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Viruses as Quasispecies: Biological Implications

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Abstract During viral infections, the complex and dynamic distributions of variants, termed viral quasispecies, play a key role in the adaptability of viruses to changing environments and the fate of the population as a whole. Mutant spectra are continuously and avoidably generated during RNA genome replication, and they are not just a by-product of error-prone replication, devoid of biological relevance. On the contrary, current evidence indicates that mutant spectra contribute to viral pathogenesis, can modulate the expression of phenotypic traits by subpopulations of viruses, can include memory genomes that reflect the past evolutionary history of the viral lineage, and, furthermore, can participate in viral extinction through lethal mutagenesis. Also, mutant spectra are the target on which selection and random drift act to shape the long-term evolution of viruses. The biological relevance of mutant spectra is the central topic of this chapter.

1
Introduction: How a Theory Met Reality and Vice Versa

The historical overview of evolutionary virology by John Holland in the closing chapter of this volume documents the many indications of genetic and phenotypic instability of RNA viruses, and the growing suspicion over the second half of the twentieth century that something was fundamentally different between RNA genetics and DNA genetics. The first evidence that an RNA virus consisted of distributions of mutant genomes, and that the wild type existed only as a weighted average of many different sequences, was obtained by sampling genomic sequences of biological clones of bacteriophage Qβ by Charles Weissmann and colleagues in Zurich (review in Holland, this volume). Weissmann presented these experimental results to Manfred Eigen and his colleagues at a Max Planck Institute meeting in Klosters (Switzerland) in 1978. Eigen had developed the first theoretical treatment of a system that replicated with limited fidelity, so that the replication process regularly generated mutant copies of the templates (Eigen 1971). This theory, later to become known as quasispecies theory (Eigen and Schuster 1979), was developed to understand self-organization and adaptability of early life forms on earth. In seeing the experimental results on phage Qβ, Eigen exclaimed "Quasispecies in reality!" and this represented the beginning of a productive interaction between theoretical biophysics and experimental virology (further information on these early encounters is given in Domingo et al. 1995).

Quasispecies theory is covered in the chapters by Biebricher and Eigen and by Wilke et al. in this volume, and here we review biological implications of quasispecies dynamics for RNA viruses. The extended definition of quasispecies currently used by virologists is the following: "Viral quasispecies are dynamic distributions of non-identical but closely related mutant and recombinant viral genomes subjected to a continuous process of genetic variation, competition and selection, and which act as a unit of selection" (Domingo 1999). This definition incorporates general principles of Darwinian evolution, whose effects, in the case of viruses, can be observed within short time periods (usually days and weeks) both in natural hosts, and in model systems such as alternative hosts in vivo or in cell cultures. Short-term evolution of viruses underlies virus adaptation to compartments within infected organisms, that may contribute to time-dependent changes of disease symptoms and to disease progression. This is particularly evident with RNA viruses, and several examples are discussed in this volume, and others are regularly reported in the current literature on virology. Some terms (such as "fitness", "environment", etc.) used in this and other chapters, and that have been approached mainly with viral infections in cell culture, may seem abstract and distant

from the real world of viral diseases. Yet they are not. Fitness values express a replication capacity, and competitive growth occurs in infected hosts when variant forms of the same virus meet to penetrate a compartment of a differentiated organism, or to replicate in the same cell subset. Environment may mean specific cell types or cell assemblies in tissues and organs where virus replication takes place, or a set of physiological conditions that may affect virion stability or susceptibility of cells to viral variants.

The main steps involved in middle-term and long-term RNA virus evolution have some resemblance with some of the steps that determine short-term evolution in quasispecies dynamics (Fig. 1), despite their occurring with very different space-time scales. The triggering event in these evolutionary episodes is reproduction with genetic variation. Then positive and negative selection, together with random sampling (drift) that take place within a host, or between host individuals, shape the genetic composition of the virus. Reproductive success can be quantified as relative fitness values (Quiñones-Mateu and Arts, this volume) or with epidemiological parameters such as the basic reproductive rate (or ratio) (Ro), defined as the average number of secondary cases resulting from the introduction of a single infected case into a susceptible population. Ro is a general parameter (which can be applied to demography of any type of biological entity) that can predict the long-term epidemic spread of a pathogenic agent (reviews concerning application to viral infections in Nowak and May 2000 and Woolhouse 2004). Both fitness and Ro values capture average values of fundamentally heterogeneous entities, and such averages may vary as an infection progresses or an epidemic spreads, thus adding an additional level of complexity to the interpretation of the reproductive success of a virus. While the reproductive ratio is generally used in epidemiological investigations, fitness finds its application in the comparison of the relative replication capacity of variant viruses within a single host or in cell culture.

As indicated by Biebricher (1999), evolutionary success depends on two components of the phenotype: those that determine survival and those that determine the rate of production of viable progeny, and the combination of the two is what we call fitness. Virologists have adapted growth-competition experiments to measure the relative capacity of two viruses to produce infectious progeny, thus providing estimates of relative fitness values under a given set of environmental conditions (Holland et al. 1991). Fitness can be measured in other ways (DeFilippis and Villarreal 2000), and it is virtually impossible to design a measurement that captures in full the potential evolutionary success of viruses replicating in their host organisms (Biebricher 1999; DeFilippis and Villarreal 2000). Despite limitations in the measurements and significance of fitness values for RNA viruses, variations in relative fitness, based on

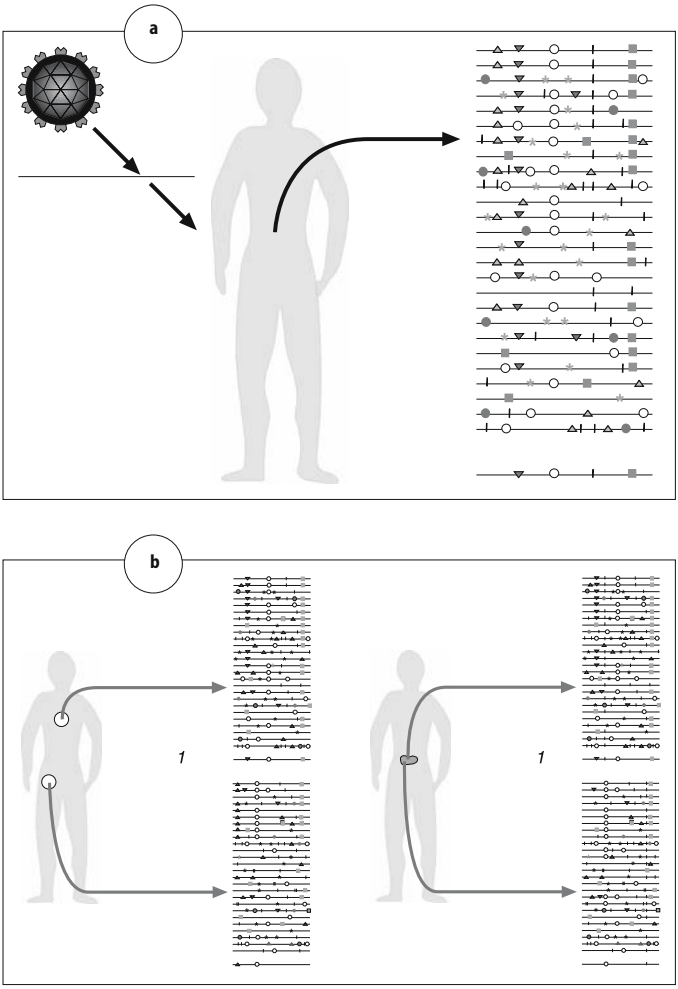

Fig. 1a,b Schematic representation of viral quasispecies in an infected host. Viral genomes are represented as *horizontal lines*, and mutations as *symbols on the lines*. **a** Upon infection with an RNA virus (even with a single particle, as depicted here, enlarged about 10^6 times), viral replication leads to a mutant spectrum of related genomes, termed viral quasispecies. Nucleotide sequencing of the ensemble produces a consensus sequence which includes in each of its positions (nucleotide or amino acid) the residue found most frequently in the corresponding position of the distribution (mutant spectrum). **b** Different mutant distributions are found in different infected organs or at different sites of the same organ. As further discussed in the text, in real infections multiple mutant spectra that can amount to a large number of replicating (or potentially replicating) genomes (up to 10^9 or even 10^{12} per infected individual) provide highly dynamic mutant repertoire

viral yields in cell culture, have been immensely powerful in characterizing the population dynamics of RNA viruses (see references in the reviews by Domingo and Holland 1997; Quiñones-Mateu and Arts 2002; Novella 2003; and the chapters by Quiñones-Mateu and Arts and Escarmís et al., this volume). As usually performed, fitness values incorporate differences in replication capacity (both of viruses in isolation and when co-replicating in the same cells) at all stages of the viral life cycle. Restricting fitness measurements to individual events of the replication cycle (RNA synthesis, viral-specific protein synthesis, virion assembly, etc.) would entail additional ambiguities (i.e. RNA replication may be coupled to translation, translation to assembly, etc.). Quiñones-Mateu and Arts in the next chapter of this volume discuss several important implications of fitness measurements based on viral yields. Figure 2 shows a schematic view of quasispecies dynamics in relation to population size and fitness variations. But there are additional implications of the links between quasispecies theory and virus population dynamics.

2
Molecular Characterization of RNA Virus Populations

Since RNA viruses replicate as complex mutant distributions (Figs. 1 and 2), determination of the consensus nucleotide sequence (or the consensus amino acid sequence of encoded proteins) provides very fragmentary information of the genetic composition and of the evolutionary potential of a virus population. Analyses of individual genomic sequences of mutant spectra can be achieved by two alternative procedures. One is to isolate virus either from individual plaques developed on cell monolayers or from an infection following end-point dilution. Viral RNA is then subjected to reverse transcription-polymerase chain reaction (RT-PCR) amplification and nucleotide sequencing. This procedure leads a sequence that cannot be influenced by possible misincorporations introduced during the RT-PCR amplification (that can arise due to the limited copying fidelity of the enzymes used in the amplification). In sequence screening of biological clones, a bias may occur that favours representation of genomes that are more infectious (produce early cytopathology or larger plaques) in the particular cell line or primary culture chosen. A second means to characterize a virus population is to subject total RNA extracted from the biological specimen of interest to RT-PCR, followed by molecular cloning and sequencing of DNA of individual molecular clones. This procedure does not depend on infectivity of the viral RNA in a cell culture system. Here a bias may arise from the low fidelity of the enzymes used in the RT-PCR, which may result in an overestimate of the nucleotide sequence

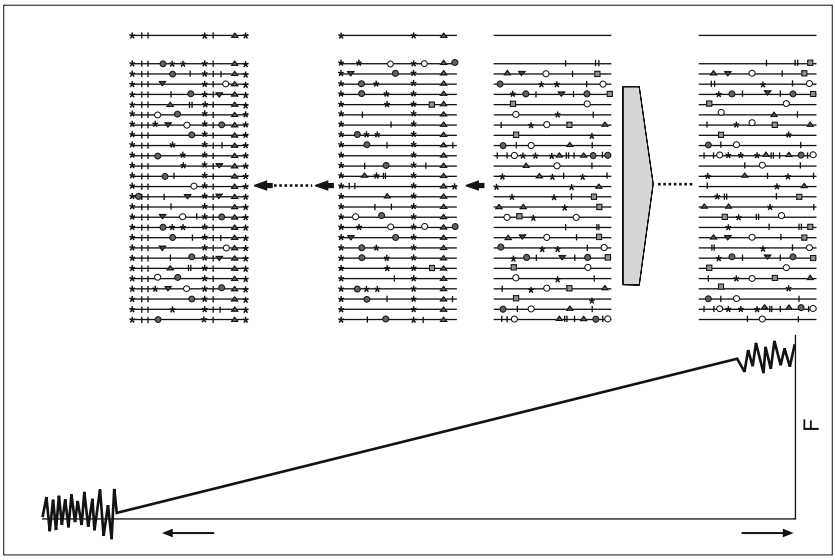

Fig. 2 Scheme of quasispecies dynamics and fitness variations. *Top*: Viral genomes are depicted as *horizontal lines* and mutations as s*ymbols on the lines* (see also Fig. 1). *Small arrows* indicate genetic bottlenecks experimentally realized as plaque-to-plaque transfers. Serial bottleneck events lead to accumulation of mutations in the consensus sequence. The *large arrow* represents replication of the quasispecies without population size limitations. *Bottom*: Fitness (F) variation associated with passage regime. Bottleneck events lead to a decreases in fitness; however, at low fitness values a fluctuating pattern with a constant average fitness is observed (Escarmís et al., this volume). Large population passages result in a fitness increase that may or may not result in a modification of the consensus sequence. Again, at high fitness, a fluctuating pattern of fitness values is observed, presumably reflecting a limitation exerted by the population size on the capacity for fitness increase (Novella et al. 1995, 1999). (Figure adapted from Domingo 1999, with permission)

heterogeneity. This can be solved by using high-fidelity polymerases, correct amplification reaction conditions (adequate pH and ionic composition, unbiased concentrations of chemically intact nucleoside triphosphates, etc.), and control experiments to determine a basal error rate for the system (Arias et al. 2001). Another bias may come from insufficient initial template RNA so that several sequences that originate from the same viral template RNA may be represented among the clones analyzed. This will generally result in an underestimate of the nucleotide sequence heterogeneity. Some simple calculations have been applied to derive the expected number of independent sequences (those that will represent different templates in the viral population) from the

last dilution of the template RNA that yields positive RT-PCR amplification (Airaksinen et al. 2003). Our rule of thumb is that when a 1:100 dilution of the template yields positive RT-PCR amplification, we proceed with the cloning and sequencing of the amplification product of the undiluted template. When the amount of template is limiting, alternative procedures (such as multiple parallel amplifications prior to cloning and sequencing) must be sought (Airaksinen et al. 2003).

It is a common practice to align at least 20 genomic (or subgenomic) sequences and to determine the mutation frequency and Shannon entropy (Table 1). In most situations, 20–100 sequences represent a tiny minority of

Table 1 The characterization of mutant spectra of viral quasispecies

1. Mutation frequency

Definition: The proportion of mutant nucleotides in a genome population. Mutant frequency may refer to the proportion of genomes harbouring a specific mutation.

Calculation: Determine the total number of mutations (counting repeated mutations only once) relative to the consensus (defined by the same set of sequences) and divide by the total number of nucleotides that have been sequenced (i.e. 10,000 when the same 500 nucleotides of 20 individual genomes have been determined). In some cases, it may be interesting for statistical reasons to count all mutations found (counting repeated mutations as many times as they occur), yielding a maximum mutation frequency.

2. Shannon entropy

Definition: The proportion of different genomes in a mutant distribution

Calculation: Normalized Shannon entropy, $Sn = -[\sum_i (p_i \times lnp_i)] / \ln N$, in which p_i is the proportion of each different sequence of the mutant spectrum, and N the total number of sequences compared.

3. Genetic distance and Hamming distance

Definitions: Genetic distance is the number of mutations that distinguish any two sequences from the population. The average for all possible pairs reflects the genetic complexity.

Hamming distance is the number of mutated positions in a genome with respect to the best adapted sequence (the most abundant) in a genome distribution. For quasispecies analysis, a list of Hamming distances (or the average for the population) can characterize the complexity of the ensemble.

Calculation: Align sequences and divide the number of mismatched positions between any two sequences by the number of identical positions. Matrices of pair-wise genetic distances are used to determine phylogenetic trees. For distantly related sequences (rarely occurring within a quasispecies), multiple sequence alignments using CLUSTAL W and adequate scoring of gaps may be needed.

Based on Eigen 1992; Volkenstein 1994; Domingo 1996; Mount 2004; Domingo et al. 2005.

the total number of viral genomes in a biological specimen, and therefore this procedure must be regarded as a very crude sampling of a viral population. In some cases, a selective agent may permit the screening of minority components of mutant spectra. For example, in studies on quasispecies memory (described in Sect. 6) analyses of mutant spectra were based on the repertoire of mutants resistant to a monoclonal antibody that neutralized the dominant FMDV population, but did not neutralize the portion of the mutant spectra of interest (Ruiz-Jarabo et al. 2002, 2003b).

Despite these limitations, determination of nucleotide sequence heterogeneities in virus populations using correct reagents and adequate controls has consistently documented that most RNA viruses (and also some DNA viruses) consist of complex mutant spectra, with an average number of 1–100 mutations per genome (Sect. 3). The degree of heterogeneity within a viral population will be influenced by the fidelity of the replication machinery, the distance (in rounds of replication) from a clonal origin, constraints for variation (both at the RNA and protein levels), and intervening bottlenecks in the evolutionary history, among other influences. Scientists should be cautious in attributing to high-fidelity RNA replication what may be a standard error level together with negative selection, which maintains an invariant consensus nucleotide sequence. The literature contains several cases of such likely misinterpretation of data. It is not surprising that comparisons of different virus–host systems have led to exceedingly broad ranges of genetic diversity within mutant spectra. This should not blur the common underlying influences and the general biological relevance of population complexity even if seemingly modest in terms of mutations per individual genome. This is justified in the next section.

3
The Importance of Being Mutant Spectra

The relevance of viruses replicating as mutant spectra is intuitively obvious since any individual mutant genome of the ensemble can potentially differ in behaviour from other individuals or from the ensemble of genomes. The importance of such a population structure is strengthened by considering five parameters that characterize a mutant distribution: average number of mutations per genome, virus population size, genome length, mutations needed for a phenotypic change, and virus fecundity (Table 2). Despite all cellular organisms being highly polymorphic genetically (in that distinct alleles from a gene are represented among individuals of one biological species), the level of heterogeneity of RNA virus populations confers a much greater adaptability

than the levels of polymorphism estimated for cells. This is a consequence of parameters 2 and 3 (listed in Table 2): as an example, a viral genome of 10,000 nucleotides has a total of 3×10^4 possible single mutants (disregarding fitness effects of mutations), which is a figure well below the population size of many natural virus populations. In contrast, the total number of possible single mutants for a mammalian genome is about 10^{10}, well above the population size of mammalian species. That is, the capacity to explore sequence space (a concept discussed in the chapter by Biebricher and Eigen in this volume, which refers to the total number of possible sequences available to a genetic system, reviewed by Eigen and Biebricher 1988) is far greater for viruses than for cellular organisms. This confers adaptability to viruses (with amply recognized biological implications) and renders viruses suitable experimental systems for probing evolutionary concepts (see Domingo et al. 2001; Flint et al. 2004, and other chapters of this volume).

One of the critical parameters in viral quasispecies is the number of mutations in an RNA virus that is needed for a phenotypic change in the virus (Table 2). Indeed, if a relevant phenotypic change (for example, a modification in host cell tropism, resistance to neutralizing antibodies or to an antiviral agent, etc.) depended on the occurrence of 50–100 mutations in a viral genome (to invent a simple example), then the quasispecies nature of RNA viruses (with the characteristic parameters we measure today; Table 2) would

Table 2 Some important parameters that influence the adaptability of viral quasispecies

1. Average number of mutations per genome in a mutant spectrum

   Generally it amounts to an average of 1–100 mutations per genome. (See text for reasons for broad range).

2. Virus population size

   Variable, but very high upper limits. An acutely infected organism may include 10^9–10^{12} viral particles at any given time. Even a single viral plaque on a cell's monolayer can yield 10^3–10^{10} particles.

3. Genome length

   3 kb–32 kb

4. Mutations needed for a phenotypic change

   Many recorded adaptive changes depend on one or a few mutations (see text).

5. Fecundity

   Variable. Average of $<$ 1–10^6 particles per cell have been measured. High fecundity promotes quasispecies dynamics, as discussed in several chapters of this volume.

Based on Domingo et al. 2001 and references therein.

be largely irrelevant for short-term adaptation. This is because in the exploration of sequence space the probability of arriving at a point corresponding to a mutant with the required 50–100 mutations is exceedingly low (10^{-200} to 10^{-400}!). Even if we relax the requirement to multiple combinations of mutations, it can be estimated from the Poisson distribution that the probability of genomes with any 50 mutations in a population of 10^{11} individuals is about 10^{-44} genomes (on a purely statistical basis, ignoring fitness effects; additional representative figures can be found in Domingo 1997). As evidenced by many experimental results with RNA viruses of virtually all families that have been examined, one or a few amino acid replacements are sufficient to modify a biologically relevant feature of a virus, as amply recorded in the literature (review in Domingo et al. 2001; examples of single mutations associated with epidemiologically relevant adaptations of arboviruses are given in the chapter by Weaver in this volume). Because phenotypically relevant mutations can be frequently represented in mutant spectra, they have the potential to become dominant (command the development of new mutant distributions) in response to environmental demands, despite the modulating effects of mutant spectra described in Sect. 4. Multiple constraints for variability have been evidenced in viral genomes (Simmonds and Smith 1999; Simmonds et al. 2004). Therefore, many variants with one or few mutations that may confer biologically relevant, adaptive traits may not be represented in a mutant spectrum (under a set of environmental conditions) due to fitness costs. It is impressive that despite such constraints (for example, genome-scale, ordered RNA structures in hepaciviruses; Simmonds et al. 2004), the level of heterogeneity and adaptive capacity of viral populations are the ones we record with unfailing continuity with any virus we examine in some detail.

A highly significant example is the occurrence of mutants with one or a few amino acid substitutions that confer decreased sensitivity to antiviral inhibitors. This is a general phenomenon – documented with many viruses since the 1960s both in vivo and in cell culture – which complicates enormously the treatment of viral disease (a recent overview can be found in Domingo 2003; see also previous versions published in *Progress in Drug Research* for a historical account). It is not the case that "suddenly" a virus strain "appears" that is resistant to an inhibitor. Resistant mutants are generated as components of mutant spectra and then may become dominant when virus replication occurs in the presence of the inhibitor. Perhaps the most dramatic example has been the selection over the years of mutants of human immunodeficiency virus type 1 (HIV-1) resistant (or with decreased sensitivity) to the antiretroviral inhibitors (targeted mainly to reverse transcriptase, protease or surface structures involved in virus fusion or cell recognition) used in clinical practice (see the chapter by Mullins and Jensen, this volume). Both experi-

mental analyses of HIV-1 populations (Coffin 1995; Nájera et al. 1995) and theoretical predictions (Ribeiro et al. 1998; Gerrish and Garcia-Lerma 2003) suggest that such mutants may preexist in HIV-1 populations, even when they have not been exposed to the inhibitors. In addition, resistant mutants that may occupy very low frequency levels in HIV-1 mutant spectra in the absence of the inhibitor may be raised to higher frequency levels in the presence of the inhibitor. The recently described phenomenon of memory in viral quasispecies, including memory subpopulations of HIV-1 in vivo (Sect. 6), supports even further a role of genome subpopulations in the response of viruses to inhibitors (Domingo et al. 2003). The fitness costs of inhibitor-resistance mutations, as well as relative fitness values of wild-type and resistant mutants in the absence and the presence of the inhibitor will influence the kinetics and degree of dominance of inhibitor-resistant mutants (treated in the chapter by Quiñones-Mateu and Arts, this volume).

Paradoxically, the presence of a mutant with a specific phenotypic trait may not be sufficient to guarantee its dominance even when a selective constraint favours that phenotypic trait. The reason for this important phenomenon again has to do with the presence of a mutant spectrum surrounding the relevant mutant, and this is discussed next.

4
Suppressive Effects of Mutant Spectra

A mutant virus potentially capable of becoming dominant in an evolving viral quasispecies (either because of its high fitness or because it harbours a selectable trait) may remain as a minority in the population, depending on the nature of the mutant spectrum in which it is immersed. This concept was first documented with a numerical example of two master sequences of small size differing in fitness by 10%, replicating near the error threshold (see the chapters by Biebricher and Eigen and by Wilke et al., this volume, and Sect. 7 in this chapter). Interestingly, the inferior mutant outgrew the fitter one when the inferior mutant was surrounded by 50 closely related mutants that were somewhat better adapted than the mutants that surrounded the fitter master. This and other numerical simulations (Swetina and Schuster 1982; review in Eigen and Biebricher 1988) suggest a strong influence of the mutant spectrum on the behaviour of any particular variant and supports the view that the target of selection is not a single species, but rather the distribution of the quasispecies as a whole (Eigen and Biebricher 1988; Perales et al. 2005).

The prediction that the mutant spectrum can affect the dominance of an individual mutant has found experimental confirmations both in vivo and in

cell culture (Table 3). The first evidence was obtained using vesicular stomatitis virus (VSV) (de la Torre and Holland 1990). A mutant spectrum of VSV suppressed replication of a mutant of superior fitness than the ensemble, unless the mutant was present above a certain frequency in the population. In another instance of remarkable practical relevance, it was found that in anti-poliomyelitis vaccine preparations, the dominant attenuated poliovirus (PV) suppressed neurological disease associated with minority virulent PV, unless the latter was present above a minimal concentration in the vaccine (Chumakov et al. 1991). Some strains of the arenavirus lymphocytic choriomeningitis virus (LCMV) (see the chapter by Sevilla and de la Torre, this volume) induce a hormone-deficiency syndrome in mice (reviewed by Oldstone 2002). Remarkably, some co-infecting nonpathogenic strains of LCMV suppressed expression of pathogenic strains in such a way that no disease was manifested (Teng et al. 1996). Studies with foot-and-mouth disease virus (FMDV) have provided two additional examples (recent reviews on FMDV in Rowlands 2003; Sobrino and Domingo 2004; Mahy 2005). FMDV serially passaged in BHK-21 cells in the presence of polyclonal antibodies directed to a specific antigenic site of the virus, generated a complex mutant spectrum of antigenic variants of low relative fitness. Such mutant spectra included biological clones that manifested high fitness when separated from the mutant cloud (Borrego et al. 1993). In the transition to error catastrophe of FMDV (Sect. 7), pre-extinction viral RNA (which is defined as viral RNA extracted from the population that, in the serial passages in the presence of mutagens, precedes the one in which virus extinction occurs) interferes with infectious RNA (González-López et al. 2004). Interference was documented by co-electroporation of cells with two RNAs and it was exerted by pre-extinction RNA but was not exerted by a defective FMDV RNA (containing an in-frame deletion), unrelated viral and nonviral RNAs, or the same pre-extinction RNA reduced in size by chemical treatment. It was proposed that due to mutations in pre-extinction viral RNA, abnormal expression patterns and expression of abnormal viral proteins may jeopardize the replication capacity of coexisting infectious RNA (González-López et al. 2004). In support of this proposal, FMDV 3Ds (polymerases) harbouring deleterious mutations have been identified in mutagenized populations (Sierra et al. 2000; Arias et al. 2005; see also Sect. 5 for additional information on possible mechanisms of suppressive effects of mutant spectra).

An alternative model, termed positive clonal interference, taken from bacterial population genetics, was proposed to explain dominance of one VSV clone over another (Miralles et al. 1999). In this model, the basis for the interference is competition among genomes carrying advantageous mutations during replication as large populations. In addition to a technical problem

derived from the likely presence of defective interfering (DI) particles in the co-infections carried out to test the model (see Domingo 2003 for a more detailed discussion of this problem), recent results with FMDV show that suppression does not require comparable fitness of the two competing populations (González-López et al. 2005), in agreement with a suppressive effect exerted by a low fitness mutant distribution on genomes displaying higher fitness (Sect. 5).

The suppression of individual genomes by mutant spectra (Table 3) confers additional biological relevance to population bottleneck events that may intervene during virus replication and evolution. In addition to effects on virus population dynamics (see the chapter by Escarmís et al., this volume), and in the relationship between virulence and transmission mode (Bergstrom et al. 1999), bottlenecks may release a portion of mutant spectrum from the complete cloud that may modulate the behaviour of minority genomes (Fig. 3). Also, some contradictory results seen in the literature on the frequency of deleterious mutations or epistatic effects of mutations in RNA viruses may in part derive from the fact that it is not possible to "freeze" a mutant to remain as the "same" individual in the course of replication: a cloud forms immediately and unavoidably. Thus, what initially was "a" genome with one or two mutations of interest will soon become a "distribution" of genomes

Table 3 Modulating effects of mutant spectra on individual virus variants

Cell culture
Mutant spectrum of vesicular stomatitis virus (VSV) suppresses a VSV variant of superior fitness (de la Torre and Holland 1990).
Low fitness antibody-escape population of foot-and-mouth disease virus (FMDV) suppresses individual antigenic variants displaying high fitness (Borrego et al. 1993).
Pre-extinction FMDV RNA interferes with infectious FMDV RNA (González-López et al. 2004).
Defective lymphocytic choriomeningitis virus (LCMV) contribute to suppression of infectivity and extinction of the virus (Grande-Pérez et al. 2005).
In vivo
Attenuated poliovirus (PV) can suppress neurological disease in monkeys, associated with virulent PV (Chumakov et al. 1991).
Nonpathogenic LCMV can suppress manifestation of the growth hormone-deficiency syndrome in mice, associated with pathogenic LCMV strains (Teng et al. 1996).

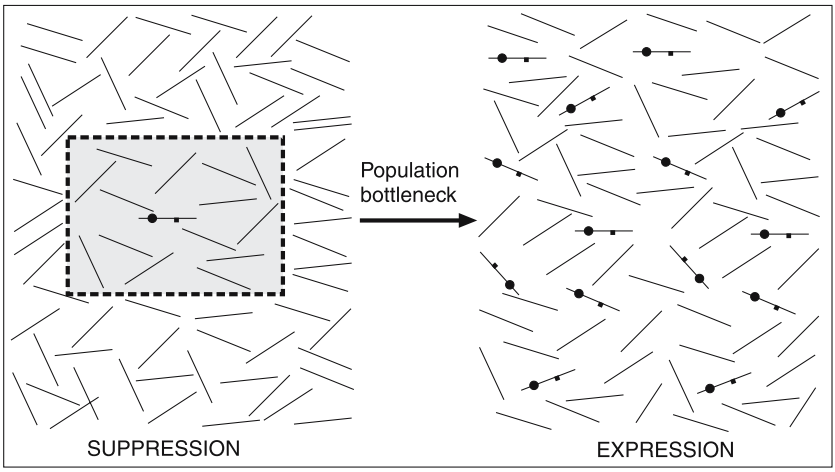

Fig. 3 Scheme of the effect of a population bottleneck on the replication of an individual viral genome. *Lines* represent a complex mutant spectrum in which individual mutations have not been indicated. An individual genome characterized by two specific mutations (*dot* and *rectangle*) is surrounded by a mutant spectrum, which suppresses its replication by the biochemical mechanisms discussed in the text (see also Fig. 4). A population bottleneck represented by the *shaded rectangle* relieves the individual genome of part of the suppressing ensemble, thereby facilitating expression of the individual genome, which may increase its frequency (*right*). Experimental evidence and references are given in the text

with additional mutations that may affect the behaviour of the genomes harbouring the initial mutations. Careful monitoring of progeny sequences and mutant spectrum complexities is needed. It is tempting to suggest that some of the uncertainties that appear to characterize virus evolution in vivo (for example, that an antibody-, CTL- or inhibitor-resistant variants may raise to dominance or not) may relate to suppressive effects of mutant spectra perturbed by intra-host bottleneck events of different intensities, prompted by the replicative characteristics of the virus in its permanent interaction with of the host. As further penetration into the fine population structure of viruses in vivo becomes technically feasible, it may be possible to elucidate some of these issues.

4.1 Consequences for Fitness Determinations

In the course of fitness determinations [usually by growth competition between the virus to be tested and a reference virus in a defined biological

environment (see the chapter by Quiñones-Mateu and Arts, this volume)] suppressive effects of mutant spectra may also influence the outcome of the competition. Indeed, as discussed in Sect. 1, the mutation rates during RNA genome replication and retrotranscription render extremely unlikely the sustained synthesis of genomes without mutations (Drake and Holland 1999) (although, interestingly, evolution appears to have adjusted mutation rates to permit the presence and survival of the nonmutated class when the latter are fitter than mutant progeny). Thus, despite the fact that during fitness determination a competition is established that should result in dominance of the most fit mutant distributions, modulating effects of mutant spectra of the type discussed in the previous section cannot be excluded, and they have been recently evidenced in the extreme case of fitness determinations of pre-extinction FMDV populations (Gonzalez-Lopez et al. 2005). The converse effect, that is, complementation among mutants (Moreno et al. 1997; Agol 2002), can also occur at the stage of fitness determination in which the two competing ensembles co-infect the same cells, and this effect has been modeled (Wilke et al. 2004) (see the chapter by Wilke et al., this volume). Taking relative virus yield in a given environment as a measure of fitness is justified because first, no virus genome ever exists as a "single nucleotide sequence"; and second, when it exists (as in the very beginning of a formation of a plaque on a cell monolayer) it rapidly becomes a mutant spectrum in the first infected cell. Thus, initial RNA replication rates are fictitious for these reasons and others summarized in Sect. 1, supporting the adequacy of fitness values given as relative viral yields (see Sect. 1 and the chapter by Quiñones-Mateu and Arts, this volume).

5
Interactions Within Mutant Spectra: A Proposal

It is a rooted tradition, not only among virologists but also in some schools of population genetics, to view the individual as the standard unit of selection, although this is a debated subject (as a general discussion of this topic see Lewontin 1970; Williams 1992). In this section, we argue that since RNA viruses consist of mutant spectra, and components of the spectra may (and often do) deviate genotypically and phenotypically from other components of the same spectrum, the behaviour of an individual may (and often will) be influenced by surrounding individuals that we call "the ensemble" (extreme cases of suppression of individual variants are discussed in Sect. 4). We further suggest that this feature of viral quasispecies is consistent with many observations made in classical viral genetics.

Viral interference provides a useful introduction to our argument. Interference referred initially to the inhibition of multiplication exerted by one virus on an entirely different virus entering the same cell (see reviews in White and Fenner 1986; Youngner and Whitaker-Dowling 1999; Condit 2001). This led to the distinction between homologous and heterologous interference, depending on the relatedness of the two viruses under analysis. Interference acquired its major impetus with the discovery of defective, noninfectious genomes (generally deletion mutants) that interfered with the corresponding standard virus and mediated the establishment of persistent infections of some RNA viruses (see reviews in Huang and Baltimore 1970; Perrault 1981; Holland 1990). The interfering genome, whether infectious or noninfectious, often has a higher affinity than the standard (interfered) virus for a viral or host protein (or other host component). Here a conceptual overlap with the concept of fitness exists (García-Arriaza et al. 2005). Most fitness measurements involve a phase of intracellular competition between the two viruses to be tested. This is because even in competitions started at low MOI there will be a second round of cell infection that, depending on the initial yield, may involve high MOI. This point has been discussed by (Novella 2004) regarding complementation, and it applies to fitness assays as well. When intracellular competition occurs, the difference in progeny production may have the same molecular basis as interference. This has been manifested in recent studies that have shown a fitness-dependent interference by a bipartite version of the FMDV genome (García-Arriaza et al. 2004, 2005). We arrive at the dilemma that either fitness values should be determined avoiding any type of intracellular competition, or it must be accepted that fitness differences (generally measured for mutants of the same virus) are influenced by intracellular competition events akin to those underlying viral interference. Intracellular competition could be avoided either by each virus infecting cells on separate culture dishes or animal hosts, or by restricting replication to the initial round of progeny production following infection at low MOI; both requirements pose technical problems.

Interference may be mediated by interferon, which evokes a general antiviral state in a cell, as well as by a number of interactions between the interfering and interfered viruses (White and Fenner 1986; Youngner and Whitaker-Dowling 1999; Condit 2001; Agol 2002). Concerning possible interference between mutants of the same virus (the form that may be most relevant to quasispecies behaviour), interference may be associated with multiple, different mutants which, even if present individually at low frequency, may have collectively the effect of a dominant-negative mutant. The latter may act through different mechanisms: for example, in the "rotten apple" hypoth-

esis, a defective polypeptide produced by the mutant may enter a multimeric complex, inhibiting its activity. In the "road-block" hypothesis, a defective function (protein or regulatory region) may sequester a factor (or a site) required for replication of both viruses. In the "all things in moderation" hypothesis, imbalances in gene dosage may lead to decreases in efficiency of steps in the life cycle of both viruses. In the "direct competition" hypothesis, both viruses compete for some viral or host factor. According to the "attractive genome" hypothesis, the dominant-negative virus may possess sites that bind required factors, and such sites may be more abundant or show higher affinity than the sites in the wild-type virus. These different hypotheses have been taken from the summary by Youngner and Whitaker-Dowling (1999), and we propose that at least some of these mechanisms may underlie modulating effects observed within mutant spectra of viral quasispecies (Fig. 4). This was proposed to explain the interfering activity of pre-extinction FMDV RNA (González-López et al. 2004) and it emphasizes increasing evidence of the critical role that defective genomes may have in dictating virus behaviour (Grande-Pérez, et al., 2005; see also the chapter by Escarmís et al., this volume). Within mutant spectra, multiple potentially dominant-negative mutants (when present at high frequency) may have a synergistic effect in preventing standard virus replication by one or several of the proposed mechanisms (Youngner and Whitaker-Dowling 1999). These negative effects on replication of individual mutants may be favoured by the multifunctional nature of viral proteins (both in mature and precursor forms), increasingly recognized for many viruses (Cornell et al. 2004; Flint et al. 2004). Parallel concepts can be applied both to modulating effects of mutant spectra (Table 4) and to the transition to error catastrophe (González-López et al. 2004; Grande-Pérez et al., in press; see also Sect. 7). Mutants with complementing or interfering capacity have been referred to as cooperators or defectors, respectively, during processes of competition inside a host (Turner and Chao 1998; Novella 2004; Novella et al. 2004). What we know of quasispecies' capacity to attain different fitness levels suggests that the same altered viral protein may be advantageous or disadvantageous depending on the dominant genomes in the mutant distribution (Fig. 4).

Thus, our proposal is that a mutant spectrum of a replicating viral quasispecies constitutes a "genetic microcosmos" in which interactions among components of a mutant spectrum may include effects similar to those previously characterized between well defined mutants of a virus or between two different viruses. What is observed to occur "between populations" is now extended to interactions among "components of a population". Experiments are now in progress to further test the validity of this proposal.

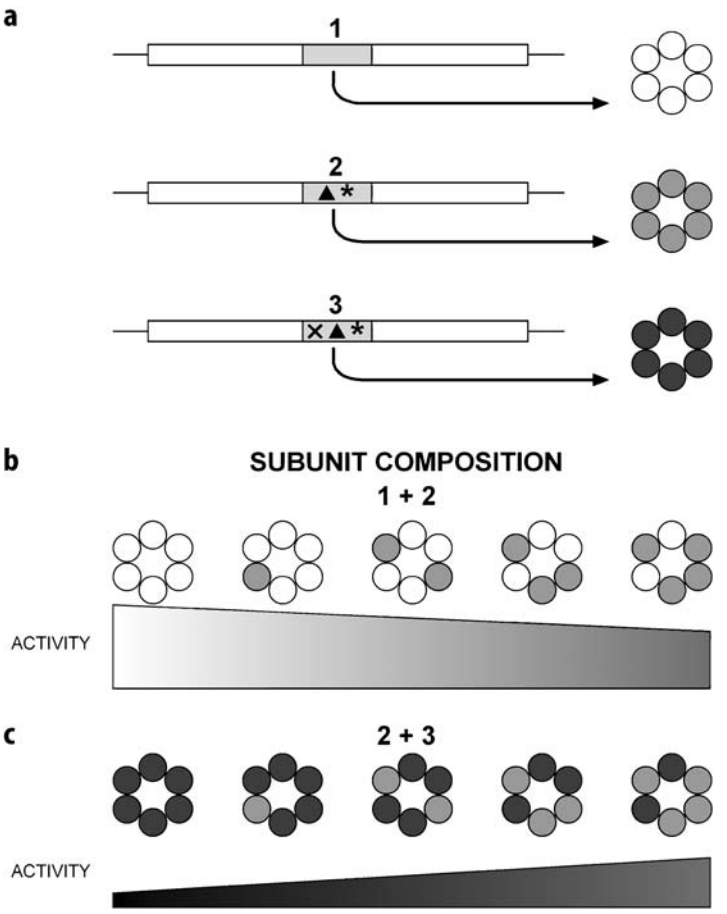

Fig. 4a–c Scheme of the effect of a *trans*-acting viral gene product on the replicative efficacy of a viral quasispecies. **A** Three forms of the same viral gene (1, 2, 3; *shaded box* in genome; symbols represent mutations) encode a protein with decreasing biological activity (*white to dark grey*) that acts as a homopolymeric hexamer (needed to produce viral progeny). **B** In a mutant spectrum in which 1 and 2 are expressed, increasing proportions of gene 2 will result in a decrease in activity of the hexameric protein, and, consequently of viral yield. **C** In a mutant spectrum in which 2 and 3 are expressed, increasing proportions of gene 3 will result in a decrease in activity of the hexameric protein, and, consequently of viral yield. Note that the same viral subunit can act as a negative modulator (**B**) or a positive modulator (**C**) depending on the activity of the accompanying subunit. In real viral quasispecies, modulating effects are far more complex than illustrated here because a range of different activities in *trans*-acting products may be present, several viral and host gene products may participate in heteropolymeric complexes, viral proteins are often multifunctional, and regulatory signals on the viral RNA may be involved

6
Additional Roles for Minorities: Memory in Viral Quasispecies

Viral quasispecies may possess in their mutant spectra minority genomes that reflect those that were dominant at an earlier phase of quasispecies evolution. This has been shown experimentally with FMDV in cell culture (Ruiz-Jarabo et al. 2000, 2002; Arias et al. 2001, 2004; Baranowski et al. 2003) and HIV-1 in vivo (Briones et al. 2003; Briones et al., personal communication). The experiments with FMDV took advantage of two well-studied classes of mutants: an antigenic variant selected with a neutralizing mAb and a very unusual variant that contained in its genome an internal polyadenylate tract, generated upon repeated plaque-to-plaque passage of FMDV clones (Escarmís et al. 1996; see also the chapter by Escarmís et al., this volume). When populations dominated by either one of these mutants were serially passaged in cell culture, revertant viruses with the wild-type sequence (that is, without the amino acid substitution that led to antigenic variation and absence of the internal polyadenylate tract, respectively) became dominant. However, genomes with the initially dominant markers did remain in the mutant spectrum not at a basal level accounted solely by mutational pressure, but at levels 10- to 100-fold higher. A scheme of implementation of memory is shown in Fig. 5 and the main features of quasispecies memory are summarized in Table 4.

Two types of memory were distinguished during HIV-1 infections in vivo. One was the "replicative" memory as defined with FMDV, and the other was a "cellular" or "anatomical" memory derived from the retroviral life cycle with

Table 4 Main features of quasispecies memory

Replicative memory
Memory genomes derive from genomes that were dominant at an early phase in the evolution of the same viral population.
Memory genomes are present at higher frequencies (in the cases studied 10 to 100 times) than expected from mere mutational pressure exerted on parental genomes.
Memory genomes are lost upon subjecting virus to a population bottleneck.
Memory genomes increase in fitness in parallel with the dominant genomes, as replication proceeds (Red Queen hypothesis).
Memory genomes may decrease in frequency as virus replicates.
Cellular or anatomical memory
Found when viral reservoirs (displaying no replication or limited replication) are activated and contribute to the composition of an evolving quasispecies.

Based on Ruiz-Jarabo et al. 2000, 2002, 2003b; Briones et al. 2003; Arias et al. 2004.

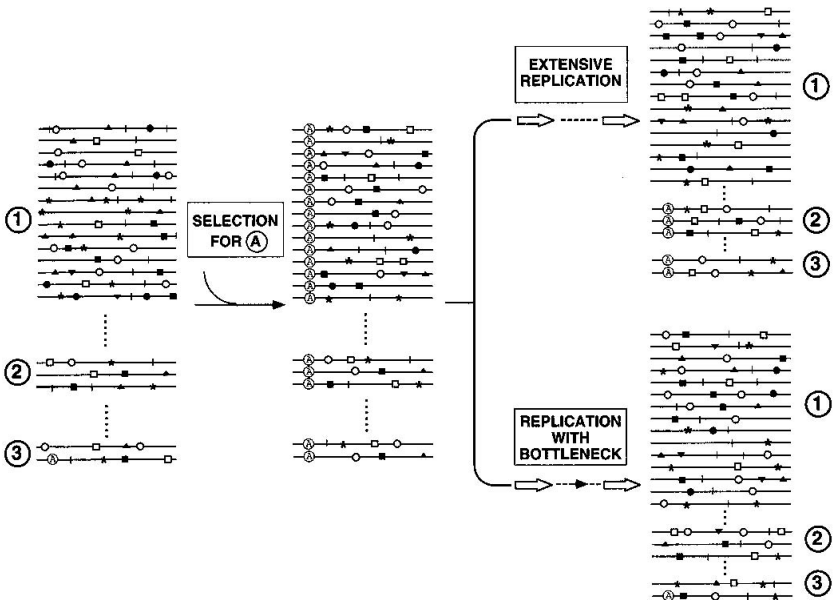

Fig. 5 Schematic representation of implementation, maintenance and loss of memory genomes in a viral quasispecies. Genomes of the mutant spectra are depicted as *horizontal lines*, and mutations as *symbols on the lines*. The mutant spectra have been divided in three levels: level 1 includes those genomes found most frequently in the mutant distribution; level 2 includes a frequency class potentially occupied by memory genomes; and level 3 gathers the less abundant genomes that occur as a result of mutational pressure and the competitive rating of all components of the mutant spectrum. Initially genomes with the genetic marker A belong to level 3. When genomes with marker A are selected, they occupy the entire quasispecies. Upon further replication, when genomes with A display a selective disadvantage with respect to genomes lacking A, genomes with the A marker will eventually become undetectable in the consensus sequence of the quasispecies. However, genomes with marker A may remain as memory genomes in level 2 because in the course of selection their fitness increased (*upper right* mutant distribution). When bottleneck passages (of sufficient intensity to exclude level 2 genomes) intervene, memory is lost and genomes with A occupy again level 3 as in the initial mutant distribution (*bottom right* mutant distribution). In real viral quasispecies, mutants are ranked to form many frequency levels (perhaps a continuum of frequency levels) rather than 3, as assumed here for simplification. Experimental evidence and references on implementation, maintenance and decay of quasispecies memory in cell culture and in vivo are given in the text and in Table 4. (Based on Domingo 2000, with permission)

integration of proviral DNA in cellular DNA, which results in reservoirs that may re-emerge at a later stage of infection (Briones et al. 2003; Briones et al., personal communication). Reemergence (or raise to dominance) of ancestral

(minority) genomes in vivo has been documented with several viral infections (Borrow et al. 1997; Wyatt et al. 1998; Karlsson et al. 1999; Briones et al. 2000; Lau et al. 2000; García-Lerma et al. 2001; Imamichi et al. 2001; Kijak et al. 2002; Charpentier et al. 2004), providing additional support for the concept of memory in viruses both in cell culture and in vivo.

The experiments to investigate the possible presence of memory in viral quasispecies (Ruiz-Jarabo et al. 2000) were motivated by features of virus evolution that fit those of complex adaptive systems (see reviews in Gell-Mann 1994; Frank 1996; Solé and Goodwin 2000). A characteristic of such systems is that they vary, yet they have continuity in the form of some built-in (heritable) component, and are endowed with mechanisms to respond to external stimuli. There is a continuous interplay between the components of the system and the interacting environment. Examples of complex adaptive systems are neurological activity in memory and learning, and the immune system of vertebrates in which long-lived memory T cells may be expanded when the organism is again exposed to an antigen identical (or related) to the one that triggered the initial response. Such potential to act quickly in response to an environmental demand has a functional parallel in the presence of memory genomes in viral quasispecies. Indeed, memory in viral quasispecies may serve the adaptive scheme of viruses by providing a molecular reservoir capable of reacting swiftly to a selective constraint which has been previously experienced by the same population, provided no bottlenecks intervened (Table 4). All evidence suggests that immune or other internal physiological responses in hosts are neither uniform nor continuous, and that memory may help confronting discontinuous or fluctuating challenges that require dominance of related viral genome subsets. Minority genomes present at higher than basal levels, associated with mutational pressure, need not be related only to memory. They may occur as a consequence of the fitness values of randomly generated variants that, although not sufficient to drive the genome subset to dominance, may allow the subset to remain at levels similar to those associated with memory. All these possibilities provide an incentive to develop methods to diagnose the presence of minority genomes in viral populations in vivo, for example for a more personalized and adequate antiviral treatment plan (i.e. to avoid antiretroviral inhibitors for which mutations that contribute to resistance are present at memory levels) (reviewed in Domingo et al. 2003).

6.1
Memory and Long-Term Evolutionary History: A Distinction

There are two concepts related to memory in viruses that should be distinguished. One is a form of long-term memory, not erased by population

bottlenecks, that has gradually shaped the genetic structure and replication strategy of viruses. A present-day virus can be viewed as a "reservoir of memories" of all influences that have conformed its present organization and biological potential, in constant co-evolution with hosts that themselves are an "outcome of history". In contrast, the quasispecies memory discussed in previous paragraphs is an immediate, short-term consequence of the recent evolutionary history of a virus, independently of the historical events that gradually shaped its current configuration.

7
Virus Entry into Error Catastrophe: An Antiviral Strategy Derived from Quasispecies Dynamics

Theoretical studies summarized in the chapters by Biebricher and Eigen and by Wilke et al., this volume, led to the very significant prediction that there must be a limit to the error rate that a replicating system can tolerate if the genetic information is to be maintained (Swetina and Schuster 1982; Eigen and Biebricher 1988; Nowak and Schuster 1989; Eigen 2002). This limit is termed the error threshold and its value depends on the complexity of the genetic information conveyed by the system. Here the term "complexity" means the information encoded by a genome (regulatory regions and number of different open-reading frames), which must be distinguished from the number of different sequences for any regulatory region or open-reading frame represented in a population of genomes; the latter is often referred to as complexity of the mutant spectrum or quasispecies complexity. For RNA viruses, which generally encode little or no redundant information, complexity (in its first meaning) can be equated with genome length. It may be expected that the most complex RNA viruses (such as the coronaviruses, with 27–32 kb genomes) may display higher average copying fidelities than the least complex RNA genomes. In this respect, it will be interesting to establish whether a domain found in the SARS and other coronavirus genomes, which corresponds to a nuclease activity, can provide a proofreading–repair function during coronavirus replication (Snijder et al. 2003).

The interactions among components of mutant spectra (Sect. 5) also provide a biochemical basis for impairing viral replication to the point of leading to the information meltdown typical of error catastrophe. Interestingly, the proposed negative interactions (for example, among *trans*-acting products as discussed in Sect. 5 and depicted schematically in Fig. 4) also favour a more likely replicative collapse when the number of open-reading frames is high (higher complexity in the sense of number of genes encoding proteins). Un-

der mutational pressure, more gene products can be deficient and contribute to replicative incompetence. This is a biochemical counterpart of the genetic basis of error catastrophe.

Measurements of LCMV RNA and infectivity levels during enhanced mutagenesis of steady-state persistent infections of the virus in cell culture have defined two viral extinction pathways. One of them occurs at low mutagenic activities, and it is mediated by defective genomes which drive the infective genomes towards extinction; both experimental results and a computational model support the lethal defection model of virus extinction, with a prominent role of the mutant spectrum (Grande-Pérez et al. 2005).

There is considerable experimental evidence that RNA viruses replicate with an error rate which is close to the error threshold for maintenance of genetic information. In several viral systems, it has been documented that an increase in mutation rate by added chemical mutagens results in decreases in infectivity, and in some cases it may lead to virus extinction (Holland et al. 1990; Lee et al. 1997; Loeb et al. 1999; Crotty et al. 2000, 2001; Loeb and Mullins 2000; Sierra et al. 2000; Lanford et al. 2001; Pariente et al. 2001; Contreras et al. 2002; Grande-Pérez et al. 2002; Airaksinen et al. 2003; Ruiz-Jarabo et al. 2003a; Severson et al. 2003; reviews in Eigen 2002; Anderson et al. 2004; Domingo 2005b). Two lines of study are particularly promising regarding a possible clinical application of error catastrophe. One concerns the mechanism of antiviral activity of the nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1, 2, 3-triazole-3-carboxamide; Rib), a licensed drug extensively used in clinical practice, which shows antiviral activity against a number of DNA and RNA viruses (Snell 2001; see also Airaksinen et al. 2003 and references therein). Rib is phosphorylated by cellular enzymes to its mono-, di- and triphosphate forms and it exerts its antiviral activity through different mechanisms. One of the mechanisms unveiled by Crotty and colleagues, working with poliovirus, is enhanced mutagenesis as a result of incorporation of ribavirin monophosphate (Rib MP) into poliovirus RNA by the viral polymerase (Crotty et al. 2000, 2001; Graci and Cameron 2002). It has also been shown that Rib MP can be incorporated by the hepatitis C virus (HCV) and coxsackievirus B3 polymerase (Maag et al. 2001; Vo et al. 2003; Freistadt et al. 2004). This raises the intriguing possibility that benefits of Rib (alone but mainly in combination with interferon α or its derivatives) for treatment of chronic HCV infections (McHutchison et al. 1998, among many other studies) may be partly due to its mutagenic activity (see also the chapter by Pawtlosky, this volume, for alternative mechanisms of Rib action on HCV infection). If this were established (by many ongoing studies), it would mean that the principle of virus entry into error catastrophe as an antiviral activity would already have been successfully documented without experts being aware of the underlying mechanisms.

A second line of promising research was launched by de la Torre and his collaborators by showing that the base analogue 5-fluorouracil (FU) prevented the establishment of a persistent lymphocytic choriomeningitis infection in mice (Ruiz-Jarabo et al. 2003a). FU is mutagenic for a number of RNA viruses (Pringle 1970; Eastman and Blair 1985; Sierra et al. 2000 and references therein) and is used to treat some types of human cancer (Parker and Cheng 1990). The demonstration that it can be a potent antiviral agent in vivo constitutes a proof-of-principle that is extremely encouraging for the prospect of a clinical application of virus entry into error catastrophe (for recent reviews of error catastrophe as an antiviral strategy, see Anderson et al. 2004 and Domingo 2005b). This recent field of research illustrates how decisive is to cultivate basic research in its many facets to arrive at potential practical applications, often in unpredictable ways.

8 Concluding Remarks

The studies summarized in this and other chapters of this book emphasize two growing concepts in the studies of infectious diseases that apply not only to viral infections, but also to bacterial, fungal and parasitic infections, as well as to cancer cells. One is the increasing experimental evidence in support of the remarkable statement by Theodosius Dobzhansky, written three decades ago, that "Nothing in biology makes sense except in the light of evolution" (Dobzhansky 1973). Dealing with evolutionary concepts is not rooted in the tradition of many schools of biochemistry and molecular biology. However, recent developments point to a decisive contribution of evolutionary events as part of the interactions between hosts and infectious agents. Justification of this statement includes the recognition of pathogen variation and adaptability in disease emergence and reemergence (Smolinski et al. 2003), as well as in pathogenesis (this volume and many references included in it). Evolutionary events underlie selection of viruses resistant to antiviral agents, and of bacteria resistant to antibiotics (for example, Welsh 2003). The list could go on for any pathogen whose replication cycles last orders of magnitude less than those of the hosts they infect (see the overview of molecular mechanisms of pathogen adaptation in Domingo 2005a).

A concept that originated in physics and that is gradually penetrating many fields of science (including biology and infectious diseases) is complexity and emergence (Cowan et al. 1994; Solé and Goodwin 2000). What this concept entails is that certain phenomena whose occurrence depends on interactions among many individual influences cannot be accounted for by the sum of

the individual contributions. Disease emergence offers an example: although we know (or strongly suspect with a reasonable basis) that human mobility, demographic changes and urbanization favour human-to-human pathogen transmission, that many agents are potentially zoonotic and that in many areas of the world there is close contact between humans and animals, it was not possible to predict the AIDS epidemic. At most, it can be predicted that because of (at least) thirteen different factors (Smolinski et al. 2003) (each one including, in turn, multiple sub-factors!) it is very likely that in the coming years new infectious diseases will emerge. Can studies on complexity be of use to infectious diseases? Probably, but the way such help can be translated into practical results is hardly graspable at the moment. This is not so, however, with regard to considering evolutionary concepts in designing antiviral strategies, since important successes (for example, highly active antiretroviral treatments to control HIV infections, or the prospects of lethal mutagenesis as an antiviral strategy) have been a consequence of understanding and trying to counter the evolutionary potential of pathogens.

The need of a holistic view of biology with a focus on evolution, emergence and complexity has been emphasized by biologists working in areas other than infectious diseases, such as general evolution and developmental biology (Woese 2004). A point has been reached that encourages a transdisciplinary approach to problems of biological complexity, including viral quasispecies. The challenge is to obtain detailed molecular information provided by current biochemistry and molecular biology and to integrate it with the concepts of complexity and evolution, with the aim of providing a more complete picture of the problems we face.

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