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CHAPTER

4

Viral Quasispecies: Dynamics, Interactions, and Pathogenesis*

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ABSTRACT

Quasispecies theory is providing a solid, evolving conceptual framework for insights into virus population dynamics, adaptive potential, and response to lethal mutagenesis. The complexity of mutant spectra can influence disease progression and viral pathogenesis, as demonstrated using virus variants selected for increased replicative fidelity. Complementation and interference exerted among components of a viral quasispecies can either reinforce or limit the replicative capacity and disease potential of the ensemble. In particular, a progressive enrichment of a replicating mutant spectrum with interfering mutant genomes prompted by enhanced mutagenesis may be a key event in the sharp transition of virus populations into error catastrophe that leads to virus extinction. Fitness variations are influenced by the passage regimes to which viral populations are subjected, notably average fitness decreases upon repeated bottleneck events and fitness gains upon competitive optimization of large viral populations. Evolving viral quasispecies respond to selective constraints by replication of subpopulations of variant genomes that display higher fitness than the parental population in the presence of the selective constraint. This has been profusely documented with fitness effects of mutations associated with resistance of pathogenic viruses to antiviral agents. In particular, selection of HIV-1 mutants resistant to one or multiple antiretroviral inhibitors, and the compensatory effect of mutations in the same genome, offers a compendium of the molecular intricacies that a virus can exploit for its survival. This chapter reviews the basic principles of quasispecies dynamics as they can serve to explain the behavior of viruses.

*Dedicated to Manfred Eigen on the occasion of his 80th birthday, for the insights that his pioneer studies have represented for virology.

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FROM EARLY REPLICONS TO PRESENT-DAY RNA VIRUSES

The quasispecies theory of molecular evolution was first proposed to describe the error-prone replication, self-organization, and adaptability of primitive replicons such as those thought to have populated the earth some 4000 million years before the present (Eigen, 1971, 1992; Eigen and Schuster, 1979; see Chapter 1). Quasispecies was formulated initially as a deterministic theory involving mutant distributions of infinite population size in equilibrium. Extensions and generalizations to ensembles of genomes of finite population size replicating in changing environments have been developed (Eigen, 2000; Wilke *et al.*, 2001a, 2001b; Saakian and Hu, 2006). Virologists use the term "viral quasispecies" to mean complex distributions of non-identical but closely related viral genomes subjected to genetic variation, competition and selection, and which act as a unit of selection (reviewed in different chapters of Domingo, 2006). More simple and general, a quasispecies has been defined as a population of similar genomes (Nowak, 2006). Quasispecies dynamics is most clearly manifested in systems such as RNA viruses that display short duplication times, generally high fecundity, and error-prone replication, traits that have been maintained despite a probable ancient origin of most extant RNA viruses in coevolution with a cellular world. Increasing numbers of careful analyses of viral populations have supported quasispecies dynamics for animal and plant RNA viruses (for recent examples see Ge *et al.*, 2007; Zhang *et al.*, 2007 and references included in these articles; see also other chapters of this book).

As discussed by Villarreal in Chapter 21, there are two main hypotheses regarding the origin of RNA viruses and other RNA genetic elements: that they are remnants of an ancient RNA world, or that they are modern derivatives of cells, originated in cellular RNAs that acquired autonomous replication. Viroids and other subviral RNA replicons may be direct descendants of early RNA (or RNA-like) replicons that preceded an organized cellular world (Robertson *et al.*, 1992) (Chapter 2). Cells and viruses share a considerable number of essential functional domains or modules: polymerases, proteases, enzymes involved in nucleotide and nucleic acid metabolism, etc. However, on the basis of key proteins involved in viral replication, that are absent in cells, and also based on the evidence of extensive genetic exchange between diverse viruses, the concept of an ancient virus world has been proposed (Koonin *et al.*, 2006). A primordial pool of genetic elements could have been the ancestor of viral and cellular genes. Cells and viruses share a ubiquitous ability to modify, lose, or acquire new genes or gene segments through genomic rearrangements, insertions, deletions, and other recombination events. Shuffling of functional modules among cells, viruses, and other replicons (plasmids, episomes, transposons, retrotransposons) is probably a frequent occurrence through fusion, transfection, conjugation, and other types of horizontal gene transfers (Botstein, 1980; Hickey and Rose, 1988; Zimmern, 1988; Davis, 1997; Holland and Domingo, 1998; Bushman, 2002). Sequence comparisons strongly suggest that all extant viruses have deep, ancient evolutionary roots (Gorbalenya, 1995; Villarreal, 2005) (Chapter 21).

ERROR-PRONE REPLICATION NECESSITATES LIMITED GENETIC COMPLEXITY TO PROTECT AGAINST ERROR CATASTROPHE

One of the critical features that distinguishes cells from viruses is the difference in the complexity of their genetic material, even after accounting for repeated DNA in animal and plant cells. Complexity in this case means the amount of genetic information encoded in their genetic material. A typical mammalian cell includes a number of chromosomes amounting to a total of about 3×10^9 base pairs (bp) of DNA. The chromosomal DNA of *Escherichia coli* has a complexity of about 4×10^6 bp. In contrast, RNA viruses have genomes in the range

of 3.0×10^3 to 3.2×10^4 nucleotides. Point mutation rates for eukaryotic cells have been estimated to be in the range of $10^{-10}-10^{-11}$ substitutions per nucleotide (s/nt), while for bacterial cells, values may reach up to 10^{-9} s/nt (Friedberg *et al.*, 2006). Mutation rates for a number of genomic sites of RNA viruses, determined using both genetic and biochemical procedures, are in the range of $10^{-3}-10^{-5}$ s/nt (Drake, 1993; Drake *et al.*, 1998; Drake and Holland, 1999; Domingo, 2007) (Chapter 7). Despite mutation rates varying with a number of environmental parameters, the above values mean that, in the process of RNA replication or retrotranscription, each progeny genomic molecule of about 10 kb will contain on average 0.1 to several mutations. These determinations of mutation rates and frequencies suggest that even the viral progeny of a single infected cell will be genetically heterogeneous (Domingo *et al.*, 1978; Holland *et al.*, 1982; Temin, 1989, 1993; Domingo, 2006, 2007; see also other chapters of this book).

Penetration into the composition of mutant spectra, either by determining the nucleotide sequence of many clones from the same population, or by other "diving" strategies, has quantitated large genotypic and phenotypic diversity within mutant spectra (Duarte *et al.*, 1994a, 1994b; Nájera *et al.*, 1995; Marcus *et al.*, 1998; Pawlotsky *et al.*, 1998; Quiñones-Mateu *et al.*, 1998; Wyatt *et al.*, 1998; Fernandez *et al.*, 2007; Garcia-Arriaza *et al.*, 2007; Ge *et al.*, 2007; Zhang *et al.*, 2007). Diversity can extend to multiple mutant and recombinant genomes within an infected organ, and even within a single infected cell. Diversity of genetic forms is a prerequisite for evolution, including the major transitions undergone by our biosphere (Eigen, 1992; Maynard Smith and Szathmary, 1995). RNA viruses have an exhuberant diversity to offer as a substrate for evolution. A virus population, by virtue of consisting of dynamic mutant spectra rather than a defined genomic sequence, has the potential to adapt readily to a range of environments.

One of the predictions of quasispecies dynamics of RNA viruses is the existence of an error threshold, defined as an average copying fidelity value at which a transition between an organized mutant spectrum and sequences lacking information contents occurs (reviewed in Eigen and Schuster, 1979; Eigen and Biebricher, 1988; Biebricher and Eigen, 2005; Nowak, 2006; Chapters 1 and 9). This transition has been coined "entry into error catastrophe," a term first used by L. Orgel to describe errors during protein synthesis that could contribute to a collapse of cellular regulatory networks in the process of aging (Orgel, 1963). Both, the concept expressed by Orgel and the one applied to genetic information of viruses address deterioration of meaningful information with a biological consequence, due to errors in an informational macromolecule. The error threshold relationship establishes a limitation for the maximum complexity of genetic information that can be stably maintained by a replicon displaying a given copying accuracy (Chapter 1). Theoretical calculations of the range of mutation rates that should be compatible with maintenance of the information carried by the simple RNA bacteriophages were compatible with the mutation rates and frequencies found experimentally (compare Batschelet *et al.*, 1976; Domingo *et al.*, 1976, 1978, with Eigen and Schuster, 1979; Eigen and Biebricher, 1988). In addition to intrinsic copying fidelity levels of viral polymerases, other biochemical features of virus replication may have evolved to preserve a minimal replication accuracy. It has been hypothesized that the "rule of six" (genome of polyhexameric length) in Mononegavirales that edit their phosphoprotein mRNA, may have evolved to prevent the negative effects of illegitimate editing that could result in error catastrophe (Kolakofsky *et al.*, 2005). Some biological systems exploit enhanced mutagenesis as a defense mechanism against invading molecular parasites. A mechanism known as "repeat-induced point mutations (RIP)" operates in some filamentous fungi such as *Neurospora crassa* resulting in the production of mutations in repeat DNA copies that penetrate into the cells (Bushman, 2002; Galagan and Selker, 2004). Also, the APOBEC3 family of cytidine

deaminases are innate immunity factors that induce hypermutation in retroviral DNA. Such activities can be regarded as a form of natural "error catastrophe" against retroviral genomes (see Chapter 8). Thus, a mutagenesis-based antiviral approach to drive virus to extinction has a parallel in natural mechanims which have contributed to the survival of organisms in the face of perturbing molecular parasites.

Increased genetic complexity as is embodied in cells required a correspondingly higher copying accuracy of the genetic material. This appears to have been accomplished with a number of pathways for post-replicative repair mechanisms as well as with the acquisition of a 3′–5′ proofreading-repair exonuclease activity by most cellular DNA polymerases (Goodman and Fygenson, 1998). No evidence of a 3′–5′ exonuclease activity in viral RNA polymerases and reverse transcriptases has been obtained from either biochemical or structural studies with viral enzymes (Steinhauer *et al.*, 1992; Ferrer-Orta *et al.*, 2006). A possible exception was presented in an early report by (Ishihama *et al.*, 1986) showing that the influenza virus RNA polymerase was able to remove excess GMP residues added to a capped oligonucleotide primer. A 3′-end repair mechanism has been described in a satellite RNA of the plant virus turnip crinkle carmovirus, involving synthesis of short oligoribonucleotides by the viral replicase using the 3′-end of the viral genome as template, and, probably, template-independent priming at the 3′-end of the damaged RNA to generate wild-type, negative strand, satellite RNA (Nagy *et al.*, 1997). Also, some coronaviruses encode a polymerase which includes a 3′–5′ exonucleolytic activity (i.e. nsp14 of SARS) (Minskaia *et al.*, 2006). In the coronavirus murine hepatitis virus, mutations in the MSP14 exoribonuclease decreased replication fidelity (Eckerle *et al.*, 2007).

Virus Entry into Error Catastrophe and its Application to Lethal Mutagenesis

The limitations imposed on average mutation rates to maintain the genetic information transmitted by simple RNA replicons (Swetina and Schuster, 1982; Eigen and Biebricher, 1988; Nowak and Schuster, 1989) (Chapter 1) encouraged the first experiments to investigate whether chemical mutagenesis was detrimental to RNA virus replication. The first studies indicated that chemical mutagenesis could increase the mutation frequency by at most three-fold at defined genomic sites of poliovirus (PV) and vesicular stomatitis virus (VSV) (Holland *et al.*, 1990), and 13-fold in the case of a retroviral vector (Pathak and Temin, 1992). Also, increased mutagenesis had an adverse effect on fitness recovery of VSV clones (Lee *et al.*, 1997). These early results suggested that RNA viruses replicate near the error catastrophe threshold, with a copying fidelity that allows a generous production of error copies.

Additional studies in cell culture and *in vivo* have established that enhanced mutagenesis can result in virus extinction (reviewed in Anderson *et al.*, 2004; Domingo, 2005). Loeb and colleagues coined the term "lethal mutagenesis" to refer to the loss of virus infectivity associated with the action of mutagenic agents (Loeb *et al.*, 1999). Mutagenic nucleoside analogues, some used in antimicrobial and anticancer therapy, are currently actively studied as promoters of lethal mutagenesis of viruses, including an ongoing clinical trial with AIDS patients (Harris *et al.*, 2005).

Lethal mutagenesis is attracting increasing interest, and several theoretical models have addressed the mechanisms underlying lethal mutagenesis and the relationship between the observations on viral extinction and the original concept of error catastrophe (several models are reviewed in Chapter 1, and one model is described in Chapter 9). Key to the validation of these models as applied to RNA viruses is the experimental finding that a low viral load and low replicative fitness (relative replication capacity) favor extinction (Sierra *et al.*, 2000; Pariente *et al.*, 2001), and that a mutagenic activity (not merely an inhibitory activity) is necessary to achieve extinction (Pariente *et al.*, 2003). This was shown by absence of extinction when the virus was subjected to equivalent inhibitory activities

with cocktails of non-mutagenic inhibitors (Pariente *et al.*, 2003). However, since low viral loads favor extinction, the inhibitory activity that is associated with the action of some mutagenic agents may contribute to lethal mutagenesis. In this respect, a combination of a mutagenic nucleoside analogue and the antiretroviral inhibitor AZT was required to extinguish high fitness HIV-1 during infections in cell culture (Tapia *et al.*, 2005). Even strong reductions in population size of highly debilitated foot-and-mouth disease virus (FMDV) and lymphocytic choriomeningitis virus (LCMV) populations did not result in virus extinction unless a mutagenic activity intervened (Sierra *et al.*, 2000; Pariente *et al.*, 2001; Pariente *et al.*, 2003). A second finding to be considered in the development of theoretical models is the negative interference exerted by mutants that either coinfect the cells along with standard virus, or are generated inside the cell by mutagenesis. The interfering activity of such "defector" genomes as contributing to viral extinction has been documented both experimentally with FMDV and LCMV, and by *in silico* simulations (González-López *et al.*, 2004; Grande-Pérez *et al.*, 2005b; Perales *et al.*, 2007). Production of a fraction of non-infectious hepatitis C virus (HCV) in infected patients as a result of ribavirin (1-β-D-ribofuranosyl-1,2,3-triazole-3-carboxamide) therapy is a key parameter in the models of HCV clearance following treatment with ribavirin and interferon alpha (IFN-α) (Dixit *et al.*, 2004; Dahari *et al.*, 2007) (see Chapter 15).

An argument that has been used to deny a connection between lethal mutagenesis and the transition into error catastrophe has been the absence of hypermutated molecules in mutagenized populations of RNA viruses. However, any hypermutated genome transiently generated during mutagenesis is unlikely to be replication-competent and to be included in any sampling of viral genomes. This has been recognized by us (Grande-Pérez *et al.*, 2005a) and others (Perelson and Layden, 2007). Despite this, a genome with a mutation frequency lying in the lower range of typically hypermutated genomes was identified in a population of 5-fluorouracil (5-FU)-treated LCMV (Grande-Pérez *et al.*, 2005a). The absence or very low frequency of hypermutated genomes in standard genome samplings of pre-extinction viral populations cannot constitute an argument against a mutagenesis-driven transition into error catastrophe.

Concerning the relationship between the concept of error catastrophe and extinction of viruses by lethal mutagenesis, M. Eigen pointed out the following: (i) dependence of copying fidelity on sequence context and the type of mutagen; (ii) fitness landscape of the quasispecies distribution, including the perturbing effects of specific types of mutants that may arise during mutagenesis (as discussed above); (iii) participation of multiple viral functions (not only RNA replication) in determining the replicative collapse of the system. As pointed out by Eigen, "Theory cannot remove complexity, but it shows what kind of 'regular' behavior can be expected and what experiments have to be done to get a grasp on the irregularities" (Eigen, 2002).

In line with the application of the error threshold relationship to real viruses (Eigen, 2002), it is obvious that virus extinction will not occur through "evaporation" into the entire sequence space theoretically available to a viral genome. This is physically impossible. As mutagenesis progresses during viral replication myriads of end-point genomes harboring lethal or highly deleterious mutations will impede further expansions into sequence space by such genomes. This is a consequence of the multiple viral functions (not only RNA replication) that affect replicative competence (Eigen, 2002). These differences between the mechanisms that mediate extinction of real viruses and the original concept of error catastrophe can be expressed by distinguishing "phenotypic" and "extinction" thresholds from an "error theshold," as has been done in some theoretical treatments (for example, Huynen *et al.*, 1996; Manrubia *et al.*, 2005). Apart from these rather obvious adaptations of error catastrophe to a real biological system, the experimental studies carried out in the laboratory of one of us (E.D.) do not provide any basis to dissociate lethal mutagenesis from error catastrophe, as initially developed by

Eigen, Schuster, and colleagues, and even less to consider that the approach to error catastrophe will impede viral extinction. In the section on "Intra-mutant spectrum suppression can contribute to lethal mutagenesis" in this chapter, we summarize our current view on the mechanisms that underlie virus extinction through lethal mutagenesis based on experimental results, and the main challenges facing, in our view, this new antiviral strategy.

INTRA-POPULATION COMPLEMENTATION AND INTERFERENCE IN VIRAL QUASISPECIES: MUTANT DISTRIBUTIONS AS THE UNITS OF SELECTION

A viral quasispecies can have a biological behavior that is not predictable from the behavior of its components considered individually. Several observations with viruses as they replicate in cell culture or *in vivo* suggest that intra-population interactions can modulate the replicative capacity of the ensemble of mutants or of individual mutants introduced in a spectrum of mutants. Fitness of biological clones of bacteriophage Qβ (Domingo *et al.*, 1978) and of VSV (Duarte *et al.*, 1994a) was lower than the fitness of the average populations from which the clones were derived. These quantifications of clonal fitness suggest that an ensemble of related mutants may collectively acquire a selective replicative advantage, perhaps because competent gene products may complement suboptimal or defective products expressed by subsets of components of the mutant spectrum. Specific mutants, including deleterious and lethal mutants, can be maintained in viral populations *in vivo*, and can be transmitted to susceptible hosts (Moreno *et al.*, 1997; Yamada *et al.*, 1998; Aaskov *et al.*, 2006; Vignuzzi *et al.*, 2006).

A seemingly opposite manifestation of the internal interactions within viral quasispecies is the suppression of the replication of specific mutants by the surrounding mutant spectrum. This possibility was suggested by theoretical models according to which a simple replicon of inferior fitness to another could nevertheless dominate the population by virtue of being surrounded by a more favorable mutant spectrum (Swetina and Schuster, 1982) (reviewed in Eigen and Biebricher, 1988; Nowak, 2006) (Chapter 1). The first experimental documentation of this prediction with real viruses was by de la Torre and Holland who showed that a standard VSV population interfered with the replication of a VSV clone of superior fitness, unless the latter was present above a certain frequency in the population (de la Torre and Holland, 1990). Suppressive effects of this type have been subsequently documented in several virus-host systems (reviewed in Domingo, 2006). Remarkable examples include suppression by attenuated PV of neuropathology in monkeys associated with virulent PV present in the vaccine preparation (Chumakov *et al.*, 1991), suppression of pathogenic LCMV by non-pathogenic variants (Teng *et al.*, 1996), the lowered replication rates of drug-resistant viruses (Crowder and Kirkegaard, 2005), and complementing-interfering effects of specific FMDV mutants (Perales *et al.*, 2007).

The Mutant Spectrum as a Determinant of Viral Pathogenesis. Picornaviral Polymerase Mutants

The complexity of the mutant spectrum of a virus (that is, the average number of mutations that distinguish the individual components of the mutant distribution) can affect the course of viral disease and the response to treatment. Most notably, prolonged persistence of HCV infection correlated with high mutant spectrum complexity (Farci *et al.*, 2000); other aspects of quasispecies behavior of HCV were reviewed in Domingo and Gomez, 2007) (see also Chapter 15).

Studies with a PV mutant with an amino acid substitution in the viral polymerase which increases about five-fold its template-copying fidelity have been particularly revealing. The mutant PV produces a narrower mutant

spectrum (with a lower average number of mutations per genome) than wild-type PV. In infections of susceptible mice (transgenic for the human PV receptor) the mutant replicated in the animals but failed to reach the brain and to produce the neuropathology that was associated with the infection with wild-type PV (Pfeiffer and Kirkegaard, 2005; Vignuzzi *et al.*, 2006). Remarkably, restoration of the standard mutant spectrum complexity by subjecting the mutant PV to 5-FU-induced mutagenesis led to a neuropathogenic mutant spectrum (Vignuzzi *et al.*, 2006). Moreover, Sabin's attenuated PV vaccine shows relatively low mutant frequency compared with wild-type strains, and this observation could be due to differences in polymerase fidelity (Vignuzzi, personal communication; see also Chapter 6). These observations are highly relevant (Biebricher and Domingo, 2007). Foremost, the results show the biological relevance of high mutation rates, in that they may affect pathology by allowing the virus to reach specific target organs, thereby increasing viral loads and chances of transmission. The observed phenotypic transitions of PV demand consideration of the virus as a quasispecies, since PV behavior could not be explained by taking into account consensus genomic nucleotide sequences alone. We come to the conclusion that virus evolution can affect viral pathogenesis in at least two ways (Domingo, 2007): (i) The information for increased pathology or for adaptation to multiple environments can be contained in the genetic material of the virus (in most of its individual clones) irrespective of the mutant spectrum to which they belong (Kimata *et al.*, 1999; Greene *et al.*, 2005, among other examples). (ii) The information for increased pathology can be contained in a distribution of mutants as such, as documented above for HCV and PV. Again, these observations reinforce the biological advantage of high mutation rates for the long-term survival of RNA viruses, and the consideration of entire quasispecies as the units of selection (see also Domingo, 2006, 2007, and other chapters of this volume).

The PV polymerase mutant displaying higher fidelity than the wild type was obtained by passaging the virus in the presence of increasing concentrations of the nucleoside analogue ribavirin (Pfeiffer and Kirkegaard, 2003; Vignuzzi *et al.*, 2006). The amino acid replacement in the polymerase (G64S) is located away from the catalytic domain of the enzyme, and an action at a distance was invoked to explain the general effect of this substitution on the copying fidelity (Arnold *et al.*, 2005) (see Chapter 6). A mutant of FMDV, selected also by passaging the virus in the presence of increasing concentrations of ribavirin, displayed higher fitness than the wild-type virus when virus replication took place in the presence of ribavirin but not in its absence (Sierra *et al.*, 2007). This phenotypic change was mapped to amino acid substitution M296I in the viral polymerase, and the mutant enzyme displayed decreased capacity to use ribavirin triphosphate as substrate (instead of GTP or ATP), but did not show an apparent alteration of general template-copying fidelity (Sierra *et al.*, 2007). Substitution M296I is located at a loop whose flexibility seems to be required to adapt its conformation and interactions to the size and shape of template residues and incoming nucleotide substrates. Ile at this position may restrict the loop flexibility and affect nucleotide recognition (Ferrer-Orta *et al.*, 2007). M296 is quite distant from the site (G62) where the equivalent, ribavirin-selected substitution in PV lays. These results suggest that in the picornaviral polymerase multiple sites (perhaps domains) might be involved either in specific interactions with nucleotide analogues or in recognition of nucleotide substrates.

Comparison of the structure of the FMDV polymerase complexed with RNA (Ferrer-Orta *et al.*, 2004), and with RNA and a number of nucleotides and nucleotide analogues (Ferrer-Orta *et al.*, 2007) has documented the involvement of multiple amino acids of the FMDV polymerase in the recognition of nucleotides. Several interactions are key to catalysis, as shown by modification of the polymerase activity of the corresponding mutants produced by site-directed mutagenesis. Interestingly, some interactions are

common to standard nucleotides and nucleotide analogues, while other interactions are specific for a given nucleotide analogue (Ferrer-Orta *et al.*, 2007). These results suggest that multiple sites in the polymerase can modulate substrate recognition, thereby affecting the fidelity properties of picornaviral (and probably other) polymerases (Arnold *et al.*, 2005; Ferrer-Orta *et al.*, 2007). These and other recent studies on the mechanism of substrate discrimination by viral RNA polymerases and reverse transcriptases are providing important information that may help in the design of drugs able to lower the copying fidelity of viral polymerases to facilitate lethal mutagenesis (see also Chapter 6).

Intra-Mutant Spectrum Suppression can Contribute to Lethal Mutagenesis

Populations of RNA viruses subjected to increased mutagenesis by nucleoside analogues display decreases in specific infectivity due to accumulation of viral genomes harboring deleterious or lethal mutations (Crotty *et al.*, 2001; Grande-Pérez *et al.*, 2002, 2005b; Airaksinen *et al.*, 2003; González-López *et al.*, 2004, 2005; Arias *et al.*, 2005). Mutagenized, pre-extinction FMDV RNA interfered with the replication of standard FMDV RNA, resulting in a delay and in a decrease in the production of progeny virus (González-López *et al.*, 2004). Since the interfering FMDV displayed at least a 0.1-fold fitness relative to the standard FMDV (González-López *et al.*, 2005), the suppression observed could not be due to mechanisms invoking competition between genomes of comparable replication capacity (such as positive clonal interference). It was suggested that the expression (normal or aberrant) of altered viral proteins could contribute to the suppression of replication of standard FMDV, and also to the extinction of FMDV RNA. To test this hypothesis, a number of capsid and polymerase mutants of FMDV were examined regarding their capacity to interfere with standard FMDV, in experiments involving coelectroporation of cells with the relevant RNAs (Perales *et al.*, 2007). The results showed that an excess of several replication-competent mutants caused a strong and specific interference on FMDV replication. Furthermore, mixtures of some capsid and polymerase mutants evoked a very strong, synergistic interference (Perales *et al.*, 2007). Notably, some of the mutants tested had been isolated from mutagenized FMDV populations in their way towards extinction. These results with FMDV are in agreement with observations on enhanced mutagenesis of LCMV which resulted in populations in which the loss of infectious progeny production preceded the loss of replicating viral RNA (Grande-Pérez *et al.*, 2005b). A deleterious effect on infectivity exerted by defective LCMV genomes was also supported by numerical simulations using realistic parameters of LCMV replication (Grande-Pérez *et al.*, 2005b).

The picture emerging from the studies with FMDV and LCMV is that the transition towards viral extinction associated with lethal mutagenesis can have at least two phases: an initial one, with a limited input of mutations in the viral genomes, in which a subset of defective genomes that have been termed "defectors" interfere with replication of standard genomes, and can contribute to viral extinction. This is termed the "lethal defection model" of virus extinction, proposed on the basis of experiments with LCMV (Grande-Pérez *et al.*, 2005b), and supported by the strong interference on FMDV replication exerted by combinations of specific capsid and polymerase mutants of FMDV (Perales *et al.*, 2007). In a second phase, as the number of mutations per genome increases due to continuing mutagenesis, the proportion of lethal mutations increases, resulting in further decreases in specific infectivity (González-López *et al.*, 2005; Grande-Pérez *et al.*, 2005b). In Chapter 6, Cameron and colleagues describe elegant experiments that show that low-fidelity mutants of poliovirus manifest an acceleration of the onset of lethal mutagenesis. Genomes with either deleterious or lethal mutations have been isolated from mutagenized FMDV and LCMV populations

on their way towards extinction (Sierra *et al.*, 2000; Pariente *et al.*, 2001; Arias *et al.*, 2005). Some detrimental mutations may be maintained in the viral populations by complementation and whenever the genomes harboring them increase in frequency they may exert an interfering activity provided that the type of genetic lesion belongs to the interfering class (Perales *et al.*, 2007). Interestingly, some genomes harboring multiple mutations (for example a triple mutant in the polymerase of FMDV) that render the genome replication-incompetent may differ in a single nucleotide position from a replication-competent, strongly interfering mutant (Arias *et al.*, 2005; Perales *et al.*, 2007). Viral genomes with interfering or lethal mutations may occupy proximal or distant positions in sequence space, relative to the standard, non-mutated genome. Thus, there might be a gradual but overlapping transition between a phase of dominance of interfering mutants and a phase of increasing presence of lethal mutants, until a replicative collapse and virus extinction occur, in agreement with the theory of error catastrophe (see Chapter 1 for a discussion of the contribution of lethal mutants to error catastrophe). Recent biochemical data have documented that viral proteins are frequently multifunctional and that they often form oligomeric complexes. Thus, mutated forms of a given protein may affect multiple viral functions and result in inactive protein complexes (several examples can be found in Mesters *et al.*, 2006; Sobrino and Mettenleiter, 2008). Abnormal behavior of altered viral proteins may be one of the molecular mechanisms underlying virus transition into error catastrophe, very much in line with the cascade of events initially proposed as a model for aging (Orgel, 1963).

The transition of FMDV and LCMV towards extinction by lethal mutagenesis occurred with a 10^2- to 10^3-fold decrease in specific infectivity (PFU/total viral RNA), and without a modification of the consensus sequence of the population (González-López *et al.*, 2004; Grande-Pérez *et al.*, 2005a) in agreement with results with poliovirus (Crotty *et al.*, 2001). Loss of infectivity was very sharp, and extinction occurred generally after 1–20 passages, depending on viral fitness and the mutagen-inhibitor combination treatment (compare the extinction kinetics in Sierra *et al.*, 2000; Pariente *et al.*, 2001, 2003; Grande-Pérez *et al.*, 2005a). Extinction can be preceded by minimal increase in the average mutation frequency of the mutant spectra (Crotty *et al.*, 2001; Grande-Pérez *et al.*, 2005b; Tapia *et al.*, 2005). These experiments have not provided evidence that as the mutational load in the viral genome increases, the virus acquires resistance to extinction. It remains to be seen whether the presence of M296I in the FMDV RdRp, which was selected by ribavirin, confers any significant resistance to lethal mutagenesis. The vulnerability of FMDV to extinction by lethal mutagenesis offers a significant contrast with the resistance of FMDV to extinction despite accumulation of mutations as a result of plaque-to-plaque transfers (Escarmis *et al.*, 2002, 2008; Lazaro *et al.*, 2003). The key difference between the two scenarios is that resistance to extinction (despite accumulation of mutations accompanying serial bottleneck events) results from the selection for a next transfer of a virus able to replicate thanks to the presence of compensatory mutations. This is in contrast to mutagenesis of a complex population whose suppressive effects do not allow the rescuing of replication-competent individuals (Manrubia *et al.*, 2005).

The course of events preceding viral extinction that we have outlined here has a number of experimentally testable predictions, currently under study. Clarification of the mechanisms underlying virus extinction may help in the design of improved protocols of administration of mutagenic agents and antiviral inhibitors for lethal mutagenesis. In our view, the main challenges facing progress in lethal mutagenesis are: (i) finding and design of new mutagenic base or nucleoside analogues that target viral (but not cellular) polymerases, that can be used in combination with antiviral inhibitors; (ii) evaluation of how widespread is the occurrence of mutagen-resistant virus mutants, and whether lethal mutagenesis may fail either because of the presence of

mutagen-resistant mutations (Pfeiffer and Kirkegaard, 2003; Sierra *et al.*, 2007) or other mechanisms (Sanjuan *et al.*, 2007); (iii) understanding of the molecular basis of template-copying fidelity of nucleic acid polymerases, and the design of drugs that can lower specifically the copying fidelity of viral polymerases; (iv) the application of lethal mutagenesis to model systems *in vivo* (Ruiz-Jarabo *et al.*, 2003a; Harris *et al.*, 2005). Concerning possible applications of lethal mutagenesis *in vivo,* measurements of the "critical drug efficacy"—as developed for treatments of infections by HIV-1 and HCV (Callaway and Perelson, 2002; Y. Huang *et al.*, 2003; Dahari *et al.*, 2007)—for mutagen-inhibitor combinations, should guide in establishing protocols adequate for viral clearance, to avoid stabilization of viral levels at a therapy-induced set point.

FITNESS AND ITS MODULATION BY VIRAL POPULATION SIZE

One of the consequences of the quasispecies dynamics of RNA viruses is fitness variations in a constant environment triggered by changes in viral population size. Fitness is a complex parameter that measures the degree of adaptation of a living organism or simple replicons to a specific environment (as general reviews see Williams, 1992, and Reznick and Travis, 1996). For viruses, fitness values have been measured as the relative ability of two competing viruses to produce infectious progeny (Holland *et al.*, 1991; reviewed in Domingo and Holland, 1997; Quiñones-Mateu and Arts, 2006). In the standard protocol, competitions are started by infecting cells or organisms with a mixture of a reference wild-type virus (given arbitrarily a fitness value of 1) and the virus to be tested, in known proportions. The progeny viruses are used to initiate a second round of infection, and the process is repeated a number of times (serial infections). Then, the logarithm of the proportion of the two competing viruses at each passage defines a fitness vector, the slope of which is the logarithm of the fitness of the test virus relative to the reference virus (Figure 4.1). The two competing viruses must be distinguishable by some phenotypic trait (e.g. a clear difference in the ability to replicate in the presence of an antibody or a drug)

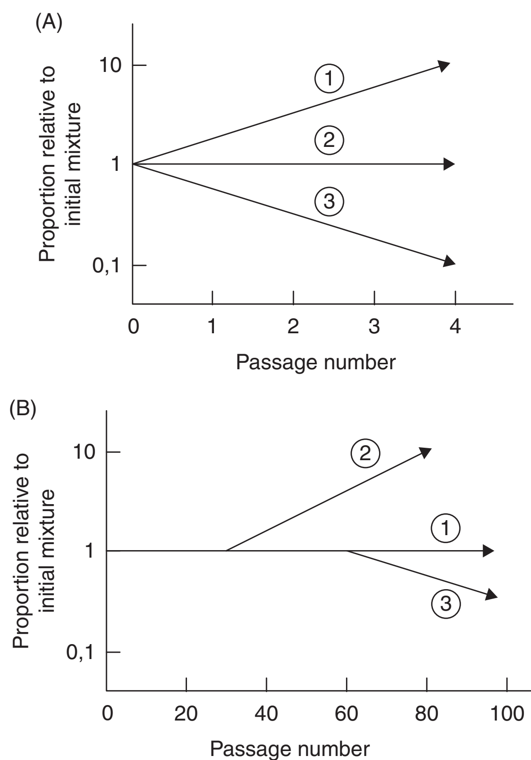

FIGURE 4.1 Schematic representation of fitness vectors and some patterns of fitness variation. (A) Plot of the proportion of the test virus and the reference virus, relative to the initial mixture, as a function of passage number. The plot gives a fitness vector. The test virus can show higher relative fitness than the reference virus (line 1), equal fitness (neutrality, line 2), or lower fitness than the reference virus (line 3). See text for comments and literature references. (B) Possible outcomes of a competition between two neutral variants. The two variants may co-exist for many generations (line 1). Occasionally one variant may displace the other in a rather unpredictable manner (lines 2 and 3), in agreement with the competitive exclusion principle of population genetics. Further information and references are given in the text.

or by some genetic change, such as nucleotide substitutions that allow the proportion of the two viruses to be determined by densitometry of a sequencing gel or by their specific amplification by real time reverse transcription-polymerase chain reaction (RT-PCR) using discriminatory oligonucleotide primers.

Fitness determinations of viruses subjected to different passage regimes have established an important effect of population size of the virus involved in the infections, on fitness evolution.

Fitness Decrease Upon Bottleneck Passages. Viral Virulence May Not Correlate with Fitness

Animal viruses are likely to undergo genetic bottlenecks during transmission; most of the evidence suggesting bottleneck effects comes from sequence analysis of infected hosts (for instance, Frost *et al.*, 2001), but Pfeiffer and Kirkegaard demonstrated bottlenecks during PV transmission from inoculated sites to the brain in transgenic mice expressing the human PV receptor (Pfeiffer and Kirkegaard, 2006). In addition, there is direct evidence demonstrating that plant viruses experience significant bottlenecks during movement from the site of infection (Ali *et al.*, 2006; Jridi *et al.*, 2006) (see Chapter 12).

RNA virus populations subjected to severe serial bottleneck events in cell culture—such as those occurring upon serial plaque-to-plaque transfers—undergo, on average, a decrease in fitness (Chao, 1990; Duarte *et al.*, 1992; Escarmís *et al.*, 1996; Yuste *et al.*, 1999; de la Iglesia and Elena, 2007). This is due to the stochastic accumulation of deleterious mutations (Figure 4.2), predicted by Müller (1964) to occur for small populations of asexual organisms lacking in mechanisms, such as sex or recombination, that could eliminate or compensate for such debilitating mutations (Maynard-Smith, 1976). Subjecting RNA viruses to repeated plaque-to-plaque transfers has all the ingredients to accentuate the effects of Müller's ratchet: a

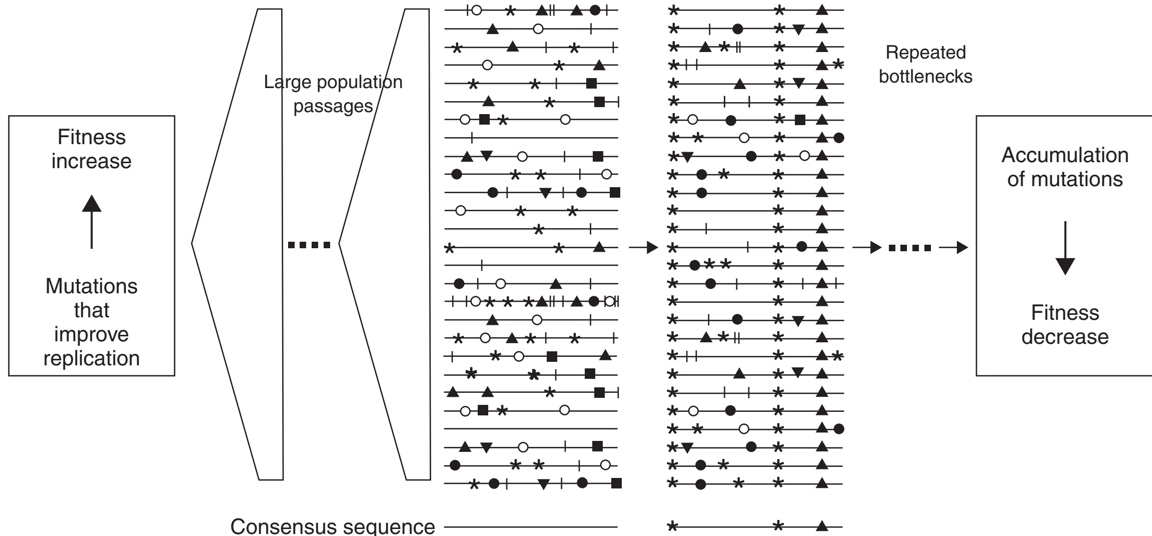

FIGURE 4.2 Schematic representation of viral quasispecies and the effect of viral population size on replicative fitness. Horizontal lines represent genomes and symbols on the lines represent mutations. Random sampling of genomes (bottleneck events, small arrows) lead to accumulation of mutations and fitness decrease. Large population passages (large arrows) lead to increases in replicative fitness. Fitness losses or gains depend on the initial fitness of the viral population and the size of the bottleneck. See text for details and references.

viral population reduced to a single genome at the onset of plaque formation (extreme genetic drift), and high mutation rates.

A study by Novella *et al.* (1995c) using VSV established that the extent of fitness loss for any given bottleneck size depends on the initial fitness of the viral clone under study. The higher the initial fitness, the less severe must the bottleneck be to avoid fitness losses. Debilitated viral clones often gain fitness even when subjected to considerable bottlenecking (Novella *et al.*, 1995a, 1995c). Rather constant, stable fitness values could be attained by choosing the appropriate bottleneck size, although occasional fitness jumps were observed (Novella *et al.*, 1996).

Escarmís *et al.* (1996, 2008) examined the genetic lesions associated with Müller's ratchet by determining genomic nucleotide sequences of FMDV clones prior to and after undergoing repeated (up to 409) plaque-to-plaque transfers. The result was that fitness loss was associated with unusual mutations that had never been seen in natural FMDV isolates or laboratory populations subjected to passages involving large viral populations. Particularly striking were an internal polyadenylate extension preceding the second functional AUG initiation codon of the FMDV genome, and amino acid substitutions at internal capsid residues. Additions or deletions of nucleotides have been frequently observed at homopolymeric tracts, particularly on pyrimidine runs in templates copied by proofreading-repair-deficient polymerases (Kunkel, 1990; Bebenek and Kunkel, 1993). The experimental results suggest that only when the repeated bottlenecks limit the action of negative selection (elimination or decrease in proportion of low fitness genomes) can such internal polyadenylate extensions (and other deleterious mutations) be maintained in the FMDV genome (Escarmís *et al.*, 1996, 2006). In contrast, sequence analysis of VSV genomes subjected to plaque-to-plaque passages did not show unusual mutations, with the possible exception of mutations in the RNA termini, which are uncommon in viruses evolving in regimes of acute replication (Novella and Ebendick-Corpus, 2004).

Fitness decrease upon subjecting FMDV to plaque-to-plaque transfers was biphasic: an initial decrease was followed by a highly fluctuating pattern with a constant average fitness value. The fluctuating pattern followed a Weibull statistical distribution (Weibull, 1951; Lazaro *et al.*, 2003). A Weibull distribution describes disparate physical and biological processes. In the case of plaque-to-plaque transfers of a virus this type of distribution probably results from the multiple host–virus interactions that occur as the virus life cycle is completed, and alterations of such interactions as mutations accumulate in multifunctional viral proteins (Lazaro *et al.*, 2003). The studies of evolution of FMDV when subjected to many repeated serial bottleneck transfers revealed a remarkable resistance of the virus to extinction despite a linear accumulation of mutations in its genome (Escarmís *et al.*, 2002), as well as the existence of multiple evolutionary pathways for fitness recovery (Escarmís *et al.*, 1999) (see also "Intra-mutant spectrum suppression can contribute to lethal mutagenesis," above).

Fitness has often been considered a component of parasite virulence, defined as the capacity of parasites to inflict damage upon their hosts. Indeed, very frequently an increase in viral fitness parallels an increase of virulence. However, a comparative quantitative analysis of fitness and virulence (cell-killing capacity) of an FMDV clone subjected to plaque-to-plaque transfers, and of its parental clone, revealed that fitness and virulence can be two unrelated traits (Herrera *et al.*, 2007). The molecular basis for the different trajectories followed by fitness and virulence resided in the fact that fitness was affected by mutations anywhere in the viral genome while determinants of cell-killing capacity were multigenic but restricted to some specific genomic regions of the viral genome. As a consequence, the random accumulation of mutations associated with bottleneck transfers had a more negative impact on fitness than on virulence of this FMDV clone (Herrera *et al.*, 2007). That viral fitness and virulence can follow different trajectories is supported

by several observations with animal and plant viruses. VSV populations that were subjected to a regime of persistent infection in sandfly cells showed overall decrease in both fitness and virulence in mammalian cells, but the decrease in virulence continued throughout the experiment, while the decrease in fitness peaked at intermediate passages and was followed by some degree of recovery (Zárate and Novella, 2004). Simian immunodeficiency virus SIVmac239 attains similar high viral loads in the sooty mangabey and the rhesus macaque, yet it is only virulent for the rhesus macaque (Kaur *et al.*, 1998). At an epidemiological level, greater fitness of historical versus current HIV-1 isolates was taken as evidence of HIV-1 attenuation over time, assuming a direct correlation between fitness and virulence (Arien *et al.*, 2005). However, no trend towards HIV-1 attenuation since the time of introduction of the virus into Switzerland was observed (Muller *et al.*, 2006). These and other studies with viral and non-viral parasites (reviewed in Herrera *et al.*, 2007) suggest that evolution in nature can drive parasites to attain virulence levels that are not necessarily coupled to fitness. This distinction between fitness and virulence should be taken into consideration in the formulation of models for parasite virulence.

Fitness Gain Upon Large Population Passages: Limitations, Exclusions, Memory and Molecular Transitions

In contrast to bottleneck passages, large population infections generally result in fitness gains of RNA viruses (Martinez *et al.*, 1991; Clarke *et al.*, 1993; Novella *et al.*, 1995b; Escarmís *et al.*, 1999). Fitness increase in this case is expected from a gradual optimization of mutant spectra when their different components, arising by mutation and in some cases also by recombination, are allowed unrestricted competition in a constant environment (Figure 4.2). High replicative fitness may help a virus to overcome selective constraints—including antiviral agents or immune responses (Quiñones-Mateu *et al.*, 2006; Grimm *et al.*, 2007)—and to delay extinction by lethal mutagenesis (Sierra *et al.*, 2000; Pariente *et al.*, 2001).

When the relative fitness of the evolving quasispecies reaches a high value, even quite large population sizes can constitute an effective bottleneck and prevent continuing fitness increase (Novella *et al.*, 1999a, 1999b). This limiting high fitness level was manifested by stochastic fluctuations in fitness values expected from random generation of mutations in a continuously evolving mutant swarm. These perturbations illustrate how difficult it is to attain a true population equilibrium even when viruses replicate in a constant environment. A rare combination of mutations—one that may occur only once over many rounds of viral replication—may transfer one genome and its descendants to a distant region of sequence space, and trigger the dominance of one viral subpopulation over another, thereby disrupting a period of population equilibrium. In competitions between two VSV clones of similar fitness coexisting at or near equilibrium, a rapid and unpredictable displacement of one VSV population by the other (Clarke *et al.*, 1994) provided support for a classical concept of population biology: the competitive exclusion principle (Gause, 1971). Furthermore, in the competition passages preceding mutual exclusion, both the winners and the losers gained fitness at comparable rates, in support of yet another concept of population genetics: the Red Queen hypothesis (Van Valen, 1973; Clarke *et al.*, 1994; reviewed in Domingo, 2006) (see Figure 4.1).

Parallel fitness gains were also observed for minority memory genomes and their majority counterparts in evolving FMDV quasispecies (Arias *et al.*, 2004). Memory genomes are subpopulations of genomes that remain in a replicating viral quasispecies at a frequency about 10^2- to 10^3-fold higher than the frequency that can be attributed to mutational pressure alone, and reflect those genomes that were dominant at a previous stage of the evolution of the same viral lineage (Ruiz-Jarabo *et al.*, 2000; review in Domingo, 2000). Memory has been documented with a number of genetic

markers of FMDV (Ruiz-Jarabo *et al.*, 2000, 2002, 2003b) and HIV-1 (Briones *et al.*, 2003, 2006), and similar results have been described for VSV (Novella *et al.*, 2007). Memory is a consequence of fitness variations inherent to quasispecies dynamics, likely to exert its main influence on the composition of mutant spectra that have been subjected to various alternating selective pressures (Domingo, 2000).

Relative viral fitness may depend on the multiplicity of infection (m.o.i.) used during selection or competition. High m.o.i. promotes coinfection, and the higher the level of coinfection the more likely that complementation will take place. Complementation effectively hides beneficial (and deleterious) variation from the effects of selection (Sevilla *et al.*, 1998; Wilke and Novella, 2003; Wilke *et al.*, 2004). In addition, high m.o.i. effects may relate to the use of alternative receptors or to interfering interactions occurring within the mutant spectra of viral quasispecies (Sevilla *et al.*, 1998; Perales *et al.*, 2007) (see section on "Intra-population complementation and interference in viral quasispecies: mutant distributions as the units of selection").

Defective viruses can be maintained in the course of high m.o.i. passages by complementation. An extensively documented case is the generation and maintenance of helper-dependent defective-interfering (DI) RNA and particles, which follow the process of mutation, competition and selection typical of quasispecies dynamics (Holland *et al.*, 1982; Roux *et al.*, 1991). Other types of defective genomes can also be maintained in viral populations by complementation (Charpentier *et al.*, 1996; Moreno *et al.*, 1997; Yamada *et al.*, 1998). Some defective genomes can be transmitted from infected into susceptible hosts, rendering the maintenance of defective genomes by complementation an event of potential epidemiological significance (Aaskov *et al.*, 2006).

A striking, extreme case of complementation between defective genomes was provided by evolution of standard FMDV towards two defective forms that were infectious and killed cells by complementation in the absence of standard FMDV (García-Arriaza *et al.*, 2004, 2005, 2006). These studies have provided evidence of a continuous dynamics of generation of defective FMDV genomes harboring in-frame internal deletions within genomic regions encoding *trans*-acting proteins, giving rise to swarms of genomes with non-identical, related deletions (García-Arriaza *et al.*, 2006). Each virion encapsidates only one type of defective genome and, therefore, the same cell must be infected by at least two different particles to permit complementation and formation of progeny defective genomes (Manrubia *et al.*, 2006). The high m.o.i.-dependent evolution of FMDV towards two defective forms that can complement each other has been regarded as experimental support of a first step in a process towards viral genome segmentation. Interestingly, multipartite segmented genomes are rare among the animal and bacterial viruses but are frequent among plant viruses, and the latter are characterized by high m.o.i. as they spread in their host plants (Lazarowitz, 2007).

The main conclusion we derive from the results summarized in the preceding paragraphs is that even in a relatively constant biological and physical environment, as is usually provided by *in vitro* cell culture systems, the degree of adaptation of viral quasispecies may undergo remarkable quantitative variations, prompted by the stochastic generation of mutant genomes, and different opportunities for competitive optimization of mutant spectra.

FITNESS VARIATIONS IN CHANGING ENVIRONMENTS

The experiments of fitness variation of viruses in cell culture summarized in the previous section have been instrumental in defining some basic influences that guide fitness evolution of viral quasispecies. However, in their replication in a natural setting, viruses encounter multiple and changing environments, and they often have to cope with conflicting selective constraints. Because of polymorphisms in key host proteins involved in cellular and humoral

immune responses, and in many other cell surface antigens, viruses do not face the same selective constraints in different individuals of the same host species. Biological environments are heterogeneous and vary with time within each infected individual. Furthermore, a considerable number of viruses are capable of infecting different host species, extending even further the range of environments they face.

Arboviruses that replicate in mammalian and insect hosts constitute a classical example of obligate environmental alternacy *in vivo* (Scott *et al.*, 1994; Weaver, 1998) (Chapter 16). Early work documented that extensive replication of viruses in insect cells led to attenuation of infectivity for mammalian cells (Peleg, 1971; Mudd *et al.*, 1973). Prolonged persistence of VSV in sandfly cells cultured at low temperatures resulted in several orders of magnitude greater fitness in insect cells than in mammalian cells (Novella *et al.*, 1995a; Zárate and Novella, 2004). In contrast, acute VSV replication in sandfly cells led to fitness increase in mammalian cells (Novella *et al.*, 1999a), and replication of West Nile virus in mosquito cells resulted in populations that, while not improved, showed no fitness losses in vertebrate cells (Ciota *et al.*, 2007). Thus, we cannot assume selective differences between insect and mammalian cells types, and when we observe tradeoffs, these may be due to different strategies of replication (persistent versus acute), not to difference in cell type *per se*.

A single passage of sandfly cell-adapted VSV in mammalian cells led to an increase in fitness in mammalian cells to near original values. It would be interesting to test whether this capacity for fitness shift would be similar for non-arboviral RNA viruses able to grow in insect cells in culture. VSV adapted to sandfly cells was highly attenuated for mice. Again, a single passage in mammalian cells restored the virulence phenotype *in vivo* (Novella *et al.*, 1995a).

Several groups have studied the evolutionary consequences of alternating environments during arbovirus replication (reviewed in Wilke *et al.*, 2006; Ciota *et al.*, 2007). The overall results showed that extensive alternating replication between mammalian and insect cells led to fitness improvement in both environments; the only exception was VSV adapted to alternation between persistent insect replication and acute mammalian replication: adaptation during alternation is dominated by the persistent environment and there is fitness loss in the mammalian environment (Zárate and Novella, 2004) (for details, see Chapter 16).

Studies of fitness variations *in vivo* have been approached in at least three ways. Some studies have involved growth-competition experiments between two viruses replicating in host organisms. In other studies, the outcome of competitions between viruses that were isolated *in vivo* has been analyzed in primary or established cell cultures. In yet another line of research, the effect of fitness variations in cell culture on the replicative potential of viruses *in vivo* has been examined.

Carrillo *et al.* (1998) isolated two variant FMDVs present at low frequency in the course of replication of a clonal virus preparation in swine. One of the variants was a MAb-resistant mutant (MARM), while the other was isolated from blood during the early viremic phase of the acute infection. The ability of the two variants to compete *in vivo* with the parental clonal population was examined by coinfection of swine with mixtures of the parental clone and each of the two variants individually. None of the two variants became completely dominant in a single coinfection *in vivo*, but fitness differences were clearly documented. The parental FMDV clone manifested a selective advantage over the MARM in that the parental clone was dominant in most lesions (vesicles) in the diseased swine. In contrast, the parental clone and the variant from the early viremic phase were about equally represented in the lesions of the animals infected with equal amounts of the two viruses (Carrillo *et al.*, 1998).

The lentivirus equine infectious anemia virus (EIAV) experiences continuous quasispecies fluctuations during persistent infections in horses (Clements *et al.*, 1988). EIAV quasispecies were characterized in a pony

experimentally infected with a biological clone of the virus. New quasispecies were associated with recurrent episodes of disease. A large deletion in the principal neutralizing domain of the virus was identified during the third febrile episode and became dominant during the fourth febrile episode. This drastic genetic change did not appear to diminish significantly the fitness of EIAV *in vivo* and in cell culture (Leroux *et al.*, 1997). The complexity of sequential EIAV populations *in vivo*, was characterized with a non-hierarchical clustering method to analyze quasispecies, termed PAQ (partition analysis of quasispecies) (Baccam *et al.*, 2001). This procedure to dissect the composition of mutant spectra should allow the recognition of subpopulations within viral quasispecies as they evolve towards fitness gain or loss.

Fitness Variations in Viral Disease Emergence and Reemergence. The Case of Human Influenza Virus

The multiple environments in which viruses have to replicate *in vivo* may promote the selective expansion of subpopulations from viral quasispecies thereby leading to variant viruses that display altered relative fitness in different host organs, as compared with their parental populations. Such variations in the potential replicative capacity constitute one of the ingredients that may affect the emergence and reemergence of viral disease (reviews in Smolinski *et al.*, 2003; Peters, 2007). The genetic lottery of blind variation through mutation, recombination, and genome segment reassortment is played in the face of a background of multiple ecological, sociological, and demographic factors. In recent decades viral disease emergences that have affected humans have occurred at a rate of about one per year. Salient examples are acquired immune deficiency syndrome (AIDS), severe acute respiratory syndrome (SARS), encephalitis associated with West Nile virus, the expansion of dengue fever, or periodic influenza pandemics (Smolinski *et al.*, 2003; Peters, 2007).

Multiple genetic changes may favor the adaptation of a virus to a new host. Once adaptation has taken place, the adapted virus may lose or maintain the pathogenic potential for the former (donor) host (as an example of maintenance of virulence for a donor and recipient host in FMDV see Nuñez *et al.*, 2007). A core (or basal) genetic composition of a viral pathogen may be in itself a predictor of pathogenic potential, as profusely documented with natural or laboratory-generated, attenuated variants of many viral pathogens. To take influenza A virus and the threat of a human influenza pandemics as examples, out of the 16 hemagglutinin (H) and 9 neuraminidase (N) subtypes circulating among animal reservoirs, some potentially threatening forms being more carefully kept under surveillance include H_5N_1, H_7N_7, H_9N_2, and H_2N_2 viruses. The expansion of the H_5N_1 subtype among wild and domestic avian species and human contacts since 2005 has resulted in over 300 human cases in nearly 50 countries, with more than 50% deaths, as well as the killing of millions of poultry (Wright *et al.*, 2007). Key parameters for an avian influenza virus to give rise to a human influenza pandemic include acquisition of receptor-recognition specificity for human cells, and the capacity for efficient human-to-human transmission (Parrish and Kawaoka, 2005; Suzuki, 2005). This capacity can be expressed as the basic reproductive ratio (Ro) which is the average number of infected contacts from each infected host (review in Nowak and May, 2000). "Epidemiologic fitness" has been used to describe (through samplings of definitory genomic sequences, diagnostic surveys, etc.) the capacity of a virus to become dominant (relative to related viruses or variants) during epidemic outbreaks (Domingo, 2007). In the case of human influenza virus, the acquisition of high epidemiological fitness depends on multiple gene products. Critical substitutions in H may modify the receptor-binding specificity of influenza viruses, and such substitutions have been found in minority subpopulations of influenza virus in several surveys. In one study, two substitutions

in H identified in a human influenza virus from a fatal human case, were shown to modify the receptor-binding preference of H of a H_5N_1 virus from sialic acid-α2,3 galactose (associated with replication in avian hosts) to both sialic acid-α2,3 galactose and sialic acid-α2,6 galactose, both associated with binding to human-type receptors, each expressed preferentially in different sites of the human respiratory tract (Auewarakul *et al.*, 2007). Thus, in influenza virus, and probably many other pathogenic viruses, both epidemiologic fitness and replicative fitness are multigenic traits (Grimm *et al.*, 2007).

Several studies have compared the amino acid sequence of multiple influenza virus proteins to search for markers (amino acid substitutions) of human isolates and human pandemic strains (from 1918, 1957, 1968 and recent human H_5N_1 isolates). In one such proteomics survey, several amino acid changes located in PB2, PA, NP, M1, and NS1 distinguished avian influenza viruses from their human counterparts (Finkelstein *et al.*, 2007). Some markers were conserved in the influenza viruses that caused the 1918, 1957, and 1968 pandemics. Other studies have identified HA and PB2 as critical for adaptation of avian virus to humans, that may occur by a step-wise process reflected in acquisition of diagnostic amino acid markers. Evidence of human-to-human transmission of avian influenza virus H_5N_1 has been obtained in some family case clusters but not in others (Yang *et al.*, 2007). Influenza constitutes the paradigm of a viral disease which, favored by a continuum of genetic variation, reemerges periodically to cause pandemics, and for which extensive epidemiological surveillance is currently in operation.

Fitness and Drug Resistance in HIV-1

An increasing number of measurements of viral fitness involve human immunodeficiency virus 1 (HIV-1) variants isolated from quasispecies replicating *in vivo*. Particularly relevant are fitness comparisons among multiple mutants harboring amino acid substitutions related to resistance to reverse transcriptase and protease inhibitors (see also Chapter 14).

HIV-1 Reverse Transcriptase (RT) Inhibitors

Since the discovery of AZT (3′-azido-3′-deoxythymidine, zidovudine) as an effective inhibitor of HIV replication (Mitsuya *et al.*, 1985), drug therapy has been widely used in the treatment of AIDS. The loss of therapeutic effect due to the acquisition of resistance was recognized for AZT in 1989, when Larder and colleagues showed that HIV isolates from patients with advanced HIV disease became less sensitive to the drug during the course of treatment (Larder *et al.*, 1989). High-level resistance to AZT is achieved through the accumulation of several mutations including M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E (for a review, see Larder, 1994). The first substitution arising during AZT treatment is usually K70R, followed by T215Y. The K70R mutation appears frequently, since it requires only one nucleotide change, and does not have a major impact on viral fitness (Harrigan *et al.*, 1998). The simultaneous presence of Leu41 and Tyr215 in the viral RT-coding region confers high-level resistance to AZT, without having a major effect on viral fitness. In contrast, other combinations of AZT resistance mutations (e.g. M41L/K70R) confer reduced replication capacity (Jeeninga *et al.*, 2001). Interestingly, transmitted HIV-1 carrying D67N or K219Q evolve rapidly to AZT resistance *in vitro* (selecting for K70R) and show a high replicative fitness in the presence of zidovudine (García-Lerma *et al.*, 2004). On the other hand, L210W improved infectivity and relative fitness of an M41L/T215Y mutant in the presence of AZT, but decreased infectivity and relative fitness when introduced into a D67N/K70R/K219Q background (Hu *et al.*, 2006).

Drug-resistant mutations occur in the mutant spectra of HIV-1 quasispecies from untreated patients (Nájera *et al.*, 1995). The replacement of Tyr215 by Cys, Asp, or Ser has been observed *in vivo* in the absence of zidovudine treatment (Goudsmit *et al.*, 1997;

Yerly *et al.*, 1998). In the absence of inhibitor, T215S and T215D confer a small but significant advantage over the wild-type virus, as determined *in vitro* in growth competition experiments. However, the replicative advantage conferred by T215S was lost in the presence of zidovudine-resistance mutations such as M41L and L210W (García-Lerma *et al.*, 2001).

Other nucleoside inhibitors of HIV-1 RT are listed in Table 4.1. High-level resistance to the nucleoside analogue 3TC (2′, 3′-dideoxy-3′-thiacytidine, lamivudine) is rapidly achieved by the substitution M184 V, located at the YMDD motif, which is part of the catalytic core of the enzyme. During 3TC treatment, the substitution M184I appears first, but then

TABLE 4.1 Amino Acid Substitutions Associated with HIV-1 Resistance to Antiretroviral Drugs

Inhibitors	Amino acid substitutions associated with drug resistance[a]
Nucleoside analogue RT inhibitors	
Zidovudine (AZT)	M41L, D67N, K70R, L210W, T215Y/F, K219Q/E
Didanosine (ddI)	K65R, L74V
Lamivudine (3TC)	(E44D/V118I), K65R, M184V/I
Stavudine (d4T)	M41L, D67N, K70R, V75T, V118I, L210W, T215Y/F, K219Q/E
Zalcitabine (ddC)	K65R, T69D, L74V, M184V
Abacavir	K65R, L74V, Y115F, M184V
Emtricitabine	(K65R/Q151 M), M184V/I
Tenofovir	K65R, K70E
Multiple nucleoside analogues	(i) M41L, D67N, K70R, L210W, T215F/Y, K219Q/E; (ii) A62V, V75I, F77L, F116Y, Q151M; (iii) Insertions between codons 69–70 (e.g. T69SSS, T69SSG, T69SSA, etc.), M41L, A62V, K70R, L210W, T215Y/F
Non-nucleoside analogue RT inhibitors	
Nevirapine	L100I, K101P, K103N/S, V106A/M, V108I, Y181C/I, Y188C/L/H, G190A/C/E/Q/S/T
Delavirdine	K103H/N/T, V106M, Y181C, Y188L, G190E, P236L
Efavirenz	L100I, K103H/N, V106M, V108I, Y188L, G190A/S/T, P225H
PR inhibitors[b]	L10I/R/V, **G48V**, I54L/V, A71T/V, G73S, V77I, V82A, I84V, **L90M**
Saquinavir	
Ritonavir	L10I/R/V, K20M/R, V32I, L33F, M36I, M46I/L, I54V/L, A71V/T, V77I, **V82A/F/S/T, I84V**, L90M
Indinavir	L10I/R/V, K20M/R, L24I, V32I, M36I, **M46I/L**, I54V, A71T/V, G73A/S, V77I, **V82A/F/S/T, I84V**, L90M
Nelfinavir	L10F/I, **D30N**, M36I, M46I/L, A71T/V, V77I, V82A/F/S/T, I84V, N88D/S, **L90M**
Amprenavir	L10F/I/R/V, V32I, M46I/L, I47V, **I50V**, I54M/V, **I84V**, L90M
Lopinavir	L10F/I/R/V, G16E, K20I/M/R, L24I, V32I, L33F, E34Q, M36I/L, K43T, **M46I/L**, **I47A/V**, G48M/V, I50V, **I54L/V**/A/M/S/T, Q58E, L63T, A71T, G73T, T74S, **V82A**/F/S/T, I84V, L89I/M
Atazanavir	L10F/I/V, K20I/M/R, L24I, L33F/I/V, M36I/L/V, M46I/L, G48V, **I50L**, I54L/V, L63P, A71I/T/V, G73A/C/S/T, V82A/F/S/T, **I84V, N88S**, L90M
Tipranavir	L10I/S/V, I13V, K20M/R, **L33F/I/V**, E35G, M36I/L/V, K43T, M46L, I47V, I54A/M/V, Q58E, H69 K, T74P, **V82L/T**, N83D, **I84V**, L90M
Darunavir	V11I, V32I, L33F, I47V, **I50V**, I54L/M, G73S, L76V, I84V, L89V
Fusion inhibitors	G36D/E/S, I37T/V, V38A/M/E, Q40H, N42T, N43D/K/S
Enfuvirtide	

[a] For additional information, see (Clark *et al.*, 2007; Clotet *et al.*, 2007; Johnson *et al.*, 2007).

[b] Primary resistance mutations are shown in bold. Most PR inhibitors (saquinavir, indinavir, amprenavir, lopinavir, atazanavir, tipranavir, and darunavir) are usually prescribed in combination with a low dose of ritonavir, that has a boosting effect on the PR inhibitor concentration in plasma.

it is lost due to the outgrowth of the M184V-containing viruses (Keulen *et al.*, 1997). Growth competition experiments showed a selective advantage of viruses with Val184 over those with Ile184. The low efficiency of 3TC-resistant HIV-1, carrying RT mutations M184V or M184I, has been attributed to the low processivity of the mutant RT (Back *et al.*, 1996), which was accentuated in peripheral blood mononuclear cells (PBMCs) (Keulen *et al.*, 1997).

Other nucleoside analogue resistance mutations (e.g. K65R, K70E, or L74V) also have a significant impact on viral fitness, which correlates with a defect in RT processivity (Sharma and Crumpacker, 1997; Miller *et al.*, 1998; Sharma and Crumpacker, 1999; White *et al.*, 2002). The presence of K65R together with L74V or M184V has a strong deleterious effect on viral replication, due to the poor ability of K65R/L74V to use natural nucleotides relative to the wild type (Deval *et al.*, 2004), or to the negative impact of the simultaneous presence of K65R and M184V on the RT's processivity, as well as in the initiation of reverse transcription (White *et al.*, 2002; Frankel *et al.*, 2007). These observations are consistent with the low prevalence of the K65R mutation among isolates from antiretroviral-drug experienced patients, and give rational support to the benefit in combining mutations that impair virus replication.

Drug combinations are very effective in blocking HIV replication, leading to a more than 10 000-fold reduction of viral load. Early studies showed that multiple drug resistance to AZT and other inhibitors can be achieved through the accumulation of mutations appearing in monotherapy (Schmit *et al.*, 1996; Shafer *et al.*, 1998). However, the response of a viral quasispecies to multiple constraints (e.g. different antiviral drugs) is often difficult to predict. Simultaneous treatment with AZT and ddI led to viruses with reduced sensitivity to AZT, ddC, ddI, ddG, and d4T (Shirasaka *et al.*, 1995; Iversen *et al.*, 1996). The resistant viruses contained substitutions A62V, V75I, F77L, F116Y, and Q151 M. Substitution Q151 M, which results from two nucleotide changes, is the first to appear and confers partial resistance to AZT, ddI, ddC, and d4T. Fitness assays involving the determination of replication kinetics or growth competition experiments showed that mutations at codons 62, 75, 77, and 116 improved the replication capacity of the resistant virus (Maeda *et al.*, 1998; Kosalaraksa *et al.*, 1999).

With the increasing complexity of the antiretroviral regimens, novel mutational patterns conferring resistance to multiple antiretroviral drugs have been identified. Thus, HIV-1 variants with insertions or deletions in the "fingers" subdomain of the RT have been found in patients failing therapy with multiple RT inhibitors (Mas *et al.*, 2000; for a recent review, see Menéndez-Arias *et al.*, 2006). The presence of the amino acid changes T69S and T215Y in the RT, together with a dipeptide insertion between positions 69 and 70 (usually Ser-Ser), and the subsequent accumulation of additional mutations (e.g. M41L, A62V, T69S, and K70R) leads to the emergence of virus displaying high-level resistance to thymidine analogues (Matamoros *et al.*, 2004; Cases-González *et al.*, 2007). Dual infection/competition experiments revealed that in the presence of low concentrations of AZT, removal of the two serine residues forming the dipeptide insertion in a multidrug-resistant isolate does not cause a detrimental effect on the replication capacity of the virus (Quiñones-Mateu *et al.*, 2002). However, in the absence of drug, the insertions improved the fitness of virus-carrying thymidine analogue mutations (e.g. M41L, L210W, T215Y, etc.). Although, multidrug-resistant mutants are able to maintain high viral loads in the presence of antiretroviral therapy, it should be noted that *in vivo* wild-type HIV variants outcompete those bearing the insertion, as demonstrated when therapy is interrupted (Briones *et al.*, 2000; Lukashov *et al.*, 2001).

Non-nucleoside RT inhibitors bind to a hydrophobic cavity which is 8–10 Å away from the polymerase active site, and lined by the side-chains of Tyr181, Tyr188, Phe227, and Trp229 (Kohlstaedt *et al.*, 1992). High-level resistance appears quickly after treatment and involves amino acid changes in residues

located at the inhibitor binding site (Table 4.1). Again, resistance mutations often lead to reduced *in vitro* replication capacity. Examples are the nevirapine-resistance mutation V106A and the delavirdine-resistance mutation P236L that impair RNase H activity (Gerondelis *et al.*, 1999; Archer *et al.*, 2000; Dykes *et al.*, 2001; Iglesias-Ussel *et al.*, 2002; Collins *et al.*, 2004), as well as several mutations at codons 138 and 190, whose effects appear to be related to impaired DNA synthesis and RNase H degradation (Pelemans *et al.*, 2001; Huang *et al.*, 2003; Collins *et al.*, 2004; Wang *et al.*, 2006).

HIV-1 Protease (PR) Inhibitors

The HIV-1 PR is a homodimeric enzyme composed of two polypeptide chains of 99 residues. The substrate binding site is located at the interface between both subunits. The side-chains of Arg8, Leu23, Asp25, Gly27, Ala28, Asp29, Asp30, Val32, Ile47, Gly48, Gly49, Ile50, Phe53, Leu76, Thr80, Pro81, Val82, and Ile84 form the substrate-binding pocket and can interact with specific inhibitors (Wlodawer and Vondrasek, 1998), such as those used in the clinical treatment of AIDS. Approved PR inhibitors share relatively similar chemical structures and cross-resistance is commonly observed in the clinical setting (Menendez-Arias, 2002). It is not unexpected that many resistance mutations affect residues of the inhibitor-binding pocket of the PR (Table 4.1). Studies carried out *in vivo* and *in vitro* have shown that several amino acid substitutions involved in drug resistance may have a deleterious effect on viral fitness. Examples are D30N, I47A, I50V, G48V, and V82A (Eastman *et al.*, 1998; Martinez-Picado *et al.*, 1999; Kantor *et al.*, 2002; Prado *et al.*, 2002; Yusa *et al.*, 2002; Colonno *et al.*, 2004).

The deleterious effects caused by drug resistance mutations can be rescued by other amino acid replacements. For example, multidrug-resistant virus arising during prolonged therapy with indinavir contained PR with the substitutions M46I, L63P, V82T, and I84V (Condra *et al.*, 1995; Martinez-Picado *et al.*, 1999). Crystallographic studies of the mutant enzyme revealed that substitutions at codons 82 and 84 were critical for the acquisition of resistance, while the amino acid changes at codons 46 and 63, which are away from the inhibitor-binding site appear as compensatory mutations (Chen *et al.*, 1995; Schock *et al.*, 1996). Although compensatory mutations within the PR-coding region increase the catalytic efficiency of the enzyme, there are other molecular mechanisms that lead to fitness recovery during PR inhibitor treatments. Examples are: (i) mutations at Gag cleavage sites that increase polyprotein processing (Doyon *et al.*, 1996; Zhang *et al.*, 1997; Pettit *et al.*, 2002), (ii) mutations that affect the frameshift signal between the *gag* and *pol* genes that lead to an increased expression of *pol* products (Doyon *et al.*, 1998), or (iii) mutations outside of the cleavage sites that could affect the conformation of the Gag polyprotein and make the cleavage sites more accessible to the viral PR (Gatanaga *et al.*, 2002; Myint *et al.*, 2004).

Novel Antiretroviral Drugs

For many years, the RT and the PR were the only targets of approved antiretroviral drugs. In 2003, enfuvirtide, a synthetic peptide that impairs virus–host cell membrane fusion, was licensed for clinical use. Resistance to enfuvirtide is mediated by amino acid substitutions at codons 36–38 of the envelope glycoprotein gp41. The amino acid sequences found at those positions in drug-sensitive viruses (DIV, SIV, GIV, or GIM) are replaced by SIM, DIM, or DTV in the drug-resistant clones (Rimsky *et al.*, 1998). As observed with PR and RT inhibitors, resistance mutations cause a fitness loss, which was estimated to be approximately 10% in replication kinetics and growth competition experiments (Lu *et al.*, 2004; Reeves *et al.*, 2005). However, it should be noted that mutations in the V3 loop of the envelope glycoprotein gp120 can also affect the viral susceptibility to enfuvirtide (Reeves *et al.*, 2002), and further studies will be necessary to evaluate its impact on viral fitness *in vivo.*

In August 2007, a CCR5 coreceptor antagonist known as maraviroc was approved for clinical use. Maraviroc has potent antiviral activity against CCR5-tropic HIV-1 variants, including primary isolates from various clades (Dorr *et al.*, 2005). Maraviroc-resistant HIV variants contained unique amino acid changes in the V3 loop (e.g. A316T and I323V) and other positions within the envelope glycoprotein, gp120, but continued to be phenotypically CCR5-tropic and sensitive to CCR5 antagonists in preclinical development, such as vicriviroc (Westby *et al.*, 2007).

Other antiretroviral drugs showing promising results in clinical trials are the integrase inhibitors raltegravir (licensed in October 2007) and elvitegravir. However, the information available on specific drug resistance mutations and their effects on the viral replication capacity is still preliminary.

This review of fitness effects of drug-resistance mutations in HIV-1 provides a dramatic illustration of the adaptive potential of a viral quasispecies. Acquisition of critical amino acid replacements for drug resistance, fitness effects that favor selection of compensatory mutations either in a viral enzyme or in its target substrate, occurrence of clusters of mutations for multidrug resistance are but some of the mechanisms displayed by HIV-1 to persist in the human population.

OVERVIEW

The virological significance of quasispecies theory becomes more apparent each year. Initial reports of extremely high error rates and great population diversity of RNA viruses were hotly disputed as being incorrect and inconsistent with often-observed stability in virus markers such as antigenicity, disease characteristics, host range, etc. High error rates and intra-population heterogeneity for RNA viruses are now widely accepted. Fortunately, early quasispecies theory presented a timely, remarkably prescient theoretical framework within which the behavior of replicating and evolving RNA virus populations could begin to be understood. Following elaboration by Eigen, Biebricher, Schuster, and colleagues, quasispecies-derived theory has been rapidly progressing and evolving. Its ground-breaking initial theoretical structure for exploring consequences of extreme biological error rates was informed by elegant molecular replication/mutation kinetic studies with small RNA replicons *in vitro.* Original quasispecies theory was formally applicable to these *in vitro* experiments, and was necessarily generalized, idealized, and, in many specifics, openly unrealistic for real viruses. Some simplifying assumptions not applicable to viruses in the real world include: infinite virus populations; global optima in the selective landscape; one most-fit master sequence in a single, unvarying selective landscape; fitness restricted to competition solely between one master sequence and diverse variants of equally low fitness; and, finally, omission of complexities such as replicative interference, lethal mutations, complementation, recombination, etc.

Early modeling could not reasonably encompass all real-life complexities. To attempt inclusion of all would render any model (or alternative collection of models) hopelessly unwieldy, uninformative, and poorly predictive due to requisite alternate weightings of factors. Simplified assumptions not conforming to complex realities need not detract from the ability of models to serve as starting points and guideposts toward new directions for experiment and theory. Quasispecies theory has indisputably led virology to powerful new insights, deductions, and directions. A few critics have suggested that the non-real world parameters in early quasispecies models, and the non-realistic (and foregone) conclusions that can be contrived from them, are reason to reject the general validity and broad significance of quasispecies. Such circular arguments are specious and trivial relative to the experimental and conceptual advances already-made, and yet-to-be-made, via quasispecies theory with its straightforward conclusions and more subtle implications.

Increasingly sensitive analyses of viral quasispecies in recent decades have produced

many remarkable insights. The most basic, far-reaching, awesomely predictive tenet of quasispecies theory will never be overshadowed; numerous variant genomes are bound together through extreme mutation rates, forming obligatorily co-selected partnerships in a vast, error-prone mutant spectrum from which they cannot escape, and from which they inevitably and coordinately may exert myriad, changing, ultimately unforseeable effects on all life forms. This tenet has been unquestionably and elegantly confirmed recently by the U. C. San Francisco, Stanford and Penn State groups (as reviewed above and elsewhere in this volume).

A significant postulate of early quasispecies models was that of "error catastrophe." This posits that replicase-generated quasispecies mutation rates are, through evolutionary selection, poised at, or near, an error threshold. Prolonged violation of this threshold (through replicase dysfunction, mutagens, elevated temperatures, nucleotide pool alterations, etc.) leads to virus extinction via a fast and irreversible transition, that has sometimes been equated with a phase transition in physics, as discussed in the opening chapter of this volume. Because the simplified model employed non-realistic parameters and envisioned indefinite mutational drift, critics deny the existence of error thresholds and sharp transitions to error catastrophe. No real-world virus could conform to the simplifying assumptions employed in that model, but recent data from lethal mutagenesis experiments do demonstrate devolution to error catastrophe. Historical precedent for the term "error catastrophe " lies with Orgel's suggestion in 1963 of cascading coordinate collapse of cellular information within (and between) various interdependent cellular nucleic acid and protein *trans*-networks. We also employ the term in a broad manner for lethal mutagenesis. This is especially appropriate with mutagens such as 5-FU (which modify both viral and cellular nucleic acids and their encoded functions and structures). We cannot presently rule out some roles for mutagenized cellular, as well as viral, macromolecules, during lethal mutagenesis. Regardless, complex interactions of altered viral macromolecular networks are definitely involved. Extinction is mediated by "*trans*-acting networks" among abundant lethal defector genomes. The senior author's group in Madrid demonstrated (reviewed above) that strongly mutagenized RNA virus populations do collapse to extinction via a sharp transition, but without the non-lethal, continuous mutational drift exemplified in the original quasispecies simulations. Extremely rapid extinctions are observed for low-fitness input virus strains, which transition into error catastrophe during a single round of infection/mutagenesis. Lethal mutagenesis of FMDV and LCMV is mediated by full-length, replicating, interfering, lethal defector genomes. Total (defective and viable) genomic RNA mutation frequencies are elevated to varying extents, whereas specific infectivity of total genomic RNA is decreased by several orders of magnitude without any change of RNA consensus sequence. In light of quasispecies "variant-ensemble" behavior, it is not surprising that defective genomes can predominate within *trans*-acting networks during lethal mutagenesis, and continue to replicate even after extinction of LCMV infectivity. Defector *trans*-effects can provide positive complementation in concert with, or alternation with, (orthogonal) interference. Standard concepts of virus fitness are only tangentially applicable within such collapsing *trans*-networks. Catastrophic decay of viral digital information proceeds on (at least) two levels: (1) genetic quenching due to egregious fixation of genomic mutations in a *trans*-network environment that does not always select for optimal function of self-encoded proteins, and (2) phenotypic *trans*-quenching of potentially viable genomes via altered, defector-encoded (interfering) proteins. Possible roles of RNA recombination remain to be explored.

Defector-driven transitions will be challenging to dissect, and no theoretical model can possibly capture even their main intricacies. During lethal mutagenesis at high multiplicities of infection, each infected cell is a single compartment in which a separate, discrete error catastrophe event may

devolve. Each discrete *trans*-network is disrupted and obscured during virus passages or RNA transfections following initial infection/mutagenesis. Multiplicity of infection (for both viable and defector virions) is clearly a crucial variable during passages. Virus strains with low replicative fitness (and mutagen-debilitated genomes) theoretically should be (and are) more vulnerable than highly fit strains to defector-mediated error catastrophe. Low-fitness strains cannot quickly produce high yields during temporary escape from defector networks. Future investigations with controlled compartmentalization (e.g. characterization of isolated infectious centers, micro-injection of single cells, etc.), together with molecular genetic construction/reconstruction of defined *trans*-networks will illuminate pre-extinction events.

The Madrid group has already verified that ordinary, viable FMDV variant RNAs, and mixtures of variant RNAs bearing defined mutations in the capsid and polymerase genes can exert *trans*-complementation and interference effects on standard FMDV RNA following co-electroporation of cells. Clearly, at high multiplicities of co-electroporation, such mixtures of defined variant and control RNAs generate unique, mutually supportive or suppressive (complementing/interfering) *trans*-acting networks within each individual, coinfected cell. This provides strong analogies to events during the transitions of lethal mutagenesis. The compelling differences, of course, are that the latter devolve to extinction due to: (1) mutagen-elevated mutation, AND elevated mutation-fixation rates in a poorly-selective *trans*-milieu; (2) potent *trans*-quenching of surviving-and-collapsing infectious virus via interfering (lethal defector-encoded) proteins.

Thus, the quasispecies postulate of a rapid transition to extinction has been experimentally verified, albeit the complex defector mechanisms for real viruses differ significantly from those originally modeled, as indeed recognized and anticipated by Eigen (see above). It is evident that the details of lethal mutagenesis will likely differ among families of RNA viruses (e.g. those having mono-, bi-, or multipartitite genomes, strong or weak complementation, homologous or only non-homologous recombination, naked or enveloped capsids, etc.). However, it seems probable that error catastrophe will be observed in all. Although no theoretical model can possibly capture all the ingredients involved in the replicative collapse of a mutagenized viral population, it was the original error threshold which inspired the experiments currently being performed in several laboratories.

The tenets of Eigen, Biebricher, Schuster, and colleagues, derived from first principles and tractable models, have had enormous influence in virology. This pervasive influence is in no manner weakened nor negated by original simplifying assumptions.

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REFERENCES

Aaskov, J., Buzacott, K., Thu, H.M., Lowry, K. and Holmes, E.C. (2006) Long-term transmission of defective RNA viruses in humans and Aedes mosquitoes. *Science* **311**, 236–238.

Airaksinen, A., Pariente, N., Menendez-Arias, L. and Domingo, E. (2003) Curing of foot-and-mouth disease virus from persistently infected cells by ribavirin involves enhanced mutagenesis. *Virology* **311**, 339–349.

Ali, A., Li, H., Schneider, W.L., Sherman, D.J., Gray, S., Smith, D. and Roossinck, M.J. (2006) Analysis of genetic bottlenecks during horizontal transmission of Cucumber mosaic virus. *J. Virol.* **80**, 8345–8350.

Anderson, J.P., Daifuku, R. and Loeb, L.A. (2004) Viral error catastrophe by mutagenic nucleosides. *Annu. Rev. Microbiol.* **58**, 183–205.

Archer, R.H., Dykes, C., Gerondelis, P., Lloyd, A., Fay, P., Reichman, R.C., Bambara, R.A. and

Demeter, L.M. (2000) Mutants of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase resistant to nonnucleoside reverse transcriptase inhibitors demonstrate altered rates of RNase H cleavage that correlate with HIV-1 replication fitness in cell culture. *J. Virol.* **74**, 8390–8401.

Arias, A., Ruiz-Jarabo, C.M., Escarmis, C. and Domingo, E. (2004) Fitness increase of memory genomes in a viral quasispecies. *J. Mol. Biol.* **339**, 405–412.

Arias, A., Agudo, R., Ferrer-Orta, C., Perez-Luque, R., Airaksinen, A., Brocchi, E. *et al.* (2005) Mutant viral polymerase in the transition of virus to error catastrophe identifies a critical site for RNA binding. *J. Mol. Biol.* **353**, 1021–1032.

Arien, K.K., Troyer, R.M., Gali, Y., Colebunders, R.L., Arts, E.J. and Vanham, G. (2005) Replicative fitness of historical and recent HIV-1 isolates suggests HIV-1 attenuation over time. *AIDS* **19**, 1555–1564.

Arnold, J.J., Vignuzzi, M., Stone, J.K., Andino, R. and Cameron, C.E. (2005) Remote site control of an active site fidelity checkpoint in a viral RNA-dependent RNA polymerase. *J. Biol. Chem.* **280**, 25706–25716.

Auewarakul, P., Suptawiwat, O., Kongchanagul, A., Sangma, C., Suzuki, Y., Ungchusak, K. *et al.* (2007) An avian influenza H5N1 virus that binds to a human-type receptor. *J. Virol.* **81**, 9950–9955.

Baccam, P., Thompson, R.J., Fedrigo, O., Carpenter, S. and Cornette, J.L. (2001) PAQ: partition analysis of quasispecies. *Bioinformatics* **17**, 16–22.

Back, N.K., Nijhuis, M., Keulen, W., Boucher, C.A., Oude Essink, B.O., van Kuilenburg, A.B. *et al.* (1996) Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J.* **15**, 4040–4049.

Batschelet, E., Domingo, E. and Weissmann, C. (1976) The proportion of revertant and mutant phage in a growing population, as a function of mutation and growth rate. *Gene* **1**, 27–32.

Bebenek, K. and Kunkel, T.A. (1993) The fidelity of retroviral reverse transcriptases. In: *Reverse Transcriptase* (A.M. Skalka and S.P. Goff, eds), pp. 85–102. New York: Cold Spring Harbor Laboratory Press.

Biebricher, C.K. and Domingo, E. (2007) The advantage of the high genetic diversity in RNA viruses. *Future Virol.* **2**, 35–38.

Biebricher, C.K. and Eigen, M. (2005) The error threshold. *Virus Res.* **107**, 117–127.

Botstein, D. (1980) A theory of modular evolution for bacteriophages. *Ann. NY Acad. Sci.* **354**, 484–491.

Briones, C., Mas, A., Gomez-Mariano, G., Altisent, C., Menendez-Arias, L., Soriano, V. and Domingo, E. (2000) Dynamics of dominance of a dipeptide insertion in reverse transcriptase of HIV-1 from patients subjected to prolonged therapy. *Virus Res.* **66**, 13–26.

Briones, C., Domingo, E. and Molina-París, C. (2003) Memory in retroviral quasispecies: experimental evidence and theoretical model for human immunodeficiency virus. *J. Mol. Biol.* **331**, 213–229.

Briones, C., de Vicente, A., Molina-Paris, C. and Domingo, E. (2006) Minority memory genomes can influence the evolution of HIV-1 quasispecies in vivo. *Gene* **384**, 129–138.

Bushman, F. (2002) *Lateral DNA Transfer. Mechanisms and Consequences*. New York: Cold Spring Harbor Laboratory Press.

Callaway, D.S. and Perelson, A.S. (2002) HIV-1 infection and low steady state viral loads. *Bull. Math. Biol.* **64**, 29–64.

Carrillo, C., Borca, M., Moore, D.M., Morgan, D.O. and Sobrino, F. (1998) *In vivo* analysis of the stability and fitness of variants recovered from foot-and-mouth disease virus quasispecies. *J. Gen. Virol.* **79**, 1699–1706.

Cases-Gonzalez, C.E., Franco, S., Martinez, M.A. and Menendez-Arias, L. (2007) Mutational patterns associated with the 69 insertion complex in multi-drug-resistant HIV-1 reverse transcriptase that confer increased excision activity and high-level resistance to zidovudine. *J. Mol. Biol.* **365**, 298–309.

Ciota, A.T., Lovelace, A.O., Ngo, K.A., Le, A.N., Maffei, J.G., Franke, M.A. *et al.* (2007) Cell-specific adaptation of two flaviviruses following serial passage in mosquito cell culture. *Virology* **357**, 165–174.

Clark, S.A., Calef, C. and Mellors, J.W. (2007) Mutations in retroviral genes associated with drug resistance. In: *HIV Sequence Compendium 2006–2007* (ed. by T. Leitner, B. Foley, B. Hahn, P. Marx, F. McCutchan, J. Mellors, S. Wolinsky and B. Korber), pp. 58–158. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory. Los Alamos, New Mexico, USA.

Clarke, D.K., Duarte, E.A., Moya, A., Elena, S.F., Domingo, E. and Holland, J. (1993) Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses. *J. Virol.* **67**, 222–228.

Clarke, D.K., Duarte, E.A., Elena, S.F., Moya, A., Domingo, E. and Holland, J. (1994) The red queen reigns in the kingdom of RNA viruses. *Proc. Natl Acad. Sci. USA* **91**, 4821–4824.

Clements, J.E., Gdovin, S.L., Montelaro, R.C. and Narayan, O. (1988) Antigenic variation in lentiviral diseases. *Annu. Rev. Immunol.* **6**, 139–159.

Clotet, B., Menéndez-Arias, L., Schapiro, J.M., Kuritzkes, D., Burger, D., Telenti, A., Brun-Vezinet, F., Geretti, A.M., Boucher, C.A., and Richman, D.D. (eds.) (2007) *Guide to management of HIV drug resistance, antiretrovirals pharmacokinetics and viral hepatitis in HIV infected subjects*, 7th edn. Fundació de Lluita contra la SIDA. Barcelona, Spain.

Colonno, R., Rose, R., McLaren, C., Thiry, A., Parkin, N. and Friborg, J. (2004) Identification of I50L as the signature atazanavir (ATV)-resistance mutation in treatment-naive HIV-1-infected patients receiving ATV-containing regimens. *J. Infect. Dis.* **189**, 1802–1810.

Collins, J.A., Thompson, M.G., Paintsil, E., Ricketts, M., Gedzior, J. and Alexander, L. (2004) Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. *J. Virol.* **78**, 603–611.

Condra, J.H., Schleif, W.A., Blahy, O.M., Gabryelski, L.J., Graham, D.J., Quintero, J.C. *et al.* (1995) In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* **374**, 569–571.

Crotty, S., Cameron, C.E. and Andino, R. (2001) RNA virus error catastrophe: direct molecular test by using ribavirin. *Proc. Natl Acad. Sci. USA* **98**, 6895–6900.

Crowder, S. and Kirkegaard, K. (2005) Trans-dominant inhibition of RNA viral replication can slow growth of drug-resistant viruses. *Nat. Genet.* **37**, 701–709.

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

Charpentier, N., Davila, M., Domingo, E. and Escarmis, C. (1996) Long-term, large-population passage of aphthovirus can generate and amplify defective noninterfering particles deleted in the leader protease gene. *Virology* **223**, 10–18.

Chen, I.S.Y., Koprowski, H., Srinivasan, A. and Vogt, P.K. (eds) (1995). *Transacting Functions of Human Retroviruses*. Berlin: Springer.

Chumakov, K.M., Powers, L.B., Noonan, K.E., Roninson, I.B. and Levenbook, I.S. (1991) Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. *Proc. Natl Acad. Sci. USA* **88**, 199–203.

Dahari, H., Ribeiro, R.M. and Perelson, A.S. (2007) Triphasic decline of hepatitis C virus RNA during antiviral therapy. *Hepatology* **46**, 16–21.

Davis, J.J. (1997) Origins, acquisition and dissemination of antibiotic resistance determinants. In: *Antibiotic Resitance: Origins, Evolution, Selection and Spread* (D.J. Chadweick and J. Goode, eds), pp. 15–35. New York: John Wiley.

de la Iglesia, F. and Elena, S.F. (2007) Fitness declines in tobacco etch virus upon serial bottleneck transfers. *J. Virol.* **81**, 4941–4947.

de la Torre, J.C. and Holland, J.J. (1990) RNA virus quasispecies populations can suppress vastly superior mutant progeny. *J. Virol.* **64**, 6278–6281.

Deval, J., White, K.L., Miller, M.D., Parkin, N.T., Courcambeck, J., Halfon, P. *et al.* (2004) Mechanistic basis for reduced viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations. *J. Biol. Chem.* **279**, 509–516.

Dixit, N.M., Layden-Almer, J.E., Layden, T.J. and Perelson, A.S. (2004) Modelling how ribavirin improves interferon response rates in hepatitis C virus infection. *Nature* **432**, 922–924.

Domingo, E. (2000) Viruses at the edge of adaptation. *Virology* **270**, 251–253.

Domingo, E., ed. (2005) Virus entry into error catastrophe as a new antiviral strategy. *Virus Res.* **107**, 115–228.

Domingo, E., ed. (2006) Quasispecies: concepts and implications for virology. *Curr. Top. Microbiol. Immunol.* **299**.

Domingo, E. (2007) Virus evolution. In: *Fields Virology* (D.M. Knipe, P.M. Howley *et al.*, eds 5th edn., pp. 389–421. Philadelphia: Lippincott Williams & Wilkins.

Domingo, E. and Gomez, J. (2007) Quasispecies and its impact on viral hepatitis. *Virus Res.* **127**, 131–150.

Domingo, E. and Holland, J.J. (1997) RNA virus mutations and fitness for survival. *Annu. Rev. Microbiol.* **51**, 151–178.

Domingo, E., Flavell, R.A. and Weissmann, C. (1976) In vitro site-directed mutagenesis: generation and properties of an infectious extracistronic mutant of bacteriophage Qb. *Gene* **1**, 3–25.

Domingo, E., Sabo, D., Taniguchi, T. and Weissmann, C. (1978) Nucleotide sequence heterogeneity of an RNA phage population. *Cell* **13**, 735–744.

Dorr, P., Westby, M., Dobbs, S., Griffin, P., Irvine, B., Macartney, M. *et al.* (2005) Maraviroc (UK-427,857), a potent, orally bioavailable and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob. Agents Chemother.* **49**, 4721–4732.

Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L. and Lamarre, D. (1996) Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *J. Virol.* **70**, 3763–3769.

Doyon, L., Payant, C., Brakier-Gingras, L. and Lamarre, D. (1998) Novel Gag-Pol frameshift site in human immunodeficiency virus type 1 variants resistant to protease inhibitors. *J. Virol.* **72**, 6146–6150.

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

Drake, J.W. and Holland, J.J. (1999) Mutation rates among RNA viruses. *Proc. Natl Acad. Sci. USA* **96**, 13910–13913.

Drake, J.W., Charlesworth, B., Charlesworth, D. and Crow, J.F. (1998) Rates of spontaneous mutation. *Genetics* **148**, 1667–1686.

Duarte, E., Clarke, D., Moya, A., Domingo, E. and Holland, J. (1992) Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. *Proc. Natl Acad. Sci. USA* **89**, 6015–6019.

Duarte, E.A., Novella, I.S., Ledesma, S., Clarke, D.K., Moya, A., Elena, S.F. *et al.* (1994a) Subclonal components of consensus fitness in an RNA virus clone. *J. Virol.* **68**, 4295–4301.

Duarte, E.A., Novella, I.S., Weaver, S.C., Domingo, E., Wain-Hobson, S., Clarke, D.K. *et al.* (1994b) RNA virus quasispecies: significance for viral disease and epidemiology. *Infect. Agents Dis.* **3**, 201–214.

Dykes, C., Fox, K., Lloyd, A., Chiulli, M., Morse, E. and Demeter, L.M. (2001) Impact of clinical reverse transcriptase sequences on the replication capacity of HIV-1 drug-resistant mutants. *Virology* **285**, 193–203.

Eckerle, L.D., Lu, X., Sperry, S.M., Choi, L. and Denison, M.R. (2007) High fidelity of murine hepatitis virus replication is decreased in msp14 exoribonuclease mutants. *J. Virol.* **81**, 12135–12144.

Eastman, P.S., Mittler, J., Kelso, R., Gee, C., Boyer, E., Kolberg, J. *et al.* (1998) Genotypic changes in human

immunodeficiency virus type 1 associated with loss of suppression of plasma viral RNA levels in subjects treated with ritonavir (Norvir) monotherapy. *J. Virol.* **72**, 5154–5164.

Eigen, M. (1971) Self-organization of matter and the evolution of biological macromolecules. *Naturwissenschaften* **58**, 465–523.

Eigen, M. (1992) *Steps towards Life*. Oxford: Oxford University Press.

Eigen, M. (2000) Natural selection: a phase transition?. *Biophys. Chem.* **85**, 101–123.

Eigen, M. (2002) Error catastrophe and antiviral strategy. *Proc. Natl Acad. Sci. USA* **99**, 13374–13376.

Eigen, M. and Biebricher, C.K. (1988) Sequence space and quasispecies distribution. In: *RNA Genetics* (E. Domingo, P. Ahlquist and J.J. Holland, eds), Vol. 3, pp. 211–245. Boca Raton, FL: CRC Press.

Eigen, M. and Schuster, P. (1979) *The Hypercycle. A Principle of Natural Self-organization*. Berlin: Springer.

Escarmís, C., Dávila, M., Charpentier, N., Bracho, A., Moya, A. and Domingo, E. (1996) Genetic lesions associated with Muller's ratchet in an RNA virus. *J. Mol. Biol.* **264**, 255–267.

Escarmís, C., Dávila, M. and Domingo, E. (1999) Multiple molecular pathways for fitness recovery of an RNA virus debilitated by operation of Muller's ratchet. *J. Mol. Biol.* **285**, 495–505.

Escarmís, C., Gómez-Mariano, G., Dávila, M., Lázaro, E. and Domingo, E. (2002) Resistance to extinction of low fitness virus subjected to plaque-to-plaque transfers: diversification by mutation clustering. *J. Mol. Biol.* **315**, 647–661.

Escarmís, C., Lázaro, E. and Manrubia, S.C. (2006) Population bottlenecks in quasispecies dynamics. *Curr. Top. Microbiol. Immunol.* **299**, 141–170.

Escarmís, C., Lázaro, E., Arias, A. and Domingo, E. (2008) Repeated bottleneck transfers can lead to non-cytocidal forms of a cytopathic virus. Implications for viral extinction. *J. Mol. Biol.* **376**, 367–379.

Farci, P., Shimoda, A., Coiana, A., Diaz, G., Peddis, G., Melpolder, J.C. *et al.* (2000) The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* **288**, 339–344.

Fernandez, G., Clotet, B. and Martinez, M.A. (2007) Fitness landscape of human immunodeficiency virus type 1 protease quasispecies. *J. Virol.* **81**, 2485–2496.

Ferrer-Orta, C., Arias, A., Perez-Luque, R., Escarmis, C., Domingo, E. and Verdaguer, N. (2004) Structure of foot-and-mouth disease virus RNA-dependent RNA polymerase and its complex with a template-primer RNA. *J. Biol. Chem.* **279**, 47212–47221.

Ferrer-Orta, C., Arias, A., Escarmis, C. and Verdaguer, N. (2006) A comparison of viral RNA-dependent RNA polymerases. *Curr. Opin. Struct. Biol.* **16**, 27–34.

Ferrer-Orta, C., Arias, A., Perez-Luque, R., Escarmis, C., Domingo, E. and Verdaguer, N. (2007) Sequential structures provide insights into the fidelity of RNA replication. *Proc. Natl Acad. Sci. USA* **104**, 9463–9468.

Finkelstein, D.B., Mukatira, S., Mehta, P.K., Obenauer, J.C., Su, X., Webster, R.G. and Naeve, C.W. (2007) Persistent host markers in pandemic and H5N1 influenza viruses. *J. Virol.* **81**, 10292–10299.

Frankel, F.A., Invernizzi, C.F., Oliveira, M. and Wainberg, M.A. (2007) Diminished efficiency of HIV-1 reverse transcriptase containing the K65R and M184V drug resistance mutations. *Aids* **21**, 665–675.

Friedberg, E.C., Walker, G.C., Siede, W., Wood, R.D., Schultz, R.A. and Ellenberger, T. (2006) *DNA Repair and Mutagenesis*. Washington, DC: American Society for Microbiology.

Frost, S.D., Dumaurier, M.J., Wain-Hobson, S. and Brown, A.J. (2001) Genetic drift and within-host metapopulation dynamics of HIV-1 infection. *Proc. Natl Acad. Sci. USA* **98**, 6975–6980.

Galagan, J.E. and Selker, E.U. (2004) RIP: the evolutionary cost of genome defense. *Trends Genet.* **20**, 417–423.

Garcia-Lerma, J.G., Nidtha, S., Blumoff, K., Weinstock, H. and Heneine, W. (2001) Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naive persons. *Proc. Natl Acad. Sci. USA* **98**, 13907–13912.

García-Arriaza, J., Manrubia, S.C., Toja, M., Domingo, E. and Escarmís, C. (2004) Evolutionary transition toward defective RNAs that are infectious by complementation. *J. Virol.* **78**, 11678–11685.

García-Arriaza, J., Domingo, E. and Escarmís, C. (2005) A segmented form of foot-and-mouth disease virus interferes with standard virus: a link between interference and competitive fitness. *Virology* **335**, 155–164.

García-Arriaza, J., Ojosnegros, S., Dávila, M., Domingo, E. and Escarmis, C. (2006) Dynamics of mutation and recombination in a replicating population of complementing, defective viral genomes. *J. Mol. Biol.* **360**, 558–572.

Garcia-Arriaza, J., Domingo, E. and Briones, C. (2007) Characterization of minority subpopulations in the mutant spectrum of HIV-1 quasispecies by successive specific amplifications. *Virus Res.* **129**, 123–134.

Garcia-Lerma, J.G., MacInnes, H., Bennett, D., Weinstock, H. and Heneine, W. (2004) Transmitted human immunodeficiency virus type 1 carrying the D67N or K219Q/E mutation evolves rapidly to zidovudine resistance in vitro and shows a high replicative fitness in the presence of zidovudine. *J. Virol.* **78**, 7545–7552.

Gatanaga, H., Suzuki, Y., Tsang, H., Yoshimura, K., Kavlick, M.F., Nagashima, K. *et al.* (2002) Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multitude of HIV-1 resistance against protease inhibitors. *J. Biol. Chem.* **277**, 5952–5961.

Gause, G.F. (1971) *The Struggle for Existence*. New York: Dover.

Ge, L., Zhang, J., Zhou, X. and Li, H. (2007) Genetic structure and population variability of tomato yellow leaf curl china virus. *J. Virol.* **81**, 5902–5907.

Gerondelis, P., Archer, R.H., Palaniappan, C., Reichman, R.C., Fay, P.J., Bambara, R.A. and Demeter, L.M. (1999) The P236L delavirdine-resistant human immunodeficiency virus type 1 mutant is replication defective and demonstrates alterations in both RNA 5′-end- and DNA 3′-end-directed RNase H activities. *J. Virol.* **73**, 5803–5813.

González-López, C., Arias, A., Pariente, N., Gómez-Mariano, G. and Domingo, E. (2004) Preextinction viral RNA can interfere with infectivity. *J. Virol.* **78**, 3319–3324.

González-López, C., Gómez-Mariano, G., Escarmís, C. and Domingo, E. (2005) Invariant aphthovirus consensus nucleotide sequence in the transition to error catastrophe. *Infect. Genet. Evol.* **5**, 366–374.

Goodman, M.F. and Fygenson, K.D. (1998) DNA polymerase fidelity: from genetics toward a biochemical understanding. *Genetics* **148**, 1475–1482.

Gorbalenya, A.E. (1995) Origin of RNA viral genomes; approaching the problem by comparative sequence analysis. In: *Molecular Basis of Virus Evolution* (A. Gibbs, C.H. Calisher and F. Garcia-Arenal, eds), pp. 49–66. Cambridge: Cambridge University Press.

Goudsmit, J., de Ronde, A., de Rooij, E. and de Boer, R. (1997) Broad spectrum of in vivo fitness of human immunodeficiency virus type 1 subpopulations differing at reverse transcriptase codons 41 and 215. *J. Virol.* **71**, 4479–4484.

Grande-Pérez, A., Sierra, S., Castro, M.G., Domingo, E. and Lowenstein, P.R. (2002) Molecular indetermination in the transition to error catastrophe: systematic elimination of lymphocytic choriomeningitis virus through mutagenesis does not correlate linearly with large increases in mutant spectrum complexity. *Proc. Natl Acad. Sci. USA* **99**, 12938–12943.

Grande-Pérez, A., Gómez-Mariano, G., Lowenstein, P.R. and Domingo, E. (2005a) Mutagenesis-induced, large fitness variations with an invariant arenavirus consensus genomic nucleotide sequence. *J. Virol.* **79**, 10451–10459.

Grande-Pérez, A., Lazaro, E., Lowenstein, P., Domingo, E. and Manrubia, S.C. (2005b) Suppression of viral infectivity through lethal defection. *Proc. Natl Acad. Sci. USA* **102**, 4448–4452.

Greene, I.P., Wang, E., Deardorff, E.R., Milleron, R., Domingo, E. and Weaver, S.C. (2005) Effect of alternating passage on adaptation of sindbis virus to vertebrate and invertebrate cells. *J. Virol.* **79**, 14253–14260.

Grimm, D., Staeheli, P., Hufbauer, M., Koerner, I., Martinez-Sobrido, L., Solorzano, A. *et al.* (2007) Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene. *Proc. Natl Acad. Sci. USA* **104**, 6806–6811.

Harrigan, P.R., Bloor, S. and Larder, B.A. (1998) Relative replicative fitness of zidovudine-resistant human immunodeficiency virus type 1 isolates in vitro. *J. Virol.* **72**, 3773–3778.

Harris, K.S., Brabant, W., Styrchak, S., Gall, A. and Daifuku, R. (2005) KP-1212/1461, a nucleoside designed for the treatment of HIV by viral mutagenesis. *Antiviral Res.* **67**, 1–9.

Herrera, M., Garcia-Arriaza, J., Pariente, N., Escarmis, C. and Domingo, E. (2007) Molecular basis for a lack of correlation between viral fitness and cell killing capacity. *PLoS Pathog.* **3**, e53.

Hickey, D.A. and Rose, M.R. (1988) The role of gene transfer in the evolution of eukaryotic sex. In: *The Evolution of Sex* (R.E. Michod and B.R. Levin, eds), pp. 161–175. Sunderland, MA: Sinauer.

Holland, J. and Domingo, E. (1998) Origin and evolution of viruses. *Virus Genes* **16**, 13–21.

Holland, J.J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. and VandePol, S. (1982) Rapid evolution of RNA genomes. *Science* **215**, 1577–1585.

Holland, J.J., Domingo, E., de la Torre, J.C. and Steinhauer, D.A. (1990) Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis. *J. Virol.* **64**, 3960–3962.

Holland, J.J., de la Torre, J.C., Clarke, D.K. and Duarte, E. (1991) Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. *J. Virol.* **65**, 2960–2967.

Hu, Z., Giguel, F., Hatano, H., Reid, P., Lu, J. and Kuritzkes, D.R. (2006) Fitness comparison of thymidine analog resistance pathways in human immunodeficiency virus type 1. *J. Virol.* **80**, 7020–7027.

Huang, W., Gamarnik, A., Limoli, K., Petropoulos, C.J. and Whitcomb, J.M. (2003) Amino acid substitutions at position 190 of human immunodeficiency virus type 1 reverse transcriptase increase susceptibility to delavirdine and impair virus replication. *J. Virol.* **77**, 1512–1523.

Huang, Y., Rosenkranz, S.L. and Wu, H. (2003) Modeling HIV dynamics and antiviral response with consideration of time-varying drug exposures, adherence and phenotypic sensitivity. *Math. Biosci.* **184**, 165–186.

Huynen, M.A., Stadler, P.F. and Fontana, W. (1996) Smoothness within ruggedness: the role of neutrality in adaptation. *Proc. Natl Acad. Sci. USA* **93**, 397–401.

Iglesias-Ussel, M.D., Casado, C., Yuste, E., Olivares, I. and Lopez-Galindez, C. (2002) In vitro analysis of human immunodeficiency virus type 1 resistance to nevirapine and fitness determination of resistant variants. *J. Gen. Virol.* **83**, 93–101.

Ishihama, A., Mizumoto, K., Kawakami, K., Kato, A. and Honda, A. (1986) Proofreading function associated with the RNA-dependent RNA polymerase from influenza virus. *J. Biol. Chem.* **261**, 10417–10421.

Iversen, A.K., Shafer, R.W., Wehrly, K., Winters, M.A., Mullins, J.I., Chesebro, B. and Merigan, T.C. (1996) Multidrug-resistant human immunodeficiency virus type 1 strains resulting from combination antiretroviral therapy. *J. Virol.* **70**, 1086–1090.

Jeeninga, R.E., Keulen, W., Boucher, C., Sanders, R.W. and Berkhout, B. (2001) Evolution of AZT resistance

in HIV-1: the 41–70 intermediate that is not observed in vivo has a replication defect. *Virology* **283**, 294–305.

Johnson, V.A., Brun-Vézinet, F., Clotet, B., Günthard, H.F., Kuritzkes, D.R., Pillay, D., Schapiro, J.M. and Richman, D.D. (2007) Update of the drug resistance mutations in HIV-1: 2007. *Top. HIV Med.* **15**, 119–125.

Jridi, C., Martin, J.F., Marie-Jeanne, V., Labonne, G. and Blanc, S. (2006) Distinct viral populations differentiate and evolve independently in a single perennial host plant. *J. Virol.* **80**, 2349–2357.

Kantor, R., Fessel, W.J., Zolopa, A.R., Israelski, D., Shulman, N., Montoya, J.G. *et al.* (2002) Evolution of primary protease inhibitor resistance mutations during protease inhibitor salvage therapy. *Antimicrob. Agents Chemother.* **46**, 1086–1092.

Kaur, A., Grant, R.M., Means, R.E., McClure, H., Feinberg, M. and Johnson, R.P. (1998) Diverse host responses and outcomes following simian immunodeficiency virus SIVmac239 infection in sooty mangabeys and rhesus macaques. *J. Virol.* **72**, 9597–9611.

Keulen, W., Back, N.K., van Wijk, A., Boucher, C.A. and Berkhout, B. (1997) Initial appearance of the 184Ile variant in lamivudine-treated patients is caused by the mutational bias of human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* **71**, 3346–3350.

Kimata, J.T., Kuller, L., Anderson, D.B., Dailey, P. and Overbaugh, J. (1999) Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. *Nat. Med.* **5**, 535–541.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A. and Steitz, T.A. (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**, 1783–1790.

Kolakofsky, D., Roux, L., Garcin, D. and Ruigrok, R.W. (2005) Paramyxovirus mRNA editing, the "rule of six" and error catastrophe: a hypothesis. *J. Gen. Virol.* **86**, 1869–1877.

Koonin, E.V., Senkevich, T.G. and Dolja, V.V. (2006) The ancient Virus World and evolution of cells. *Biol. Direct* **1**, 29.

Kosalaraksa, P., Kavlick, M.F., Maroun, V., Le, R. and Mitsuya, H. (1999) Comparative fitness of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 (HIV-1) in an In vitro competitive HIV-1 replication assay. *J. Virol.* **73**, 5356–5363.

Kunkel, T.A. (1990) Misalignment-mediated DNA synthesis errors. *Biochemistry* **29**, 8003–8011.

Larder, B.A. (1994) Interactions between drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase. *J. Gen. Virol.* **75**(Pt 5), 951–957.

Larder, B.A., Darby, G. and Richman, D.D. (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* **243**, 1731–1734.

Lazaro, E., Escarmis, C., Perez-Mercader, J., Manrubia, S.C. and Domingo, E. (2003) Resistance of virus to extinction on bottleneck passages: Study of a decaying and fluctuating pattern of fitness loss. *Proc. Natl Acad. Sci. USA* **100**, 10830–10835.

Lazarowitz, S.D. (2007) Plant viruses. In: *Fields Virology* (D.M. Dnipe and P.M. Howley, eds), pp. 641–705. Philadelphia: Lippincot Williams and Wilkins.

Lee, C.H., Gilbertson, D.L., Novella, I.S., Huerta, R., Domingo, E. and Holland, J.J. (1997) Negative effects of chemical mutagenesis on the adaptive behavior of vesicular stomatitis virus. *J. Virol.* **71**, 3636–3640.

Leroux, C., Issel, C.J. and Montelaro, R.C. (1997) Novel and dynamic evolution of equine infectious anemia virus genomic quasispecies associated with sequential disease cycles in an experimentally infected pony. *J. Virol.* **71**, 9627–9639.

Loeb, L.A., Essigmann, J.M., Kazazi, F., Zhang, J., Rose, K. D. and Mullins, J.I. (1999) Lethal mutagenesis of HIV with mutagenic nucleoside analogs. *Proc. Natl Acad. Sci. USA* **96**, 1492–1497.

Lu, J., Sista, P., Giguel, F., Greenberg, M. and Kuritzkes, D.R. (2004) Relative replicative fitness of human immunodeficiency virus type 1 mutants resistant to enfuvirtide (T-20). *J. Virol.* **78**, 4628–4637.

Lukashov, V.V., Huismans, R., Jebbink, M.F., Danner, S.A., de Boer, R.J. and Goudsmit, J. (2001) Selection by AZT and rapid replacement in the absence of drugs of HIV type 1 resistant to multiple nucleoside analogs. *AIDS Res. Hum. Retroviruses* **17**, 807–818.

Maeda, Y., Venzon, D.J. and Mitsuya, H. (1998) Altered drug sensitivity, fitness and evolution of human immunodeficiency virus type 1 with pol gene mutations conferring multi-dideoxynucleoside resistance. *J. Infect. Dis.* **177**, 1207–1213.

Manrubia, S.C., Escarmis, C., Domingo, E. and Lazaro, E. (2005) High mutation rates, bottlenecks and robustness of RNA viral quasispecies. *Gene* **347**, 273–282.

Manrubia, S.C., Garcia-Arriaza, J., Domingo, E. and Escarmís, C. (2006) Long-range transport and universality classes in *in vitro* viral infection spread. *Europhys. Lett.* **74**, 547–553.

Marcus, P.I., Rodriguez, L.L. and Sekellick, M.J. (1998) Interferon induction as a quasispecies marker of vesicular stomatitis virus populations. *J. Virol.* **72**, 542–549.

Martinez-Picado, J., Savara, A.V., Sutton, L. and D'Aquila, R.T. (1999) Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J. Virol.* **73**, 3744–3752.

Martinez, M.A., Carrillo, C., Gonzalez-Candelas, F., Moya, A., Domingo, E. and Sobrino, F. (1991) Fitness alteration of foot-and-mouth disease virus mutants: measurement of adaptability of viral quasispecies. *J. Virol.* **65**, 3954–3957.

Mas, A., Parera, M., Briones, C., Soriano, V., Martínez, M.A., Domingo, E. and Menéndez-Arias, L. (2000) Role of a dipeptide insertion between codons 69–70 of HIV-1 reverse transcriptase in the mechanism of AZT resistance. *EMBO J.* **19**, 5752–5761.

Matamoros, T., Franco, S., Vazquez-Alvarez, B.M., Mas, A., Martinez, M.A. and Menendez-Arias, L. (2004) Molecular determinants of multi-nucleoside analogue resistance in HIV-1 reverse transcriptases containing a dipeptide insertion in the fingers

subdomain: effect of mutations D67N and T215Y on removal of thymidine nucleotide analogues from blocked DNA primers. *J. Biol. Chem.* **279**, 24569–24577.

Maynard-Smith, J. (1976) *The Evolution of Sex*. Cambridge: Cambridge University Press.

Maynard Smith, J. and Szathmary, E. (1995) *The Major Transitions in Evolution*. Oxford: W.H. Freeman.

Menendez-Arias, L. (2002) Targeting HIV: antiretroviral therapy and development of drug resistance. *Trends Pharmacol. Sci.* **23**, 381–388.

Menendez-Arias, L., Matamoros, T. and Cases-Gonzalez, C.E. (2006) Insertions and deletions in HIV-1 reverse transcriptase: consequences for drug resistance and viral fitness. *Curr. Pharm. Des.* **12**, 1811–1825.

Mesters, J.R., Tan, J. and Hilgenfeld, R. (2006) Viral enzymes. *Curr. Opin. Struct. Biol.* **16**, 776–786.

Miller, M.D., Lamy, P.D., Fuller, M.D., Mulato, A.S., Margot, N.A., Cihlar, T. and Cherrington, J.M. (1998) Human immunodeficiency virus type 1 reverse transcriptase expressing the K70E mutation exhibits a decrease in specific activity and processivity. *Mol. Pharmacol.* **54**, 291–297.

Minskaia, E., Hertzig, T., Gorbalenya, A.E., Campanacci, V., Cambillau, C., Canard, B. and Ziebuhr, J. (2006) Discovery of an RNA virus 3′– > 5′ exoribonuclease that is critically involved in coronavirus RNA synthesis. *Proc. Natl Acad. Sci. USA* **103**, 5108–5113.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St Clair, M.H., Lehrman, S.N., Gallo, R.C. *et al.* (1985) 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proc. Natl Acad. Sci. USA* **82**, 7096–7100.

Moreno, I.M., Malpica, J.M., Rodriguez-Cerezo, E. and Garcia-Arenal, F. (1997) A mutation in tomato aspermy cucumovirus that abolishes cell-to-cell movement is maintained to high levels in the viral RNA population by complementation. *J. Virol.* **71**, 9157–9162.

Mudd, J.A., Leavitt, R.W., Kingsbury, D.T. and Holland, J.J. (1973) Natural selection of mutants of vesicular stomatitis virus by cultured cells of *Drosophila melanogaster*. *J. Gen. Virol.* **20**, 341–351.

Muller, M.J. (1964) The relation of recombination to mutational advance. *Mut. Res.* **1**, 2–9.

Muller, V., Ledergerber, B., Perrin, L., Klimkait, T., Furrer, H., Telenti, A. *et al.* (2006) Stable virulence levels in the HIV epidemic of Switzerland over two decades. *Aids* **20**, 889–894.

Myint, L., Matsuda, M., Matsuda, Z., Yokomaku, Y., Chiba, T., Okano, A. *et al.* (2004) Gag non-cleavage site mutations contribute to full recovery of viral fitness in protease inhibitor-resistant human immunodeficiency virus type 1. *Antimicrob. Agents Chemother,* **48**, 444–452.

Nagy, P.D., Carpenter, C.D. and Simon, A.E. (1997) A novel 3′-end repair mechanism in an RNA virus. *Proc. Natl Acad. Sci. USA* **94**, 1113–1118.

Nájera, I., Holguín, A., Quiñones-Mateu, M.E., Muñoz-Fernández, M.A., Nájera, R., López-Galíndez, C. and Domingo, E. (1995) Pol gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. *J. Virol.* **69**, 23–31.

Novella, I.S. and Ebendick-Corpus, B.E. (2004) Molecular basis of fitness loss and fitness recovery in vesicular stomatitis virus. *J. Mol. Biol.* **342**, 1423–1430.

Novella, I.S., Clarke, D.K., Quer, J., Duarte, E.A., Lee, C.H., Weaver, S.C. *et al.* (1995a) Extreme fitness differences in mammalian and insect hosts after continuous replication of vesicular stomatitis virus in sandfly cells. *J. Virol.* **69**, 6805–6809.

Novella, I.S., Duarte, E.A., Elena, S.F., Moya, A., Domingo, E. and Holland, J.J. (1995b) Exponential increases of RNA virus fitness during large population transmissions. *Proc. Natl Acad. Sci. USA* **92**, 5841–5844.

Novella, I.S., Elena, S.F., Moya, A., Domingo, E. and Holland, J.J. (1995c) Size of genetic bottlenecks leading to virus fitness loss is determined by mean initial population fitness. *J. Virol.* **69**, 2869–2872.

Novella, I.S., Cilnis, M., Elena, S.F., Kohn, J., Moya, A., Domingo, E. and Holland, J.J. (1996) Large-population passages of vesicular stomatitis virus in interferon-treated cells select variants of only limited resistance. *J. Virol.* **70**, 6414–6417.

Novella, I.S., Hershey, C.L., Escarmis, C., Domingo, E. and Holland, J.J. (1999a) Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells. *J. Mol. Biol.* **287**, 459–465.

Novella, I.S., Quer, J., Domingo, E. and Holland, J.J. (1999b) Exponential fitness gains of RNA virus populations are limited by bottleneck effects. *J. Virol.* **73**, 1668–1671.

Novella, I.S., Ebendick-Corpus, B.E., Zarate, S. and Miller, E.L. (2007) Emergence of mammalian cell-adapted vesicular stomatitis virus from persistent infections of insect vector cells. *J. Virol.* **81**, 6664–6668.

Nowak, M.A. (2006) *Evolutionary Dynamics*. Cambridge, MA and London: The Belknap Press of Harvard University Press.

Nowak, M.A. and May, R.M. (2000) *Virus dynamics. Mathematical Principles of Immunology and Virology*. New York: Oxford University Press.

Nowak, M. and Schuster, P. (1989) Error thresholds of replication in finite populations mutation frequencies and the onset of Muller's ratchet. *J. Theor. Biol.* **137**, 375–395.

Nuñez, J.I., Molina, N., Baranowski, E., Domingo, E., Clark, S., Burman, A. *et al.* (2007) Guinea pig-adapted foot-and-mouth disease virus with altered receptor recognition can productively infect a natural host. *J. Virol.* **81**, 8497–8506.

Orgel, L.E. (1963) The maintenance of the accuracy of protein synthesis and its relevance to ageing. *Proc. Natl Acad. Sci. USA* **49**, 517–521.

Pariente, N., Sierra, S., Lowenstein, P.R. and Domingo, E. (2001) Efficient virus extinction by combinations of a mutagen and antiviral inhibitors. *J. Virol.* **75**, 9723–9730.

Pariente, N., Airaksinen, A. and Domingo, E. (2003) Mutagenesis versus inhibition in the efficiency of extinction of foot-and-mouth disease virus. *J. Virol.* **77**, 7131–7138.

Parrish, C.R. and Kawaoka, Y. (2005) The origins of new pandemic viruses: the acquisition of new host ranges by canine parvovirus and influenza A viruses. *Annu. Rev. Microbiol.* **59**, 553–586.

Pathak, V.K. and Temin, H.M. (1992) 5-Azacytidine and RNA secondary structure increase the retrovirus mutation rate. *J. Virol.* **66**, 3093–3100.

Pawlotsky, J.M., Germanidis, G., Neumann, A.U., Pellerin, M., Frainais, P.O. and Dhumeaux, D. (1998) Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations. *J. Virol.* **72**, 2795–2805.

Peleg, J. (1971) Growth of viruses in arthropod cell cultures: applications. I. Attenuation of Semliki Forest (SF) virus in continuously cultured *Aedes aegypti* mosquito cells (Peleg) as a step in production of vaccines. *Curr. Top. Microbiol. Immunol.* **55**, 155–161.

Pelemans, H., Aertsen, A., Van Laethem, K., Vandamme, A.M., De Clercq, E., Perez-Perez, M.J. *et al.* (2001) Site-directed mutagenesis of human immunodeficiency virus type 1 reverse transcriptase at amino acid position 138. *Virology* **280**, 97–106.

Perales, C., Mateo, R., Mateu, M.G. and Domingo, E. (2007) Insights into RNA virus mutant spectrum and lethal mutagenesis events: replicative interference and complementation by multiple point mutants. *J. Mol. Biol.* **369**, 985–1000.

Perelson, A.S. and Layden, T.J. (2007) Ribavirin: is it a mutagen for hepatitis C virus?. *Gastroenterology* **132**, 2050–2052.

Peters, C.J. (2007) In: *Fields Virology* (D.M. Knipe, P.M. Howley *et al.*, eds), 5th edn. pp. 605–625. Philadelphia: Lippincott Williams and Wilkins.

Pettit, S.C., Henderson, G.J., Schiffer, C.A. and Swanstrom, R. (2002) Replacement of the P1 amino acid of human immunodeficiency virus type 1 Gag processing sites can inhibit or enhance the rate of cleavage by the viral protease. *J. Virol.* **76**, 10226–10233.

Pfeiffer, J.K. and Kirkegaard, K. (2003) A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. *Proc. Natl Acad. Sci. USA* **100**, 7289–7294.

Pfeiffer, J.K. and Kirkegaard, K. (2005) Increased fidelity reduces poliovirus fitness under selective pressure in mice. *PLoS Pathog.* **1**, 102–110.

Pfeiffer, J.K. and Kirkegaard, K. (2006) Bottleneck-mediated quasispecies restriction during spread of an RNA virus from inoculation site to brain. *Proc. Natl Acad. Sci. USA* **103**, 5520–5525.

Prado, J.G., Wrin, T., Beauchaine, J., Ruiz, L., Petropoulos, C.J., Frost, S.D. *et al.* (2002) Amprenavir-resistant HIV-1 exhibits lopinavir cross-resistance and reduced replication capacity. *Aids* **16**, 1009–1017.

Quiñones-Mateu, M.E., Mas, A., Lain de Lera, T., Soriano, V., Alcami, J., Lederman, M.M. and Domingo, E. (1998) LTR and tat variability of HIV-1 isolates from patients with divergent rates of disease progression. *Virus Res.* **57**, 11–20.

Quiñones-Mateu, M.E., Tadele, M., Parera, M., Mas, A., Weber, J., Rangel, H.R. *et al.* (2002) Insertions in the reverse transcriptase increase both drug resistance and viral fitness in a human immunodeficiency virus type 1 isolate harboring the multi-nucleoside reverse transcriptase inhibitor resistance 69 insertion complex mutation. *J. Virol.* **76**, 10546–10552.

Quiñones-Mateu, M.E. and Arts, E. (2006) Virus fitness: concept, qunatification and application to HIV population dynamics. *Curr. Top. Microbiol. Immunol.* **299**, 83–140.

Reeves, J.D., Gallo, S.A., Ahmad, N., Miamidian, J.L., Harvey, P.E., Sharron, M. *et al.* (2002) Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density and fusion kinetics. *Proc. Natl Acad. Sci. USA* **99**, 16249–16254.

Reeves, J.D., Lee, F.H., Miamidian, J.L., Jabara, C.B., Juntilla, M.M. and Doms, R.W. (2005) Enfuvirtide resistance mutations: impact on human immunodeficiency virus envelope function, entry inhibitor sensitivity and virus neutralization. *J. Virol.* **79**, 4991–4999.

Reznick, D. and Travis, J. (1996) The empirical study of adaptation in natural populations. In: *Adaptation* (M.R. Rose and G.V. Lander, eds), pp. 243–289. San Diego: Academic Press.

Rimsky, L.T., Shugars, D.C. and Matthews, T.J. (1998) Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. *J. Virol.* **72**, 986–993.

Robertson, B.H., Jansen, R.W., Khanna, B., Totsuka, A., Nainan, O.V., Siegl, G. *et al.* (1992) Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J. Gen. Virol.* **73**, 1365–1377.

Roux, L., Simon, A.E. and Holland, J.J. (1991) Effects of defective interfering viruses on virus replication and pathogenesis in vitro and in vivo. *Adv. Virus Res.* **40**, 181–211.

Ruiz-Jarabo, C.M., Arias, A., Baranowski, E., Escarmís, C. and Domingo, E. (2000) Memory in viral quasispecies. *J. Virol.* **74**, 3543–3547.

Ruiz-Jarabo, C.M., Arias, A., Molina-París, C., Briones, C., Baranowski, E., Escarmís, C. and Domingo, E. (2002) Duration and fitness dependence of quasispecies memory. *J. Mol. Biol.* **315**, 285–296.

Ruiz-Jarabo, C.M., Ly, C., Domingo, E. and de la Torre, J. C. (2003a) Lethal mutagenesis of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV). *Virology* **308**, 37–47.

Ruiz-Jarabo, C.M., Miller, E., Gómez-Mariano, G. and Domingo, E. (2003b) Synchronous loss of quasispecies memory in parallel viral lineages: a deterministic feature of viral quasispecies. *J. Mol. Biol.* **333**, 553–563.

Saakian, D.B. and Hu, C.K. (2006) Exact solution of the Eigen model with general fitness functions and degradation rates. *Proc. Natl Acad. Sci. USA,* **103**, 4935–4939.

Sanjuan, R., Cuevas, J.M., Furio, V., Holmes, E.C. and Moya, A. (2007) Selection for robustness in mutagenized RNA viruses. *PLoS Genet.* **3**, e93.

Scott, T.W., Weaver, S.C. and Mallampali, V.L. (1994) Evolution of mosquito-borne viruses. In: *Evolutionary Biology of Viruses* (S.S. Morse, ed.), pp. 293–324. New York: Raven Press.

Schmit, J.C., Cogniaux, J., Hermans, P., Van Vaeck, C., Sprecher, S., Van Remoortel, B. *et al.* (1996) Multiple drug resistance to nucleoside analogues and nonnucleoside reverse transcriptase inhibitors in an efficiently replicating human immunodeficiency virus type 1 patient strain. *J. Infect. Dis.* **174**, 962–968.

Schock, H.B., Garsky, V.M. and Kuo, L.C. (1996) Mutational anatomy of an HIV-1 protease variant conferring cross-resistance to protease inhibitors in clinical trials. Compensatory modulations of binding and activity. *J. Biol. Chem.* **271**, 31957–31963.

Sevilla, N., Ruiz-Jarabo, C.M., Gómez-Mariano, G., Baranowski, E. and Domingo, E. (1998) An RNA virus can adapt to the multiplicity of infection. *J. Gen. Virol,* **79**, 2971–2980.

Shafer, R.W., Winters, M.A., Palmer, S. and Merigan, T.C. (1998) Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients. *Ann. Intern. Med.* **128**, 906–911.

Sharma, P.L. and Crumpacker, C.S. (1997) Attenuated replication of human immunodeficiency virus type 1 with a didanosine-selected reverse transcriptase mutation. *J. Virol.* **71**, 8846–88451.

Sharma, P.L. and Crumpacker, C.S. (1999) Decreased processivity of human immunodeficiency virus type 1 reverse transcriptase (RT) containing didanosine-selected mutation Leu74Val: a comparative analysis of RT variants Leu74Val and lamivudine-selected Met184Val. *J. Virol.* **73**, 8448–8456.

Shirasaka, T., Kavlick, M.F., Ueno, T., Gao, W.Y., Kojima, E., Alcaide, M.L. *et al.* (1995) Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. *Proc. Natl Acad. Sci. USA,* **92**, 2398–2402.

Sierra, M., Airaksinen, A., González-López, C., Agudo, R., Arias, A. and Domingo, E. (2007) Foot-and-mouth disease virus mutant with decreased sensitivity to ribavirin: implications for error catastrophe. *J. Virol.* **81**, 2012–2024.

Sierra, S., Dávila, M., Lowenstein, P.R. and Domingo, E. (2000) Response of foot-and-mouth disease virus to increased mutagenesis. *Influence of viral load and fitness in loss of infectivity. J. Virol.* **74**, 8316–8323.

Smolinski, M.S., Hamburg, M.A. and Lederberg, J. (2003) *Microbial Threats to Health. Emergence, Detection and Response*. Washington DC: The National Academies Press.

Sobrino, F. and Mettenleiter, T. (2008) *Animal Viruses: Molecular Biology*. UK: Horizon Scientific Press.

Steinhauer, D.A., Domingo, E. and Holland, J.J. (1992) Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. *Gene* **122**, 281–288.

Suzuki, Y. (2005) Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. *Biol. Pharm. Bull.* **28**, 399–408.

Swetina, J. and Schuster, P. (1982) Self-replication with errors. A model for polynucleotide replication. *Biophys. Chem.* **16**, 329–345.

Tapia, N., Fernandez, G., Parera, M., Gomez-Mariano, G., Clotet, B., Quiñones-Mateu, M. *et al.* (2005) Combination of a mutagenic agent with a reverse transcriptase inhibitor results in systematic inhibition of HIV-1 infection. *Virology* **338**, 1–8.

Temin, H.M. (1989) Is HIV unique or merely different?. *J. AIDS* **2**, 1–9.

Temin, H.M. (1993) The high rate of retrovirus variation results in rapid evolution. In: *Emerging Viruses* (S.S. Morse, ed.), pp. 219–225. Oxford: Oxford University Press.

Teng, M.N., Oldstone, M.B. and de la Torre, J.C. (1996) Suppression of lymphocytic choriomeningitis virus-induced growth hormone deficiency syndrome by disease-negative virus variants. *Virology* **223**, 113–119.

Van Valen, L. (1973) A new evolutionary law. *Evol. Theory* **1**, 1–30.

Vignuzzi, M., Stone, J.K., Arnold, J.J., Cameron, C.E. and Andino, R. (2006) Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. *Nature* **439**, 344–348.

Villarreal, L.P. (2005) *Viruses and the Evolution of Life*. Washington DC: ASM Press.

Wang, J., Dykes, C., Domaoal, R.A., Koval, C.E., Bambara, R.A. and Demeter, L.M. (2006) The HIV-1 reverse transcriptase mutants G190S and G190A, which confer resistance to non-nucleoside reverse transcriptase inhibitors, demonstrate reductions in RNase H activity and DNA synthesis from tRNA(Lys, 3) that correlate with reductions in replication efficiency. *Virology* **348**, 462–474.

Weaver, S.C. (1998) Recurrent emergence of Venezuelan equine encephalomyelitis. In: *Emerging Infections* (W.M. Sheld and J. Hughes, eds), Vol. 1, pp. 27–42. Washington DC: ASM Press.

Weibull, W.J. (1951) A statistical distribution function of wide applicability. *Appl. Mech.* **18**, 293–297.

Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M. *et al.* (2007) Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J. Virol.* **81**, 2359–2371.

White, K.L., Margot, N.A., Wrin, T., Petropoulos, C.J., Miller, M.D. and Naeger, L.K. (2002) Molecular

mechanisms of resistance to human immunodeficiency virus type 1 with reverse transcriptase mutations K65R and K65R + M184V and their effects on enzyme function and viral replication capacity. *Antimicrob. Agents Chemother* **46**, 3437–3446.

Wilke, C.O. and Novella, I..S. (2003) Phenotypic mixing and hiding may contribute to memory in viral quasispecies. *BMC Microbiol.* **3**, 11.

Wilke, C.O., Ronnewinkel, C. and Martinetz, T. (2001a) Dynamic fitness landscapes in molecular evolution. *Phys. Rep.* **349**, 395–446.

Wilke, C.O., Wang, J.L., Ofria, C., Lenski, R.E. and Adami, C. (2001b) Evolution of digital organisms at high mutation rates leads to survival of the flattest. *Nature* **412**, 331–333.

Wilke, C.O., Reissig, D.D. and Novella, I..S. (2004) Replication at periodically changing multiplicity of infection promotes stable coexistence of competing viral populations. *Evolution Int. J. Org. Evolution,* **58**, 900–905.

Wilke, C.O., Foster, R. and Novella, I..S. (2006) Quasispecies in time-dependent environments. *Curr. Top. Microbiol. Immunol.* **299**, 33–50.

Williams, G.C. (1992) *Natural Selection. Domains, Levels and Challenges*. New York, Oxford: Oxford University Press.

Wlodawer, A. and Vondrasek, J. (1998) Inhibitors of HIV-1 protease: a major success of structure-assisted drug design. *Annu. Rev. Biophys. Biomol. Struct.* **27**, 249–284.

Wright, P.F., Neumann, G. and Kawaoka, Y. (2007) Orthomyxoviruses. In: *Fields Virology* (D.M. Dnipe, P.M. Howley, *et al.*, eds) 5th edn, pp. 1691–1740. Philadelphia: Lippincott Williams & Wilkins.

Wyatt, C.A., Andrus, L., Brotman, B., Huang, F., Lee, D.H. and Prince, A.M. (1998) Immunity in chimpanzees chronically infected with hepatitis C virus: role of minor quasispecies in reinfection. *J. Virol.* **72**, 1725–1730.

Yamada, K., Mori, A., Seki, M., Kimura, J., Yuasa, S., Matsuura, Y. and Miyamura, T. (1998) Critical point mutations for hepatitis C virus NS3 proteinase. *Virology* **246**, 104–112.

Yang, Y., Halloran, M.E., Sugimoto, J.D. and Longini, I.M. (2007) Detecting human-to-human transmission of avian influenza A (H5N1). *Emerging Infect. Dis.* **13**, 1348–1353.

Yerly, S., Rakik, A., De Loes, S.K., Hirschel, B., Descamps, D., Brun-Vezinet, F. and Perrin, L. (1998) Switch to unusual amino acids at codon 215 of the human immunodeficiency virus type 1 reverse transcriptase gene in seroconvertors infected with zidovudine-resistant variants. *J. Virol.* **72**, 3520–3523.

Yusa, K., Song, W., Bartelmann, M. and Harada, S. (2002) Construction of a human immunodeficiency virus type 1 (HIV-1) library containing random combinations of amino acid substitutions in the HIV-1 protease due to resistance by protease inhibitors. *J. Virol.* **76**, 3031–3037.

Yuste, E., Sánchez-Palomino, S., Casado, C., Domingo, E. and López-Galíndez, C. (1999) Drastic fitness loss in human immunodeficiency virus type 1 upon serial bottleneck events. *J. Virol.* **73**, 2745–2751.

Zárate, S. and Novella, I..S. (2004) Vesicular stomatitis virus evolution during alternation between persistent infection in insect cells and acute infection in mammalian cells is dominated by the persistence phase. *J. Virol.* **78**, 12236–12242.

Zhang, L., Huang, Y., Yuan, H., Chen, B.K., Ip, J. and Ho, D.D. (1997) Genotypic and phenotypic characterization of long terminal repeat sequences from long-term survivors of human immunodeficiency virus type 1 infection. *J. Virol.* **71**, 5608–5613.

Zhang, X., Hasoksuz, M., Spiro, D., Halpin, R., Wang, S., Vlasova, A. *et al.* (2007) Quasispecies of bovine enteric and respiratory coronaviruses based on complete genome sequences and genetic changes after tissue culture adaptation. *Virology* **363**, 1–10.

Zimmern, D. (1988) Evolution of RNA viruses. In: *RNA Genetics* (E. Domingo, J.J. Holland and P. Ahlquist, eds), Vol. 2, pp. 211–240. Florida: CRC Press Inc.