Review

Quasispecies as a matter of fact: Viruses and beyond

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ABSTRACT

We review the origins of the quasispecies concept and its relevance for RNA virus evolution, viral pathogenesis and antiviral treatment strategies. We emphasize a critical point of quasispecies that refers to genome collectivities as the unit of selection, and establish parallels between RNA viruses and some cellular systems such as bacteria and tumor cells. We refer also to tantalizing new observations that suggest quasispecies behavior in prions, perhaps as a result of the same quantum-mechanical indeterminations that underlie protein conformation and error-prone replication in genetic systems. If substantiated, these observations with prions could lead to new research on the structure–function relationship of non-nucleic acid biological molecules.

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1. Introduction: origin of the quasispecies concept

Replication with continuous generation of mutants is a feature shared by all RNA viruses characterized to date and also at least by DNA viruses whose replication is catalyzed by low fidelity DNA-dependent DNA polymerases. Until well into the second half of the XXth century evidence for continuous (rather than occasional) generation of mutants during RNA virus replication was only indirect. For several viruses it was noted that stocks of what should have been standard forms of viruses contained unusual amounts of temperature sensitive (ts) mutants, or that mutant stocks readily reverted towards the wild type phenotypes upon virus passage (several examples were reviewed in Domingo and Holland, 1988; Holland et al., 1982; Horiuchi, 1975).

Also until well into the XXth century, no theoretical treatments that dealt with error-prone replication had been developed. Classical population biology in general did not consider mutation as a prominent mechanism of variation. The discovery of genetic polymorphisms in man and *Drosophila* (Harris, 1966; Hubby and Lewontin, 1966; Lewontin and Hubby, 1966) came as a surprise and led to formulation of mutation-selection equilibrium models to explain polymorphisms, and contributed to the controversy of neutralism versus selectionism. As tools of molecular biology penetrated into population genetics, the evidence of genetic variation became more and more established. As these events developed, virology and virus evolution were ignored by population geneticists. As surprising as it may seem now, in no way replicating systems that unavoidably produced mutants at each round of genome replication were considered as attractive objects to study evolution.

The first mathematical treatment of a replication system with a regular production of error copies of a template molecule was by Manfred Eigen in his much quoted treatise published in 1971 (Eigen, 1971). That such a quantitative treatment was a necessity at the time is evidenced by the fact that Francis Crick suggested to Eigen in a breakfast discussion to undertake the project. Eigen was also inspired by the experimental work of Sol Spiegelman and colleagues on serial transfer experiments of bacteriophage Qβ RNA using purified Qβ replicase. In the transfer experiments Qβ RNA manisfested Darwinian behavior (that is, mutation, competition, and selection) *in vitro* (Mills et al., 1967). Eigen's treatment put together concepts of information theory and Darwinian evolution to propose a theory of the origin of life based on self-organization of macromolecules, their replication and adaptability in what might have been a primitive RNA world (Eigen, 1971). This theory, termed quasispecies theory, was further developed by Eigen and Schuster (1979) and has found an important application in the evolution of RNA viruses (Domingo, 2006; Domingo and Wain-Hobson, 2009; Holland, 2006; Lauring and Andino, 2010).

A point of confluence between quasispecies theory and RNA virus populations is that quasispecies theory proposes that primitive RNA or RNA-like replicons consisted of mutant distributions (mutant spectra or mutant clouds), and this is the type of population structure displayed by present day RNA genomes in general. Populations of RNA viruses were soon shown to be composed also of mutant distributions as a consequence of high mutation and replication rates, exactly following the pattern of error-prone replication that Crick had encouraged Eigen to pursue mathematically. The initial evidence of quasispecies behavior of an RNA virus was obtained with bacteriophage Qβ replicating in its host *Escherichia coli*, that exhibited high mutation rates and the typical competition and selection dynamics predicted by quasispecies theory (Batschelet et al., 1976; Domingo et al., 1976, 1978). When Eigen learned about the experimental results with bacteriophage Qβ he did not doubt that they represented an experimental confirmation of his theory (for historical accounts of the introduction

of quasispecies in virology, see Domingo et al., 2001; Domingo and Holland, 1988; Domingo and Wain-Hobson, 2009; Holland, 2006; Holland et al., 1982; Perales et al., 2010).

Quasispecies was formulated as a deterministic theory that assumed populations of replicons of infinite size, in equilibrium. The fact that viral populations are of finite size and rarely in a true population equilibrium does not imply that quasispecies is not a good theory to represent virus populations. Indeed, the critical concept of a population consisting of a mutant spectrum was represented in the deterministic derivation. Furthermore, extensions of quasispecies theory to finite populations of replicons under non-equilibrium conditions (that is, under variable fitness landscapes, see Section 5 on *Fitness*) have been derived (Eigen, 2000; Park et al., 2010; Saakian et al., 2006, 2009; Saakian and Hu, 2006; Schuster, 2010; Wilke et al., 2001). To develop first a deterministic theory in mathematically solvable terms, and then introduce stochastic components in it, is a usual procedure in theoretical biology. In fact the initial deterministic formulation of quasispecies was intended to framework the self-organization of primitive replicons, that one cannot easily justify as being in equilibrium when they occurred some 4000 million years ago (Eigen, 1992).

Quasispecies is not the only theoretical treatment of Darwinian behavior but the most adequate when high mutation rates characterize the replication system (Page and Nowak, 2002). It has been emphasized that there is no conflict between quasispecies and the concept of selection-mutation equilibrium (often termed Wright–Fisher formulation) (Musso, 2011; Wilke, 2005). It remains a bit of a mystery why quasispecies rather than other approaches of population genetics was so successful to penetrate into the study of viruses at the population level. Among several possibilities that have been previously outlined (Perales et al., 2010), we think that the main reason is the direct experimental evidence that RNA viruses replicate as mutant spectra, a fact that found its best conceptualization in quasispecies theory. Also, the presence of a mutant spectrum established an immediate connection with problems of viral disease control. Indeed a mutant spectrum readily explained the high frequency of escape mutants to many selective constraints intended to inhibit viral replication (Domingo, 1989; Domingo and Holland, 1992). The connection with control of virus disease was more distant and less intuitive with other theoretical treatments of population genetics.

It has been also previously commented how inadequate (due to an implied oversimplification) it is to refer to components of a mutant spectrum as alleles, and to a quasispecies structure as genetic polymorphism, at least if we adhere to the original definitions of allele and polymorphisms in genetics (Domingo et al., 2001; Perales et al., 2010; Spiess, 1977). Terminology might be regarded as secondary provided the type of population structure of viruses to be confronted is well understood (Perales et al., 2010). Virologists presently use the term quasispecies to mean distributions of non-identical but related genomes subjected to a continuous process of genetic variation, competition, and selection, and which act as a unit of selection (Domingo, 2006; Perales et al., 2010).

Following the Qβ work, quasispecies dynamics was documented with foot-and-mouth disease virus in cell culture and *in vivo* (Domingo et al., 1980; Sobrino et al., 1983), including the concept that antigenic heterogeneity was a consequence of genetic heterogeneity (Mateu et al., 1987, 1988, 1989; review in Domingo et al., 2003). In parallel, Holland and his colleagues interpreted the competition-selection dynamics between vesicular stomatitis virus (VSV) and their corresponding defective-interfering (DI) particles in the light of high mutation rates and quasispecies dynamics (Holland, 1984; Holland et al., 1982; Semler and Holland, 1979; Spindler et al., 1982). The studies with VSV established important connections between classical concepts of population genetics (the Red Queen hypothesis, the competitive exclusion principle, fitness

variations, etc.) and RNA virus evolutionary dynamics (reviewed in Novella, 2003).

In the present article, we first describe experimental approaches to the study of viral evolution, then we summarize some of the most striking behaviors described for RNA viruses in cell culture, and the connection of findings with observations *in vivo*. Finally we explore the application of quasispecies to non-viral systems.

2. Experimental approaches to virus evolution

2.1. The experiment as an answer or as a question

An interesting feature derived from the rapid diversification of RNA viruses is the production of diverse, and often unusual collections of phenotypes (not only genotypes) during cell culture experiments in a time-scale observable in the laboratory. Several research groups have made use of this property of RNA virus biology to learn about evolution by means of two main approaches. The first approach aims at describing the molecular mechanisms underlying viral replication and variation, and their consequences for virus adaptability. The virus itself is the object of the investigation. In a second approach, RNA viruses are considered model organisms, and are used to test classical evolutionary theories, mainly those that originated in population genetics, and that would benefit from an experimental validation. In the first approach, the experiment itself is the question, and the observed viral response would provide the answer. The second approach starts with a predefined question or hypothesis and the experiment provides the answer. Both approaches are complementary, and have provided new insights in virus evolution and in general evolution, although in the present review we will focus mainly on the first experimental approach.

2.2. The experiment as a question

Variability of RNA virus populations underlies some of the major problems related to their pathogenic potential. The width of the mutant spectrum has been associated with severity of hepatitis C virus (HCV)-associated disease, and response to antiviral treatment (Domingo and Gomez, 2007; Farci et al., 2000; Le Guen et al., 1997; Lopez-Labrador et al., 1999; Mas et al., 2004; Morishima et al., 2006; Qin et al., 2005; Rothman et al., 2005), the progression of human immunodeficieny virus (HIV) infection to AIDS (Nowak et al., 1991), or with poliovirus neurovirulence (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). Quasispecies diversity ensues from the action of error-prone polymerases, recombination events, genome segment reasortment, complementation, and a number of other molecular mechanisms (Domingo et al., 2006). Research on the impact of mutation rates and population heterogeneity on virus adaptability and survival should benefit available polymerase fidelity mutants of picornaviruses (Pfeiffer and Kirkegaard, 2005; Vignuzzi and Andino, 2010; Vignuzzi et al., 2006) and coronaviruses with either an active or inactive proofreading-repair activity (Denison et al., 2011; Eckerle et al., 2007, 2010; Levi et al., 2010). Because RNA viruses typically produce large numbers of progeny in relatively short time periods, the diversity generated by those mechanisms is explosively amplified leading to a competition between multitude of different variants and the selection of some of them in the classical Darwinian way. It must be stressed that an invariant consensus sequence does not mean absence of mutations. Rather, it means that the mutations that occur at the usual rate of 10^{-3} to 10^{-5} substitutions per nucleotide copied (Batschelet et al., 1976; Drake and Holland, 1999; Sanjuan et al., 2010) when components of the mutant spectrum replicate, lead to the same consensus. Quasispecies populations are thus complex systems with a tremendous capacity to evolve, and our knowledge of these adaptative mechanisms is still fragmentary (Manrubia et al., 2005).

Although classical theories may constitute a good starting point to study virus evolution, a predefined hypothesis that may operate during the evolution of DNA organisms, may not apply to more complex systems such as RNA virus populations. It is actually not unusual that established concepts of DNA evolution fail to match the results obtained from experiments with RNA viruses (Froissart et al., 2010; Holland et al., 1982; Sacristan et al., 2005). New theories built upon experimental observations are needed to complete the whole picture of RNA virus evolution. Research in our laboratory has often involved repeated passages of virus clones in cell culture (Fig. 1), a simple protocol which favors competition among variants, and the rescuing of interesting phenotypes, impossible to predict *a priori*. By using appropriate and contrasting passage regimes, combined with molecular biology techniques, in some cases it has been possible to find the mechanisms leading to unusual phenotypes.

2.3. Dynamics of fitness variation

Viral fitness has been typically treated as a relative parameter that quantifies the replication capacity of a virus relative to some reference isolate, in a given environment (Domingo and Holland, 1997; Martinez-Picado and Martinez, 2008; Quiñones-Mateu and Arts, 2006). Fitness is usually measured in a competition between the virus to be tested and a reference variant in cell culture or *in vivo*, and its value has been equated with that of a selection coefficient (Maree et al., 2000). It is important to note that fitness values are relative and environment-dependent. Fitness of a HIV-1 variant determined by growth-competition experiments in PBMCs does not necessarily reflect the fitness that the same isolate will have in an infected patient. Fitness of viruses *in vivo* is a determinant of viral load and the latter is often one of the correlates of disease severity (Mellors et al., 1996; Mueller et al., 2008; Srikiatkhachorn and Green, 2010). Thus, despite the complications encountered, fitness determinations *in vivo* could help predicting the disease potential of viruses (Carrillo et al., 1998). Even in the course of fitness determinations in cell culture, a mutant cloud is generated around the clonal population used for the assay. This introduces an additional indetermination regarding the behavior of the competing populations (Domingo and Holland, 1997).

One of the first observations derived from passaging viruses sequentially in cell culture was that when a virus was allowed to replicate at high multiplicity of infection (MOI) (high viral inoculum), the whole population tended to increase its fitness in an exponential manner (Escarmís et al., 1999; Novella et al., 1995). In contrast, when viruses were passaged in a regimen consisting of sequential bottleneck events, the fitness of the virus dropped drastically (Fig. 1). Fitness loss of asexual populations was already predicted by Muller (1964) and first confirmed experimentally with bacteriophage Φ6 (Chao, 1990).

In detailed experiments carried out with foot-and-mouth disease virus (FMDV), several viral clones were extensively passaged in series of up to 300 bottleneck, or plaque-to-plaque transfers (Escarmís et al., 1996, 1999, 2002, 2008, 2009). The virus fitness decreased rapidly during the first passages, but afterwards the virus fitness oscillated around a constant average value during roughly 300 passages. Several unusual mutations and extreme phenotypes were obtained upon subjecting FMDV clones to hundreds of plaque-to-plaque transfers. It is worth mentioning the evolution towards non-cytocidal forms of FMDV that could establish a persistent infection in cell culture (Escarmís et al., 2008), without an initial phase of cytopathology. The latter was necessary for the wild type FMDV to establish persistence in a few surviving cells (de la Torre et al., 1985; Herrera et al., 2008), but not for the evolved, non-cytopathic clones. In each plaque-to-plaque transfer a single infectious genome from
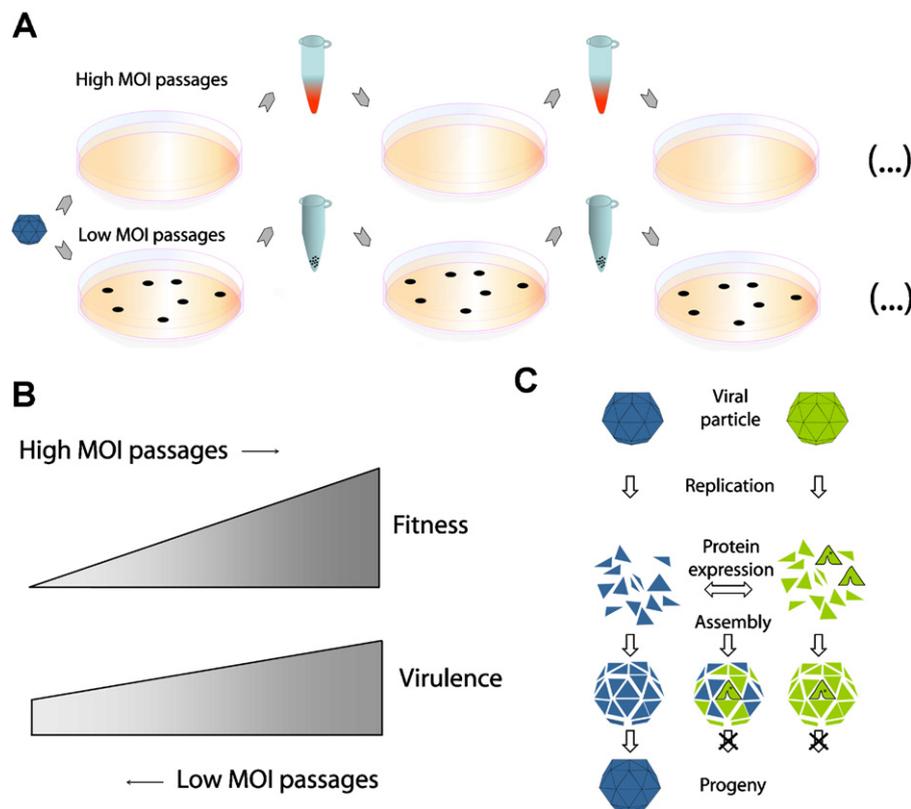
Fig. 1. (A) Schematic representation of sequential viral infections in cell culture. High MOI infections are carried out with undiluted samples from the supernatant of the previous infection. Low MOI or plaque-to-plaque transfers are carried out by diluting virus from individual lysis plaques, and transferring the virus to the next plate. (B) Fitness and virulence variations in viral populations. Serial passages of a virus involving a large population size per passage result in increased replication capacity and fitness gain (triangle). In contrast, repeated bottleneck events lead to fitness decrease. This part of the scheme is based on studies in several laboratories discussed in the text. In a comparative study of the evolution of virulence values of foot-and-mouth disease clones subjected to bottleneck events, virulence (trapezium) did not follow the same trajectory than fitness, and the molecular basis of the different trajectories was elucidated (Herrera et al., 2007). However, the relationship between fitness and virulence can be more complex, and its discussion is beyond the scope of the present article (see text for the influence of quasispecies organization as a determinant of virulence in cell culture, Ojosnegros et al., 2010a). (C) Schematic representation of a model of viral molecular interference. The viral particle depicted in green has generated progeny some of which carry a lethal (or highly disadvantageous) mutation (*), and the corresponding altered protein can be shared among its different competing mutants. Viruses incorporating this (or other) "toxic" proteins will have their fitness affected even if the mutation is not present in their genome.

the mutant spectrum is amplified to generate the virus populations in the ensuing plaque. Thus, the multiple and extreme genotypes and phenotypes identified in the studies on bottleneck passages of FMDV testify the incredible diversification potential of RNA viruses (Fig. 1).

Bottleneck events are probably abundant during the life cycle of viruses in their infected hosts (Ali et al., 2006; Betancourt et al., 2008; Domingo et al., 2008; Escarmís et al., 2006; Foy et al., 2004; Haaland et al., 2009; Li and Roossinck, 2004; Quer et al., 2005, 2008; Scholle et al., 2004; Smith et al., 2008). Given what we know now of the complexity of mutant spectra, it is necessary to further investigate the consequences of bottleneck events of different intensities when they occur within infected host organisms. However, bottlenecks are not the only mechanisms to unveil altered phenotypes. Equally rich mutant spectra are generated upon large population transfers, sometimes with striking consequences, as discussed next.

3. Interactions within mutant spectra

One of the most unexpected but also attractive findings in recent years has been that mutant spectra often do not act as independent collections of mutants, but rather as interacting sets of variant viruses subjected to group selection. These observations not only reinforce the concept of quasispecies as the unit of selection but also offer prospects of new research avenues in which the behavior

of viruses is dictated not by individuals but by connected sets of individuals. Here we underline some of these observations.

3.1. The competition-colonization trade off

The RNA virus literature includes several instances of the influence of the multiplicity of infection (MOI) on the evolution of a virus. Viruses adapted to replicate at high MOI will outcompete viruses adapted at low MOI when the competition takes place under high MOI conditions. The opposite is true in competitions under low MOI conditions (Sevilla et al., 1998; Turner and Chao, 1999). This kind of density-dependent selection suggests that RNA viral genomes may behave differently when they are rare or when they are abundant in a population (Novella et al., 2004). Since any tissue or cell culture is a discrete resource (that is, composed of individual cells), the viruses can adapt to compete either for the whole cell pool or locally within a single cell. When different viruses coinfect the same cell, a local competition is established. The replication of a variant of the population can interfere with the replication of another one, even if both variants are close in sequence space (the two genomes differ in a limited number of mutations). The degree of interference exerted can range from a simple competition for the resources with coexistence of the two competitors, to an almost complete abolition of replication of one of the competitors, as in the case of some defective viruses (Holland, 1990; Roux et al., 1991). The rationale for the existence of interference among viral mutants

is that viruses share, with a variable degree, their genetic products and cell resources in coinfected cells. When a dominant negative mutant arises (also referred to as defector or cheater) it finds the suitable molecular environment to progress.

Replication-interference mechanisms have been described in a range of different DNA and RNA viruses. Among other phenotypes, these processes include the suppression of high fitness viruses by lower fitness mutants documented with VSV (de la Torre and Holland, 1990), density-dependent selection of VSV (Novella et al., 2004), the snow drift type of replication of RNA phage Φ6 (Turner and Chao, 1999, 2003), the antigenic exchange between coinfecting mutants called phenotypic hiding, documented for VSV and influenza virus (Holland et al., 1989; Valcarcel and Ortin, 1989), suppression of virulent poliovirus by its corresponding attenuated variants (Chumakov et al., 1991) or suppression of the growth hormone deficiency syndrome induced by some strains of lymphocytic choriomeningitis virus (LCMV) by disease-negative LCMV variants (Teng et al., 1996), and finally, an innumerable list of defective virus types (Holland, 1990).

A recent study has suggested that the difference between the local competition in coinfected cells and the competition for the whole cell pool, can lead to the diversification of a single, purified FMDV clone into competitor and colonizer phenotypes (Ojosnegros et al., 2010a). Colonizers are virulent strains adapted to replicate faster and spread faster through the cell culture. At low MOI, colonizers were selected because they quickly took over the unoccupied culture (susceptible cells). At high MOI, at which local competition in multiply infected cells is frequent, competitors succeeded because of their higher interference capacity or intracellular fitness (Ojosnegros et al., 2010b). The selection of either type followed a dynamic density-dependent selection, a highly non-linear process, in agreement with previous observations on the lack of direct correlation between fitness and virulence (Herrera et al., 2007) (Fig. 1). These studies stress the importance of confections and the interaction among components of mutant spectra in shaping the virulence of viral populations. Natural variation of virulence *in vivo* may partly stem from interactions among viral subpopulations, a possibility which remains largely unexplored. An extension of the competition-colonization model simulates continuous infections, the scenario of real infections where susceptible cells are replenished instead of being exhausted as in cell culture systems. The model stresses, in a quantifiable manner, that the realistic values of variable MOI taking place in an infection can account for a high degree of interaction and even attenuation of viral infections (Delgado-Eckert et al., 2011).

3.2. Extreme case of complementation

Following our preferred approach, simply by allowing an FMDV clone to replicate serially in cell culture at high MOI, we described the evolution of the virus towards a population dominated by defective genomes that were infectious by complementation, a remarkable evolutionary transition (Garcia-Arriaza et al., 2004, 2006). The viral population resembled segmented multipartite viruses (i.e. brome mosaic virus) where each segment is packaged in a single capsid. After performing a battery of experiments involving the analyses of different steps of the virus life cycle, our observations indicated that particles harbouring smaller genomes were more stable and had longer life spans than particles with the full length genome (Ojosnegros et al., 2011). The segmented genomes became dominant in the population spontaneously without any genome engineering or experimental intervention. Increasing evidence from different virus families indicates that viral genomes are tightly packaged in their capsid shells (Gelbart and Knobler, 2009), and that capsid density may be inversely related to the stability of the particle (De Paepe and Taddei, 2006). In agreement with these

mechanistic studies, our results provide an experimental proof for the potential evolutionary benefit of increasing particle stability which represents a completely new theory for the evolution of segmented genomes (Ojosnegros et al., 2011).

The study of the segmented population of FMDV led to the design of an immunization protocol using the segmented viruses as vaccines that share features of inactivated and live-attenuated vaccines (Rodriguez-Calvo et al., 2010). The requirement of complementation for replication renders the infection self-limiting. The inoculation point may have the necessary high MOI conditions for the different segments to complement. However, after dilution through diffusion in cells next to the inoculation site and then in the blood, the probability of two particles hitting the same cell drops drastically (Manrubia et al., 2006). The preliminary vaccination tests included immunization of mice susceptible to FMDV infection and achieved 100% protection against lethal doses of the wild type virus. The immunization of swine, the FMDV natural host, induced high titers of both FMDV-specific neutralizing antibodies and activated FMDV-specific T cells in swine which correlated with solid protection against FMDV (Rodriguez-Calvo et al., 2010). Thus, virus evolution may inspire new approaches to vaccine design. This statement applies to antiviral drugs too.

4. Quasispecies and antiviral therapy

To underline concepts discussed in Section 2, genetic variability and population dynamics of highly variable RNA viruses are one of the main obstacles for prevention and control of the diseases associated with them (Domingo, 1989; Figlerowicz et al., 2003). Quasispecies dynamics of viral populations, that results in virus escaping the inhibitory activity of antiviral inhibitors due to the selection of drug-resistant viral mutants, is one of the key issues that has to be taken into account for the planning of antiviral strategies. The systematic selection of drug-escape mutants has promoted research on lethal mutagenesis as an antiviral strategy whose objective is to extinguish virus through an increase in mutation rate, thereby counteracting the adaptive capacity of RNA viruses. Several studies in cell culture and *in vivo* have supported lethal mutagenesis as a viable antiviral strategy (Anderson et al., 2004; Domingo, 2005; Eigen, 2002; Graci and Cameron, 2008), and a clinical trial in which a mutagenic pyrimidine analogue was administered to AIDS patients has been recently reported (Mullins et al., 2011).

A better understanding of the molecular basis of viral replication and of lethal mutagenesis should help unveiling new therapeutic targets both for mutagenic and non-mutagenic agents. An essential feature of quasispecies dynamics is that the quasispecies as a whole, rather than an individual genome, is the target of selection. Quasispecies represents the recognition of complex behavior of viruses having important implications for RNA virus adaptability and their pathogenic potential. Quasispecies complexity and composition may affect the response to antiviral treatments.

The behavior of mutant spectra as an ensemble is influenced by complementation and interference that are exerted among its individual components. Interference is believed to be exerted mainly through altered gene products that produce non-functional protein complexes during the virus life cycle (reviewed in Domingo, 2006; see also Section 3). Precisely the mastering of these negative interactions may determine the success of lethal mutagenesis as an antiviral therapy, since an excess of negative interfering interactions is one of the mechanisms likely to contribute to virus extinction by lethal mutagenesis (González-López et al., 2004; Grande-Pérez et al., 2005b; Iranzo and Manrubia, 2009; Perales et al., 2007). These studies demonstrated that in the transition towards extinction, mutagenized, preextinction FMDV populations

(corresponding to virus from the passage that precedes the one in which the virus goes extinct) interfered with the infectivity of standard FMDV RNA, resulting in a decrease in the production of progeny virus (González-López et al., 2004). It was also shown that the decrease of virus infectivity preceded the decrease of viral RNA in the infected cells, suggesting an increased concentration of defective genomes in the mutagenized population (Grande-Pérez et al., 2005b). This observation is in agreement with the decrease in specific infectivity (the ratio of PFU to the number of viral RNA genomes) generally observed in the transition of viruses towards extinction (Domingo et al., 2008). Interference by specific FMDV mutants was further documented in RNA coelectroporation experiments with wild type and capsid or polymerase FMDV mutants (Perales et al., 2007). The results showed that an excess of several replication-competent mutants caused a strong and specific interference on FMDV replication. Moreover, mixtures of some capsid and polymerase mutants evoke a very strong, synergistic interference, supporting the notion that, as the proportion of mutant genomes increases as a result of mutagenesis, interfering mutant genomes can contribute to virus extinction. The results evidenced also that a single amino acid substitution can be responsible for rendering a non-interfering genome interfering, and vice versa (interfering and non-interfering genomes can be neighbors in sequence space). Thus, negative interactions (interference) among components of a mutant spectrum (through *trans*-acting, altered gene products) may be attained through modest increases in mutational load (Grande-Pérez et al., 2005b; Ojosnegros et al., 2008), and may participate in virus extinction, in support of the lethal defection model of extinction. *Trans*-acting mutants of poliovirus were shown to delay replication of drug-resistant poliovirus mutants (Crowder and Kirkegaard, 2005; Spagnolo et al., 2010) (Fig. 1).

The presence of the nucleoside analogue ribavirin during viral replication enhanced the interfering activity of some specific FMDV mutants, presumably because ribavirin may contribute to the generation of additional defector genomes. In contrast, the presence of an inhibitor of the viral replication such as guanidine hydrochloride (GU) suppressed the interference exerted by the defectors mutants (Perales et al., 2009b). This important point is still under investigation, but current evidence suggest that these events are probably influenced by the dynamics of mutagenesis versus inhibition during viral genome replication, and also because GU impeded the replication of defector genomes, and diminished their interfering activity. Thus, current evidence suggests that interfering activity is important to drive the population towards extinction.

The administration of virus-specific mutagenic base or nucleoside analogues requires careful consideration of protocols when antiviral inhibitors are coadministered with the mutagenic agents. To optimize antiviral protocols that take into account these requirements, different treatment protocols were compared. FMDV was extinguished by the combined (simultaneous) or sequential administration of mutagenic agents and antiviral inhibitors, and extinction occurred with a decrease of the specific infectivity of the virus, and without alteration of the consensus sequence of the population (Domingo et al., 2008; González-López et al., 2005; Grande-Pérez et al., 2005a). However, our recent results suggest that when a mutagenic agent is involved in an antiviral treatment, a sequential administration first of the inhibitor and then of the mutagen may be more effective than the corresponding combination treatment. The advantage of such sequential therapy has been supported by experiments in cell culture and by a theoretical model for the evolution of a viral population under the action of increased mutagenesis in the presence of an inhibitor of viral replication (Perales et al., 2009b). The understanding of the interplay between mutagenesis of the viral genome and inhibition of viral replication is needed for the design of treatment protocols aimed at reducing the viral load or eliminating the virus from infected cells.

Viral population size influences the selection of inhibitor-escape mutants. The administration of a non-mutagenic inhibitor first in a sequential therapy may result in a sufficiently low viral replicative load that may prevent the selection of inhibitor-escape mutants (Perales et al., 2011a). Then, the subsequent administration of a mutagenic agent may generate defector mutants that interfere with the infectivity of wild type virus, and lead to the collapse of the replicative system. In contrast, in a combination therapy the simultaneous administration of both drugs increases the probability of selection of inhibitor-escape mutants due to the mutagen-induced error rate. Additionally, interfering mutants generated by the mutagen that contribute to extinction, may not be active in the presence of an inhibitor (Perales et al., 2009b).

Recently, the theory underlying the interaction between a mutagenic agent and antiviral inhibitor has been developed in connection with the relative advantage of sequential versus combination antiviral treatments (Iranzo et al., in press). *In silico* simulations can predict the response of a viral population to different doses of inhibitor and mutagens, and how to minimize the probability of appearance of resistant mutants. Drug doses largely determine the suitability of a sequential versus combined administration of a mutagen and an inhibitor. Application of this new theoretical model may reduce the number of experiments prior to the implementation of a treatment protocol based on lethal mutagenesis (Iranzo et al., in press).

4.1. Drug-resistant viral mutants

The rapid emergence of drug-resistant viral mutants is a general observation, to the point that such a selection is considered evidence of the specificity of the antiviral drug. The ease of selection of many resistant variants originates in quasispecies dynamics and it is favored by the pre-existence of the relevant mutants in the corresponding mutant spectra (Nájera et al., 1994, 1995, and many other examples documented since then).

Mutagenic agents are not an exception and selection of mutagen-resistant viruses may impede the extinction of the viral population. RNA viruses display different mechanisms of resistance to mutagenic nucleotide analogues as most clearly evidenced with resistance to ribavirin (Agudo et al., 2010; Arias et al., 2008; Castro et al., 2005; Pfeiffer and Kirkegaard, 2003; Sierra et al., 2007; Vignuzzi and Andino, 2010; Vignuzzi et al., 2006). When dominant in a population, a mutation that confers partial resistance to a mutagenic agent can jeopardize virus extinction by elevated doses of the same mutagen. However, we have demonstrated that a mutagen-resistant variant could still be driven towards extinction by a combination treatment involving another mutagen and a non-mutagenic antiviral inhibitor (Perales et al., 2009a). Thus, there are positive prospects that alternative inhibitors and mutagenic agents used sequentially or in combination could be used in clinical trials, following the footsteps of Loeb, Mullins and colleagues (Mullins et al., 2011).

4.2. Diving into mutant spectra in search of minority genomes

Appropriate methods to quantify the variability of RNA viruses include analyzing mutant spectrum by molecular cloning and sequencing, and more recently by using new molecular approaches such as ultra deep nucleotide sequencing (Domingo, 2006; Domingo and Wain-Hobson, 2009; Garcia-Arriaza et al., 2007; Lauring and Andino, 2011; Mardis, 2008; Mas et al., 2010; Perales et al., 2010; Webster et al., 2009; Wright et al., 2011; Zagordi et al., 2010). Despite a number of relevant publications that have used ultra deep sequencing for quasispecies analysis (reviewed in Djikeng and Spiro, 2009; Perales et al., 2010) it is important to note that appropriate correcting algorithms must be used to

exclude artefactual mutations due to the amplification and analytical procedures (Eriksson et al., 2008; Zagordi et al., 2010). When corrections are applied, accurate descriptions of mutant spectra can be achieved, and they are currently confirming the genetic complexity of viral populations *in vivo*.

Ultra deep sequencing analysis has enabled the identification of low-frequency mutations present at baseline (preexisting mutants), before drug exposure that confer resistance to protease inhibitors for HCV (Verbinnen et al., 2010), among other examples. A critical issue now is whether an application of ultra deep sequencing will become cost-effective regarding treatment planning.

Other procedures have been developed to penetrate into the composition of mutant spectra. Differential PCR amplifications have been developed for the detection and quantification of minority viral subpopulations (Garcia-Arriaza et al., 2007). Heavily mutagenized minority components generated as a result of R mutagenesis have been analyzed by differential DNA denaturation PCR (3D-PCR) that permits the selective amplification of AU-rich hypermutants produced by R mutagenesis, by modulating the PCR denaturation temperature (Suspène et al., 2005). The analysis of an A,U-rich portion of the genomic sequence space unveiled a deep mutant spectrum complexity not only of FMDV populations passaged in the presence of R, but also in its absence reinforcing the concept that the virus replicates close to an error threshold for maintenance of genetic information (Perales et al., in press). Interestingly, the major effect of R mutagenesis was to accelerate the occurrence of A,U-rich mutant clouds during the early replication rounds of the virus. A previously characterized R-resistance polymerase mutation (P44S) (Agudo et al., 2010) was present in the mutant spectrum of an FMDV not treated with ribavirin. This result in cell culture extends previous evidence of the presence of inhibitor-resistance mutations in natural viral isolates infecting patients who had never been treated with the corresponding inhibitors (Section 4.1). The substitution that confers resistance to ribavirin increased in frequency as the population was confronted with R, and this selective 3D-PCR amplification procedure has revealed additional substitutions as possible candidates to confer resistance to the drug. The application of differential amplifications have opened the possibility to characterize molecularly new defector subpopulations of RNA viruses that contribute to virus extinction by lethal mutagenesis, as well as to detect the presence of hypermutated genomes, that might occur in the transition into error catastrophe (Perales et al., in press).

5. Fitness of individuals, fitness of groups of individuals. Cell collectivities as a model

Most of the events that preside quasispecies dynamics can be traced to selection of viral subpopulations, that acquire a selective advantage as a result of an environmental change, or to subpopulations that separate from their ensemble as a result of a bottleneck event (Fig. 1). In addition, several features of viral quasispecies support the concept of a mutant ensemble being the target of selection in viruses. In our laboratory, this concept was tested experimentally by reconstructing a mutant distribution using nineteen antigenic variants of FMDV. The results showed that an ample representation of variants (and not an individual genome) was selected by a specific antibody, underlying the behavior of the quasispecies as an entire collection of mutant viruses (Perales et al., 2005). The relevance of the composition of the mutant spectrum was also documented with the presence of a molecular memory in quasispecies, which means the maintenance as minority components of genomes which are closely related to those that were dominant at an earlier phase of the same evolutionary lineage (Briones and Domingo, 2008; Ruiz-Jarabo et al., 2000). Memory confers preparedness to

a viral population to respond to a selective constraint previously experienced by the same viral population.

As discussed in Section 3, the implication of quasispecies theory that an ensemble is the target of selection (Codoner et al., 2006; Eigen, 1971; Eigen and Biebricher, 1988; Eigen and Schuster, 1979; Schuster, 2010) may be at times realized through complementing or interfering interactions. The question arises whether such quasispecies behavior exists in other biological systems (Mas et al., 2010).

The unit of natural selection is one of the most basic, complex and debated questions of evolutionary biology (Mayr, 1997). According to one of the current views, if the fitness of a group is higher that the arithmetic mean of the fitness values of the composing individuals, then the group as a whole can serve as a unit of selection. The difference in favor of the fitness of a group may be due to the interaction among the individuals that compose the group, to a division of labor, or other social actions (Mayr, 1997). Thus, an interaction among components of a mutant spectrum in a RNA virus quasispecies implies that the individuals must be linked, so that the entire population forms a cooperative structure that evolves as a single unit (Lauring and Andino, 2010). In this context, the social Darwinism model developed by Spencer (1876) was the first to cite the term "superorganism" in relation to a social organization. The term superorganism is often mentioned in relation to eusocial animals, that show a specialized division of labor, and individuals are not able to survive by themselves for extended periods of time (Queller and Strassmann, 2003). In this case, social actions or division of labor could be regarded as analogous to the interaction of the individuals (complementing interactions) described for RNA virus quasispecies (see Sections 3 and 4).

5.1. Collective behavior in bacteria

The tendency of individuals to act in a collective fashion has been increasingly evidenced for microbes generally (Foster, 2011). It is now recognized that bacteria very frequently do not exist as solitary cells, but as colonial organisms that exploit elaborate systems of intercellular communication to facilitate their adaptation to changing environmental conditions. Social behavior related to antibiotic production, virulence, motility or biofilm formation has been extensively described (Rumbaugh et al., 2009). Much of the social behavior of bacteria is regulated by cell-to-cell signals in a cell density-dependent manner known as quorum-sensing (Fuqua et al., 1994; Miller and Bassler, 2001; Nealson and Hastings, 1979). Quorum sensing is based on a two-component signal transduction phosphorelay scheme that allows microorganisms to sense the cell density, and produce a coordinated response as a whole population. Intrapopulation diversity in bacteria may result in higher levels of cooperation and modulation of virulence, as previously described for RNA virus quasispecies (Ojosnegros et al., 2010a,b; Vignuzzi and Andino, 2010; Vignuzzi et al., 2006). In fact, recent studies on the role of quorum sensing during infections support this hypothesis, and show that cooperative interactions can modulate virulence levels in bacteria. It is interesting that in mono-strain infections, the bacteria that do not participate cooperatively in quorum sensing (cheats or defectors as in the lethal defection model of RNA viruses, see Section 4) are significantly less virulent, and are more readily cleared by the immune system or by antibacterial interventions. However, during a mixed infection with a cooperating strain, the cheat has a fitness that exceeds that of the cooperating strain (Rumbaugh et al., 2009). This suggests that during *in vivo* infections, quorum sensing mutants can exploit the products of cooperators. The overall implication is that the spread of cheats within a population can significantly reduce virulence due to a breakdown in cooperation. Therefore, in addition to the infection being less virulent, these mixed infections may be easier to treat

with conventional antimicrobial therapies (Rumbaugh et al., 2009). Lethal mutagenesis of viruses can be viewed also as a cooperation failure, and it is the basis of the new antiviral treatment discussed in Section 4 (Perales et al., 2009b, 2011a).

Biofilm formation has been also viewed as a manifestation of bacterial social behavior. Biofilms are communities of microorganisms that assemble within an extracellular polymeric slime matrix (Costerton et al., 1978). Some examples of biofilms are those formed by *Pseudomonas aeruginosa*, *Legionella* or *Neisseria gonorrea*, or the dental plaque. These organic super-structures have important clinical implications as infectious agents (Costerton et al., 1978, 1987, 1999). The microbial cells growing in a biofilm are highly heterogeneous and physiologically different from the constituent cells when floating or swimming in isolation in a liquid medium (Stewart and Franklin, 2008). In addition, the mechanisms that confer antibiotic resistance in biofilms seem to be different from those based on plasmids, transposons, or mutations that confer innate resistance to individual bacterial cells (Stewart and Costerton, 2001). Different hypotheses for antibiotic resistance of biofilms have been proposed including limited diffusion of the antibiotic within the biofilm, altered chemical microenvironment, or the presence of subpopulations of microorganisms that form a unique and highly protected phenotypic state (Stewart and Costerton, 2001). These multiple resistance mechanisms are not mutually exclusive and therefore, antibiofilm therapies might have to address more than one mechanism simultaneously to be clinically effective. The existence of subpopulations with different and well defined phenotypes inside the biofilm resembles the heterogeneity described for RNA virus populations. Furthermore, biofilms have been also proposed as the target of evolutionary selection (Caldwell and Costerton, 1996), in a clear parallel with viral quasispecies.

One strategy proposed to combat biofilms is based on infection by bacteriophages. However, infection could contribute to biofilm dispersal and selection of morphotypic bacterial variants. It has been recently reported for *P. aeruginosa* and Pf4 bacteriophage that mutant biofilms that lack the phage are less virulent than the wild type (Rice et al., 2009), emphasizing again the highly complex organization of such communities.

5.2. Heterogeneity and group behavior in cancer cells

Genomic mutation rates of RNA viruses are extremely high and do not have a parallel in cells under normal physiological conditions, except in localized functional hypermutations such as in generation of immunoglobulin diversity. However, eukaryotic cells can achieve high levels of heterogeneity by mechanisms other than those used by viruses (Snijder and Pelkmans, 2011; Snijder et al., 2009). Cellular heterogeneity lies at the basis of important clinical disorders such as cancer which can be defined as a genetic disease. Groups of cells display uncontrolled growth, invasion of adjacent tissues, and sometimes metastasis (invasion of distant tissues) (Boveri, 1914; Hanahan and Weinberg, 2011; Hansemann, 1890). To achieve these properties, cancer cells change their genetic information through point mutations in their DNA, chromosomal rearrangements and/or epigenetic modifications (Hanahan and Weinberg, 2011). Mutations in cellular DNA are more frequent in tumor cells than in normal cells. A mutator phenotype of cancer cells could act as an evolutionary motor to increase the probability of finding the most advantageous phenotype for tumor growth and dispersal (Bielas et al., 2006; Loeb, 2001; Nicolson, 1987). The cellular machinery for deamination has been recently linked to this mutator phenotype (Vartanian et al., 2008). Multiple mutations affecting proteins involved in DNA binding, transcriptional regulation, or cellular signaling have been related to cancer (Futreal et al., 2004). They often result either in the gain of an oncogene or the loss of a tumor suppressor.

Identification of mutations involved in cancer development, together with high-throughput sequencing technologies (ultra-deep sequencing) are currently active fields of research and may lead to a better understanding of cancer complexity (Chin et al., 2011), again in sharp parallel with mutant spectrum complexity of viruses (Section 4). High frequency mutations involving oncogenes or tumor suppressors are usually accompanied by a complex combination of low-frequency changes, and these mutations can contribute to the phenotypic traits of cancer cells (Greenman et al., 2007). These facts indicate that cancer behaves as a complex system that displays high heterogeneity and clonal evolution (Deisboeck and Couzin, 2009; Nowell, 1976). The system evolves through mutations and epigenetic changes, following Darwinian principles of competition and selection (Maley and Forrest, 2000; Merlo et al., 2006). Maley et al. (2006) have described that clonal diversity in cancer cells could be used as a factor for predicting progression in an esophageal adenocarcinoma cancer model.

Theoretical studies have correlated genetic instability in cancer cells (Gonzalez-Garcia et al., 2002; Maley and Forrest, 2000) with quasispecies models of minimal replicators (Brumer et al., 2006; Tannenbaum et al., 2006), and with the implementation of the error catastrophe concept in cancer (Fox and Loeb, 2010; Solé and Deisboeck, 2004). All these studies point to the high genetic heterogeneity of tumor cells as the source of adaptation of cancer to overcome the immune response, to become resistant to different anti-cancer drugs, to invade adjacent tissues, and to produce metastasis and invade other organs. According to mathematical models, tumor benefits from a highly stable component: the cancer stem cells (Solé et al., 2008), that might be considered analogous to a master sequence in a viral quasispecies. In this view, tumor would embody two components, a most variable one which explores phenotypes that allow the tumor to grow and persist, and the small but also more robust component of cancer stem cells plays the role of a reservoir of stability. This strategy would work as a life insurance for the tumor, allowing progeny cells to mutate beyond the limits established for normal cell types. The two-component theory of tumor cells is reminiscent of the "pan-genome" in bacterial populations (Mira et al., 2010; Whitaker and Banfield, 2006). While in a given bacterial species all its components share a core component, subrepresentatives of the same species differ in part of their genomic content. In other terms, the genome complement of a bacterial species may be larger than present in the individual strains that constitute the species. As in the case of cancer cells, a pan-genome type of population permits flexibility for adaptation to changing environments. Indeed, horizontal gene transfers can contribute to the construction of a non-core, adaptative portion of the genome while preserving the core component that ensures survival.

The highly diverse population generated during cancer cell replication will allow the cellular ensemble to face diverse environmental challenges, including immune responses and drugs used in chemotherapy. It seems reasonable to anticipate that anti-cancer drugs will fail when administered individually, even if they display high potency (Luo et al., 2009). Great efforts have been assumed from public and private institutions to achieve highly active anti-cancer treatments (HAACT) or orthogonal therapy, the equivalent to highly active antiretroviral therapy (HAART) used in HIV-1 therapy (Luo et al., 2009). As in the case of variable RNA viruses, cancer should be treated with therapies able to overcome the selection of drug-resistant cells. In a clear parallel with the treatment of HIV-1, sequential administration of anti-cancer compounds will most likely result in treatment failure (Luo et al., 2009). Cancer therapies are considered orthogonal, and therefore they act synergistically when their anti-cancer activity is exerted in at least two different ways, such that a suppressor mutation of the first therapy cannot affect the second therapy, and vice versa. Because cancer is a

compilation of very different diseases (in the sense of triggering factors and resulting cellular phenotypes) the orthogonal therapy will vary depending upon the tumor genotype and possibly the genotype of the patient. Furthermore, because cancer therapies often imply DNA damage, they may increase the rate of emergence of tumor suppressor mutators. As an additional parallelism with RNA viruses, lethal mutagenesis has been proposed as a novel therapeutic approach for the treatment of solid tumors (Fox and Loeb, 2010).

We have extended the discussion of collective behavior of bacteria and cancer cells to illustrate the broad implications of quasispecies theory regarding the concept of unit of selection. As surprising as it may appear, even non-genetic systems may display collective, Darwinian behavior, as suggested by some recent results with prions (only-protein pathogenic agents) which are summarized next.

6. Molecular quasispecies again. The case of prions

Quasispecies theory was developed as a principle of self-organization of early life forms (Section 1) and it has since been associated with nucleic acids as conveyers of genetic information. However, other non-genetic macromolecular systems can also display heterogeneity regarding features that are key to their biological function. Recent developments in prion diseases offer a striking example. Prions are infectious agents composed only of protein, and are the causative agents of scrapie and other neurodegenerative diseases, as first proposed by Alper and Griffith (Alper et al., 1967; Griffith, 1967), and then confirmed in the seminal work by Prusiner and colleagues (Bolton et al., 1982; Prusiner, 1982). Prions propagate by transmitting a misfolded protein state of a cell-coded protein. The protein-only hypothesis of prion propagation was controversial until accumulation of evidence that included the description of prions generated in vitro (Castilla et al., 2005), as well as their propagation and further characterization in cell-free environments (Barria et al., 2009; Castilla et al., 2008), has basically settled the issue.

The recent discovery of fungal prions not associated with disease suggests that prions could constitute a new and widespread regulatory mechanism maintained through evolution (Jarosz et al., 2010; Tuite and Serio, 2010; Tyedmers et al., 2008). In parallel with viral quasispecies, prions cloned by end point dilution in cell culture can gradually become heterogeneous by accumulating protein-folding "mutants" (Li et al., 2010). Furthermore, selective pressures resulted in the emergence of variants, including drug-resistant mutants (Ghaemmaghami et al., 2009; Li et al., 2010; Mahal et al., 2010). These results suggest that not only nucleic-acid base systems can show high population heterogeneity, and can experience selective events. A protein defined by a primary structure can be folded in different ways, each one associated with a different phenotype that can be selected and further propagated. In striking similarity with nucleic acid-based replicative ensembles, prion populations show high population size and conformation heterogeneity, and recent results suggest that such heterogeneity may underlie selection and propagation capacity, a typical Darwinian behavior. How a population of this type evolves either as a sum of its components or only as molecular individualities is still largely unknown (Straub and Thirumalai, 2011).

It may seem surprising that a non-replicative feature such as protein conformation may display quasispecies behavior, the application of a theory intended to address genetic systems. However, this is not that unexpected when the ultimate molecular basis of error-prone replication of nucleic acids is considered. Mutations are the consequence of elementary events (molecular fluctuations) subjected to quantum mechanical uncertainty (Domingo et al., 1995, 2001; Eigen, 1971). Protein conformation is the final result of multiple amino acid–amino acid interactions themselves subjected to molecular fluctuations such as ionization and ionic interaction or hydrophobic contacts dependent on torsion angles of bonds which are also subjected to thermal fluctuations. Thus, it is not unexpected that a collection of related but non-identical conformations exist in any populations of proteins and that environmental factors may favor some conformations over others. The environment may also dictate the presence of minority conformations at different frequencies. Transitions among related conformation states in prions became apparent because they affect a critical trait from the point of view of human perception: disease capacity. These observations open new prospects of research on the molecular mechanisms of protein aggregation and whether a specific conformation variant can nucleate the conversion of additional representatives of that particular variant to form mutant aggregates (as a discussion of the physico-chemical basis of protein aggregation and current research problems, see Bernacki and Murphy, 2009 and references quoted therein).

7. Concluding remarks

The adaptive capacity of RNA viruses has found a direct interpretation in their populations adhering to quasispecies dynamics. Early objections to quasispecies being adequate to interpret RNA virus behavior, namely that the depth of the mutant spectrum was overestimated due to in vitro RNA amplification errors and that classic genetics was sufficient to explain virus behavior, have been totally dispelled by two lines of evidence. One is the introduction of ultra deep sequencing techniques that have confirmed the great complexity of mutant spectra previously evidenced by Sanger sequencing of biological and molecular clones of viruses. The other is the recognition of internal interactions within quasispecies that render classic Fisher–Wright formulations incomplete. The collective behavior of viruses, cell and macromolecular collectivities has been the main emphasis of this review. How such behavior can be mastered as antiviral, antibacterial, anticancer or antiprion strategies remains a great challenge for the XXIst century, that should ideally be approached by combining experimental and theoretical research with a highly transdisciplinary flavor.

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