUG
35	AG	A	–	–	–	$A_{22–24}$	–	AUG
36	AG	A	–	–	–	$A_{24–27}$	–	AUG
37	AG	A	–	$A_{17–20}$	G	$A_{8–11}$	–	AUG
38	AG	A	–	$A_{17–20}$	G	$A_{10–13}$	–	AUG
39	AG	A	–	–	–	$A_{16–19}$	–	AUG
40	AG	A	–	–	–	$A_{18–21}$	–	AUG
41	AG	A	G	$A_{16–19}$	G	$A_{13–16}$	–	AUG
42	AG	A	–	–	–	$A_{14–17}$	–	AUG
43	AG	A	–	–	–	$A_{23–25}$	–	AUG
44	AG	A	–	–	–	$A_{21–23}$	–	AUG
45	AG	A	–	–	–	$A_{24–27}$	–	AUG
46	AG	A	G	$A_{16–18}$	G	$A_{17–20}$	–	AUG
47	AG	$A_{19–21}$	G	$A_{8–9}$	G	$A_{1–2}$	–	AUG
48	AG	A	–	$A_{17–20}$	G	$A_{11–13}$	–	AUG
49	AG	A	–	$A_{18–20}$	G	$A_{12–15}$	–	AUG
50	AG	A	G	$A_{20–22}$	G	$A_{9–11}$	U	AUG

[a] Clone H^7_{30} was derived from clone H^7_1 by 30 serial plaque transfers. Clone H^7_1 was derived from a viral population obtained after 113 serial passages at high m.o.i. of C-S8c1 (Escarmís et al., 1996). Viruses from plaque transfers 1, 3, 5 and 11–17 were not analyzed.

[b] The sequence shown is that corresponding to nucleotides 1116–1125 of the FMDV C-S8c1 genome, in which a dash indicates the absence of a nucleotide, and the number of adenylate residues preceding the second functional AUG initiation codon (genomic positions 1123–1125, last column) is given as a suffix to facilitate sequence alignment. The number of adenylates have been counted from the peak pattern of the sequencing plots; a range of values indicates an heterogeneous poly A tract with the estimated minimum and maximum number of adenylates visible in the sequencing peak pattern. Numbering of nucleotides is as in Escarmís et al. (1996).

An important distinction must be made between such heterogeneity which is essential to quasispecies dynamics, and the rate of accumulation of mutations in the course of successive plaque transfers. The latter has been quantitated by determining the consensus sequence of the entire FMDV genome of the viral population in several plaques of the same lineage (Escarmís et al., 2002, unpublished results). The rate of accumulation of mutations has been estimated in about 0.25 mutations per genome and plaque transfer. Mutations accumulated linearly with plaque transfer number, even during the second phase of fitness evolution in which fitness values fluctuated around a constant value (Section 3.1). Mutations were not evenly distributed along the FMDV genome, and showed mutation clustering at some genomic regions. Clusters were located at different sites in different plaque-to-plaque lineages, and their functional basis is not known, although some possibilities (compensation of deleterious effects of an initial, triggering mutation; local decreases of polymerase copying fidelity, etc.) have been advanced (Escarmís et al., 2002).

An analysis of the nature of mutations in sequential consensus sequences revealed important differences with the mutations observed when the same parental FMDV clones were subjected to large population passages, without intervening bottlenecks. As an example, nearly 50% of the amino acid substitutions in the capsid affected internal sites, usually relatively conserved due to structural constraints (Lea et al., 1994; Mateu et al., 1994). Also, a few mutations modified the polymerase (3D) which is a highly conserved protein in FMDV. It appears as highly remarkable that no extinctions were observed until serial plaque transfer 190 (Section 3.1) when the mutation frequency relative to the parental clone was $\geq 6.5 \times 10^{-3}$ substitutions per nucleotide, a value about 10-fold larger than the average number of mutations estimated for components of the mutant spectrum of preextinction FMDV populations (Pariente et al., 2005). Critical parameters must be involved in such a disparate potential for survival.

4. Numerical models for Muller's ratchet and error catastrophe

To study the relevant parameters that determine the biphasic kinetics of fitness variation and the fitness fluctuation pattern, we carried out numerical simulations of the process with the aid of a phenomenological model (Lázaro et al., 2002). The simulations started with a single "virtual" genome which was given an arbitrary fitness value. This fitness value was proportional to the replication rate of the genome, in keeping with the definition of fitness for RNA viruses (Table 1). After a certain number of replication rounds, progeny was generated in a way analogous to virus production that leads to the development of a lytic plaque. Mutations occur during replication (Drake and Holland, 1999), resulting in a heterogeneous population (mutant spectrum) with different fitness values as documented experimentally (Section 3.3). A relevant feature of the model is that, in addition to neutral and deleterious mutations (with an occurrence rate of p per genome and replication cycle), advantageous mutations can occur with a probability of q which is smaller than p. After a certain number of replication rounds, one of the progeny genomes is chosen at random to replicate and produce a new set of progeny genomes (as in a plaque transfer), and the process is repeated as many times as desired. Upon completion of the development of each plaque, the number of genomes generated and the distribution of their fitness values are calculated. The results of these simulations are in excellent agreement with the experimental results of plaque-to-plaque transfers of FMDV clones (Section 3.1). There is a variable time interval (number of transfers) in which the number of infectious individuals decreased in a roughly exponential way. The duration of this first phase is dependent on the system parameters (fitness of the parental genome, frequency of deleterious mutations, and number of replication cycles). After this initial phase, a statistically stationary state is reached in which fitness values strongly fluctuate around a constant mean value, as observed in the experiments (Fig. 2). The more frequent the compensatory mutations are, the larger are the fluctuations in the viral yield, and the higher the average fitness of the system subjected to repeated bottleneck events. Representative simulations in which $p + q = 0.1$ and $r = 6$ (r, number of replication cycles) (Fig. 4) show that with $q = 0.005$–0.001, a good agreement with the experimental results is obtained. When a probability (d) of particle degradation is introduced (bottom four panels in Fig. 4), the system can cross the error threshold and go extinct, with a probability which is higher the lower the frequency of compensatory mutations (Fig. 5). In this view, the transition into error catastrophe occurs when the mutational pressure precludes occurrence of effectively compensatory mutations, resulting in excess replication-incompetent RNA, as directly quantified in many FMDV populations in their way towards extinction (Gonzalez-Lopez et al., 2004).

The biphasic kinetics of evolution of fitness values and fitness fluctuations were observed whenever the simulations included both, the occurrence of advantageous mutations within a certain frequency range, and the presence of an extinction threshold. The stationary state of fitness values results from an equilibrium between the trend to eliminate individuals as their fitness falls below the extinction threshold, and the selection for the subsequent transfer of a genome which is still able to replicate, probably helped by the occurrence of compensatory mutations. Thus, survival is the result of a collective property of mutant spectra: many individuals are extinguished and a minority of genetically apt individuals (generated by virtue of some classes of mutations introduced in adequate recipient genome subpopulations) permit survival. Calculations of the ratio of total genomic RNA molecules to infectious units in individual plaques gave an estimate of 10^4–10^7, depending on the relative fitness of the virus in the plaque. Remarkably, when the stationary phase of fitness values is reached, it is the distribution of

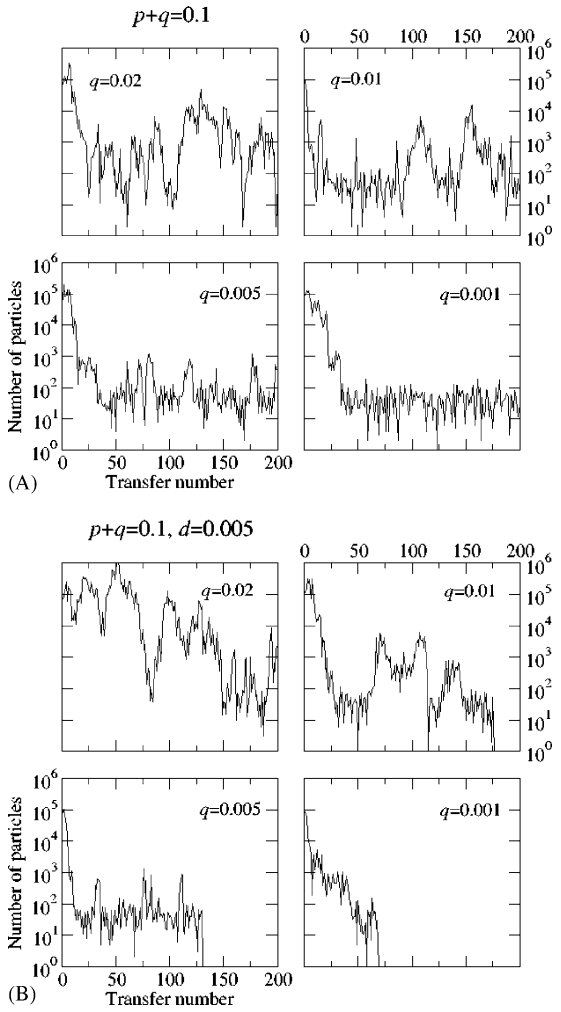

Fig. 4. Viral yield as a function of the transfer number for the numerical model described in the text. For the eight runs shown, the total number of mutations per replication cycle is kept constant, $p + q = 0.1$. As the amount of compensatory mutations (represented by the variable q) decreases, plaques with high viral yields become less frequent, and the system tends to sit close to the extinction threshold for longer time. In these simulations, the number of replication cycles allowed before the bottleneck event occurs is $r = 6$. (A) The probability that a particle disappears is $d = 0$. Despite having a low yield, complete extinction cannot occur. (B) There is a small, finite probability $d = 0.005$ per particle and replication cycle to degrade the genome and be eliminated from the system. Occasionally, this produces the complete extinction of viable particles. Note that, independently of the fitness of the seed particle, extinction can occur with probability d. In terms of the average number of transfers required, the seed particle would disappear before replicating once every $1/d$ transfers, or every 200 in this case. This number becomes smaller if the particle repeatedly fails to replicate, a situation that holds for small q. The four cases shown have been selected as representatives of the average behavior for the parameter values used.

fitness values which is fixed, not the ensemble of genomic sequences. Indeed, mutations continue to occur, a steady accumulation of mutations in the sequential consensus sequences is observed, and there is a persistent drift in the genome space (Escarmís et al., 2002, unpublished results) (Section 3.3).

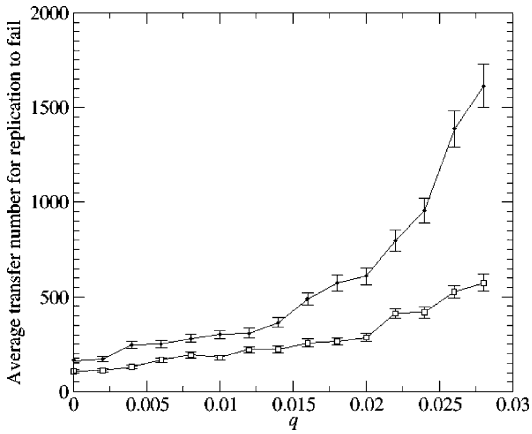

Fig. 5. Average number of transfers required for replication of the seed particle to fail. The probability of extinction decreases as the fraction of compensatory mutations increases. Two different situations are shown. In the upper curve, the probability d that a particle "dies" (or is degraded by the system) is $d = 0$. In the lower curve (open squares), this probability is positive: $d = 0.001$ per particle and replication cycle. To calculate the average values shown, we have considered both the possibility that the seed particle does not replicate and the cases where no particle survives after r replication cycles, that is to say, when actual extinction takes place. Other parameters are as in Fig. 4.

Most previous models of Muller's ratchet considered that true reversions constituted the only mechanism to compensate for the negative effects of deleterious mutations. An exception was a model of fitness evolution in finite parthenogenetic populations subjected to random drift, which considered the effect of compensatory mutations (Wagner and Gabriel, 1990). Given that the frequency of reversions is very low (except for length variation of the internal oligoadenylate tract, very few true reversions have been observed associated with fitness gain of FMDV clones or large populations (Escarmís et al., 1999, 2002)), it is nearly impossible to recover genomes that do not accumulate mutations. This is also a feature of our numerical model. It is the occurrence of compensatory mutations at a given frequency relative to the total mutation rate which maintains genomes with a sufficient fitness level for survival. It will be extremely interesting to quantitate the number of mutations that the FMDV genome can accumulate as the result of the equilibrium between deleterious and compensatory mutations, when positive selection (plaque formation) is imposed for even a larger number of transfers. Such experiments are currently in progress.

For given values of r (number of replication rounds), p (frequency of deleterious plus neutral mutations), and q (frequency of compensatory mutations), the average fitness value reached at the stationary phase is determined by the size of the population bottleneck. Hence, the fitness of the population may either increase or decrease, depending on the initial fitness and the bottleneck size (Lázaro et al., 2002). The minimal bottleneck size required to maintain fitness, dictated by the fitness of the initial population, was documented in experiments with vesicular stomatitis virus

(Novella et al., 1995b), again in agreement with our numerical model.

5. Possible biological implications of the model, and the design of error-catastrophe strategies

The strong resistance of FMDV clones to extinction despite a steady accumulation of mutations (Sections 3.1 and 3.3) in viruses evolving under an intense periodic selective pressure (the absolute requirement to give rise to a plaque on a cell monolayer) can be put into an evolutionary perspective. We know now that the mutation rate of a polymerase can vary as a consequence of structural modifications of the enzyme, produced by amino acid substitutions (Menéndez-Arias, 2002; Castro et al., 2005). Therefore, the mutation rate is a quantity that has probably been subjected to selection, and attained, in the course of evolution, values to ensure survival of the species. In model systems that consider the interaction of a quasispecies with the immune system, it has been proposed that one mutation per genome and replication round is nearly optimal to escape immune attack, with the constraint that a viable quasispecies must be maintained (Kamp and Bornholdt, 2002). Not only the immune response, but also an array of physiological responses in infected individuals, convert the environment encountered by RNA viruses in an unpredictable variable, some times involving a fluctuating pattern. Virus must confront such unpredictabilities as well as genetic bottlenecks within organisms (when few viral particles invade subsets of cells or tissues) and often in the course of transmission (which often involves small amounts of virus). A range of mutation rate values (Drake and Holland, 1999) must have evolved to ensure a continuous heterogeneity to find population subsets to adequately colonize cells under rather stressful conditions. We propose that the observed resistance to extinction of FMDV in the course of plaque-to-plaque transfers may be a reflection of the same intrinsic strength of the RNA-based genetic systems.

How can the experimental observations and the results of numerical simulations be applied to an antiviral design based on virus entry into error catastrophe? First, it must be mentioned that there is evidence that virus extinction by enhanced mutagenesis has probably played a role in clearance of hepatitis C virus (and related hepatitis-associated viruses) in treatments involving ribavirin (Lanford et al., 2001; Maag et al., 2001; Vo et al., 2003; Young et al., 2003) (further discussed by Parker, 2004; Pariente et al., 2005). Furthermore, treatment of mice with FU prevented the establishment of a persistent LCMV infection in the animals (Ruíz-Jarabo et al., 2003; de la Torre, 2005). These lines of evidence encourage extended applications of error catastrophe in vivo. Therefore, it is of interest to summarize what the available data suggest would be an effective means to use mutagenic agents and antiviral inhibitors to clear virus in vivo.

5.1. Some tentative guidelines for antiviral designs based on error catastrophe: the need to combine population dynamics and biochemistry

According to the initial formulation of the error threshold relationship (Swetina and Schuster, 1982; reviews in Eigen, 2002; Eigen and Biebricher, 1988; Biebricher and Eigen, 2005) the maximum information that can be maintained in a genome depends on the superiority (selective advantage) of the master (dominant) sequence over the mutant spectrum. Such a superiority is denoted by σ_0, and $\ln \sigma_0$ is the numerator in the error threshold relationship (defined in the glossary of Table 1). The value of σ_0 will be larger the larger the relative fitness of the mutant distribution (Eigen and Biebricher, 1988; Schuster and Stadler, 1999). Therefore, in agreement with the experimental results with FMDV (Pariente et al., 2001, 2003; Sierra et al., 2000), viral extinction by mutagenic agents should be favored when the virus displays low relative fitness in the infected organisms. Although viral subpopulations with very different relative fitness may coexist in an infected organism, in principle viruses with lower fitness may be found at early phases of infection (when quasispecies optimizations have not yet been attained (Novella et al., 1995a)), or transiently when the virus had to respond to a selective constraint such as to overcome an inhibitory activity or a strong immune response (Borman et al., 1996; Nijhuis et al., 1999). This would suggest that a mutagenic treatment should be initiated early during an infection (i.e. soon after a potentially chronic infection is diagnosed) or upon viral load reduction when a combination treatment with multiple inhibitors is implemented (i.e. highly active antiretroviral therapy for HIV-1-infected patients (Ho, 1995; Le Moing et al., 2002; Van Vaerenbergh et al., 2002)). If our view is correct, it would be important to apply a strong mutagenic treatment before virus replication permits selection – and ensuing optimization (through compensatory mutations) – of inhibitor-resistant mutants. Independently of these considerations, but reinforcing them, is that in model studies, low viral load in itself (irrespectively of the associated relative fitness) favored extinction (Pariente et al., 2001, 2003; Sierra et al., 2000). This is due probably not only to the fact that a lower viral mass will generally be easier to eliminate (and so it will be by an immune response operative during treatment), but also because the mutant repertoire (and thus the adaptive potential) is restricted when the viral load is small. It has not been investigated whether there would be an advantage in initiating simultaneously a combination treatment involving mutagens and inhibitors versus administration of either inhibitors or mutagens first, followed shortly by administration of the other component. It is not clear whether the noise inherent to any real viral infection (with compartmentalized viral subpopulations displaying different fitness, changing viral loads and mutant classes, different drug concentrations at different virus replication sites within an organism (Müller et al., 2004)) will render of any consequence small differences in the timing of application of mutagens and inhibitors. This point

will clearly need further investigation when a possible clinical application is envisaged. Independent theoretical models also support a synergy between mutagens and antiviral inhibitors as a treatment design (Gerrish and Garcia-Lerma, 2003).

To achieve synergy between mutagens and inhibitors will necessitate of addressing a number of important issues regarding the biochemical behavior of the drugs. Specifically, it will be important to identify the transporter molecules that mediate drug entry into the cells and the cellular compartments where virus replication takes place (Pastor-Anglada et al., 2005). Mutagens and inhibitors will affect metabolic pathways in ways that may alter nucleotide pools, in themselves an influence on viral replication and on mutagenesis of the viral genome. It will also be extremely relevant to find new mutagenic agents (or derivatives of the existing ones) that are specific for viral polymerases, and that show low toxicity for cells and organisms. Structural biology and toxicology will also have a role to play, emphasizing both the interdisciplinary and the transdisciplinary nature of the undertaking.

These considerations underline also the profound consequences that an understanding of the nature of viral populations and quasispecies dynamics may have in the design of new strategies to control viral disease. To turn error catastrophe into a practical application is a difficult but exciting challenge.

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