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# Viral Quasispecies Evolution

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## INTRODUCTION

Viral quasispecies evolution refers to the fact that RNA viral populations consist of mutant spectra (or mutant clouds) rather than genomes with the same nucleotide sequence. Mutant spectra and not individual genomes are the target of evolutionary events. Quasispecies evolution is decisively influenced by high mutation rates (rate of nucleotide misincorporation per nucleotide copied) during viral replication and in some cases also by molecular recombination and genome segment reassortment. Mutation rates are such that it is unlikely to produce inside any infected cell a progeny viral RNA molecule identical to its immediate parental template. Viral genomic sequences would rapidly expand in sequence space and lose biological information were it not for continuous elimination of unfit genomes, a process known as negative selection. Mutant spectra are the source of virus adaptability because they constitute dynamic (continuously changing) repositories of genotypic and phenotypic viral variants.

Major events in the biology of RNA viruses, such as their capacity to change their cell tropism or host range or to overcome internal or external selective constraints (immune responses, antiviral agents, etc.), have their origin in the repertoire of variants present and arising in mutant spectra. Major difficulties for disease prevention and treatment stem from quasispecies dynamics, and we examine strategies that have been proposed to overcome the adaptive potential of RNA viruses. Mutant clouds are not mere aggregates of independently acting mutants. Rather, internal interactions of cooperativity or interference can be established among components of a mutant spectrum, mainly through their expression products. As a consequence of such interactions, an

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ensemble of mutants (not an individual mutant) can frequently determine the biological behavior of a viral population. Recognition of intraquasispecies interactions has influenced research on an antiviral strategy that aims at extinguishing viruses through intensification of negative intrapopulation interactions, which may contribute to deterioration of viral functions. This new strategy is termed lethal mutagenesis, and it is gradually finding its way toward a clinical application.

This review is centered on the principles of viral quasispecies and their relevance for the behavior of viruses, with emphasis on medical implications. Field observations and experiments in cell culture and *in vivo* are reviewed and discussed, with the main objective of establishing concepts relevant to the understanding of viruses that display error-prone replication. We address the quasispecies-derived mechanisms that mediate adaptability for persistence, both within individual hosts and also at the host population level. Highly variable RNA viruses are among the most important human, animal, and plant pathogens, and the penultimate section covers quasispecies dynamics for three salient human pathogens: human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), and hepatitis C virus (HCV). In the conclusion of the article, extensions of quasispecies to nonviral systems and some possible course of events and future developments are addressed.

Some terms and parameters relevant to the characterization of viral quasispecies are given in Table 1.

**TABLE 1** Some terms and parameters relevant to the characterization of viral quasispecies

| Term (definition) | Comment or implication |
|---|---|
| Mutation rate[a] (the frequency of occurrence of a mutation during genome replication) | It is a biochemical event, independent of the fitness of parental and mutated genomes |
| Mutation frequency[b] (the proportion of mutations [any mutation, a mutation at a specific genome site, or a mutation type] in a population of genomes) | It is a population number, dependent on the relative fitness of the genomes harboring the mutations relative to nonmutated genomes |
| Rate of evolution[c] (the number of mutations that accumulate in viral genomes as a function of time) | The key difference from the mutation rate and frequency is that it includes a time factor |
| Mutant spectrum or mutant cloud[d] (the ensemble of genomes that constitute a viral quasispecies) | Its complexity and composition are highly relevant biologically |

[a] Expressed as substitutions per nucleotide copied. Several genetic and biochemical procedures have been used to determine mutation rates, with a general agreement that for RNA viruses they are in the range of $10^{-3}$ to $10^{-5}$ mutation introduced per nucleotide copied (see text for references).

[b] Expressed as substitutions per nucleotide. Mutation frequencies are influenced by many biochemical and environmental factors, with one being the fidelity of the viral polymerases, which determines the mutation rate (see text).

[c] Often expressed as substitutions per site per year. It may refer to intrahost or interhost replication of viral populations.

[d] The complexity of a mutant spectrum can be calculated from nucleotide sequences obtained either by classic molecular cloning and Sanger sequencing or by ultradeep sequencing. The classic method involves biological or molecular cloning, partial or complete nucleotide sequence determination, sequence alignment (generally 10 to 100 sequences per sample), and calculation of average genetic distances (average number of mutations that distinguish any two genomic sequences), mutation frequency (number of different mutations divided by total number of nucleotides sequenced), and Shannon entropy (proportion of sequences that are identical). Ultradeep sequencing involves amplification of short nucleotide sequences from a viral population and derivation of corrected reads (generally $10^5$ to $10^6$ sequences per genomic region) to calculate complexity (see text).

## THEORETICAL AND EXPERIMENTAL ORIGINS OF QUASISPECIES

### Quasispecies Theory and Its Adequacy for Viruses

Virologists adopted the term "quasispecies" from a theory on the adaptability of self-replicative entities that might have been key components at the origin of primitive forms of life. A pioneer study by Manfred Eigen on a quantitative treatment of the evolution of biological macromolecules (encouraged by Francis Crick and inspired by early *in vitro* RNA replication experiments by Sol Spiegelman and colleagues) had the merit of integrating for the first time concepts of information theory with Darwinian natural selection (237). The study represented the first theoretical treatment of self-instructive behavior required for template activity, as a necessity for the origin of inheritable information. According to Eigen's theory, a master copy of the self-replicative molecule produces mutant versions with a certain probability distribution. The production of mutant copies is dependent on a quality factor that determines the fraction of copying processes that leads to an exact copy of the template and the fraction that leads to error copies of the template. Eigen used the term "comet tail" to refer to error copies, and the terms "quasispecies" and "mutant spectrum," now used in virology, were introduced later (240–242). Thus, the initial study by Eigen was a quantitative treatment of error-prone replication, an important achievement in evolutionary biology intended to explain the events involved in the origin of life (237). The theory represented the introduction of a molecular view into evolutionary biology.

Quasispecies theory was further developed by Manfred Eigen and Peter Schuster in a series of theoretical papers that defined quasispecies as steady-state mutant distributions, dominated by a master sequence that displays the highest replication rate among the components of the mutant spectrum (243). Furthermore, the theory defined "hypercycles" as a principle of natural self-organization, to integrate different quasispecies into higher-order organizations that facilitated evolution into more complex forms, ideally combining the coding capacity of nucleic acids with the catalytic activities of proteins (243).

In addition to defining in quantitative terms a replicative system with a high mutational input, quasispecies theory established a condition needed to ensure the stable conservation of genetic information. Such a condition was formulated as an error threshold relationship. The error threshold means that for any given complexity (amount of nonredundant genetic information conveyed by a replicative system), there is a maximum error rate compatible with the maintenance of that genetic information. The error threshold value depends on the replication accuracy and the fitness of the dominant or master sequence relative to the mean fitness value of the error copies (243). The error threshold corresponds to the (mean) mutation rate (per site and replication) at which the frequency of the master sequence becomes very small and vanishes for practical purposes (Fig. 1) (789). The two fundamental equations of quasispecies theory are the dynamics of error

$$\frac{dx_i}{dt} = (A_i\, Q_i - D_i)x_i + \sum_{k=1,k\neq i}^{n} W_{ik}\, x_k - \Phi_i$$

$$\nu < \nu_{max} = \frac{\ln \sigma_0}{1-\bar{q}} = \frac{\ln \sigma_0}{\bar{p}} \quad \text{and} \quad \bar{p} < \bar{p}_{max} = \frac{\ln \sigma_0}{\nu}$$

**FIG 1** The two fundamental equations of quasispecies theory (243). The first equation describes the concentration of mutant i as a function of time, $x_i(t)$, and accordingly, $x_k(t)$ describes the concentration of mutant k. $A_i$, $D_i$, and $W_{ik}$ are reaction rate parameters for the replication of i, for the degradation of i, and for the error-prone synthesis of i on k being the template, respectively. The factor $Q_i$ expresses the fraction of correct replications producing i through copying of template i. $\Phi_i$ is a function which describes the flux of molecules as a consequence of the embedding of the replication-mutation system in some environment. In a simple flow reactor, $\Phi_i$ would be proportional to the concentration of i. This equation describes the dynamics of mutant generation within mutant spectra, as represented schematically in Fig. 2, 3, and 5. Extensions of the original equation have been developed, as described in references quoted in the text. The second equation is the error threshold relationship, in which $\nu_{max}$ is the maximum genetic complexity that can be maintained during replication, $\sigma_0$ is the selectivity or superiority of the master sequence relative to the sequences of the mutant spectrum, and $\bar{q}$ is the average copying fidelity of the replicative system, with $1 - \bar{q}$ being the average error rate per site and replication. The equation shows two important conditions: (i) the existence of a maximal sequence length $\nu_{max}$ for constant replication accuracy (left side) and (ii) the existence of a maximal error rate for constant sequence length (right side). Exceeding the limiting values leads to a breakdown of inheritance. The second case is of particular importance in virology since a drug-induced increase of the mutation rate may drive a virus population beyond the error threshold. This lies at the basis of lethal mutagenesis, depicted schematically in Fig. 12 and discussed in the text.

copy production and the error threshold relationship (Fig. 1). They convey concepts that have exerted a great influence in describing, understanding, and at times even predicting the behavior of viral populations, including strategies for controlling virus disease (reviewed in references 187, 192, 211, 236, 238, and 466).

Quasispecies theory as formulated initially was a "deterministic" theory. That is, when numerical values are assigned to a number of parameters, the system described by the equation that relates the different parameters is predictable. Deterministic theoretical models are usually articulated first because they can be solved mathematically. Determinism in quasispecies means that the theory assumed mutant distributions of infinite size in equilibrium (239, 243). Some geneticists have argued that the originally deterministic nature of quasispecies theory invalidated its adequacy for RNA viruses. However, it must be considered that a common procedure in theoretical studies is to extend deterministic models to incorporate stochastic (chance) events and that many generalized theories in physics, chemistry, and biology must be modified to fit real systems. In the case of quasispecies, several extensions to finite replicon (meaning any type of replicating entity) populations under nonequilibrium conditions (that is, in variable fitness landscapes) have been elaborated by Eigen and other authors (17, 234, 605, 608, 611, 636, 712–714, 741, 794, 863). The extensions to finite populations of the core concepts embodied in quasispecies theory justify even more the current use of the term "quasispecies" in virology (206, 373, 652). It is unlikely that the primitive replicons that quasispecies theory intended to represent fulfilled the conditions of equilibrium.

Viral quasispecies are perceived differently by physicists, chemists, biologists, and medical experts in infectious diseases. To physicists, a quasispecies is regarded as a cloud in sequence space, and viral quasispecies represented an experimental verification of the theory. To chemists, quasispecies are distributions of related but nonidentical nucleotide sequences, a definition familiar to virologists. To biologists a quasispecies is the target of selection, and the term does not imply a modification of the biological species concept (despite some ambiguity in the early literature on quasispecies). To experts in infectious diseases, quasispecies are the dynamic swarms of mutant viruses (particularly drug-resistant mutants) that they have to confront during antiviral therapy (these views on quasispecies and the adequacy of the term quasispecies in virology were previously discussed [206]).

Quasispecies theory and the supporting mathematical equations (Fig. 1) are closely connected with equations that describe other models of evolutionary dynamics (625). There is no fundamental conflict between quasispecies and the mutation-selection models of population genetics (578, 861). However, in contrast to population genetics, quasispecies theory has no problems in including epigenetic effects or RNA interference in the process of template copying, provided the mechanisms are known. Alternative terms to quasispecies, such as intrahost variation, intrahost diversity, mixture of mutants, nucleotide degeneracy, hyperploidy, heterospecies, or heteropopulations, have been proposed (commented on in reference 206). Any terminology is acceptable provided it transmits the true nature of viral populations. However, alternative terms fail to recognize quasispecies theory as the conceptual origin of mutant spectra. We do not consider it necessary to revisit arguments on why quasispecies theory has been more influential on experimental virology than classical population genetics (a point discussed in references 619 and 652). Our aim is to analyze some of the biological implications of viruses replicating as highly complex mutant distributions, including how this point of view on viral populations can contribute to the control of viral disease. We acknowledge considerable literature on theoretical aspects connected with quasispecies, which comprises extensions of quasispecies theory to finite populations in variable environments, effects of replication models on the distribution of mutations, dynamics of infection, coinfection, and virus spread, compartmentalization of infection, cooperation versus defection, attenuation and virulence, complexity, self-organization, and error catastrophe. Despite their importance, we quote only a limited number of theoretical studies either to assist in the interpretation of experimental observations or to reinforce mutual support between theory and experiment in reaching a key conclusion.

## Early Research on Reverse Genetics of Bacteriophage Qβ and Quasispecies Dynamics

During the second half of the 20th century, RNA viruses became well established as research objects following the discovery of RNA bacteriophages (492). Soon after, there were indications that their genetics might be unusual in the sense that some phenotypic traits (temperature sensitivity, plaque size, etc.) did not remain invariant (for review of evidence of RNA virus variability from the pregenomics era, see reference [203]). Some of these early suggestions concerned the RNA bacteriophages that infect *Escherichia coli*, notably MS2 (the first biological entity whose entire genome was sequenced [278]) and Qβ, an attractive system to study RNA genome replication, regulation of gene expression, and formation of RNA viral particles (855). Specifically, in 1969, Raymond C. Valentine and colleagues reported that a stock of bacteriophage Qβ

contained 8% temperature-sensitive mutants (821). They concluded that "replication errors constantly replenished the mutant pool" and that "roughly one base in $3 \times 10^4$ was misread." Early evidence of phenotypic variation of RNA bacteriophages was reviewed by Horiuchi (382). In the course of the first nucleotide sequencing studies, two different 5′-terminal sequences were found in bacteriophage Qβ (182), and Charles Weissmann, Martin Billeter, and colleagues suggested that "an apparently phenotypically homogeneous phage stock might contain multiple variants at various sites on the RNA" (856).

The first calculation of a mutation rate for an RNA virus and the demonstration of quasispecies dynamics using bacteriophage Qβ were possible because Weissmann and colleagues were developing in the 1970s the methodology that Weissmann coined "reverse genetics." It was based on the efficient copying of Qβ RNA by purified Qβ replicase *in vitro* to synthesize a full-length complementary (minus) strand which, in turn, could be copied into infectious plus strands. The unique properties of Qβ replicase allowed a stepwise synthesis of the minus strand. A mutagenic nucleotide analogue could be introduced into a preselected position of the minus-strand RNA to give rise to a plus strand with a mutation at the preselected site (283). An infectious extracistronic mutant was constructed, and it reverted to the wild-type sequence upon passage in *E. coli* (199). Serial passages of biological clones of the mutant virus alone and growth competition experiments between wild-type and mutant clones permitted an estimate of the mutation rate for the relevant G→A transition, which yielded $10^{-4}$ mutation per genome doubling (53). This mutation rate was $10^4$-fold higher than the values that at the time had been estimated for some DNA bacteriophages and $10^6$- to $10^8$-fold higher than values for bacteria and fungi (219). A high mutation rate was consistent with the previously noted phenotypic plasticity of RNA bacteriophage populations, which marked a difference from the DNA bacteriophages.

In the course of the analyses of wild-type and extracistronic mutant Qβ RNAs by $T_1$-oligonucleotide fingerprinting (a two-dimensional electrophoretic separation of the RNase $T_1$-digested products of labeled RNA, which was the only method available at the time to screen for the presence of mutations), it was observed that in some viral clones (a population derived from a single genome), typical, well-resolved oligonucleotides in the fingerprint were occasionally altered (had shifted their position or were lost, or new oligonucleotides were acquired). The modifications in the fingerprint suggested that mutations occurred with high frequency at multiple positions in the viral genome. This possibility was confirmed by control experiments in which biological clones were shown to diversify into multiple mutant clones upon passage in *E. coli* (215). It was concluded that "a Qβ phage population is in a dynamic equilibrium, with viable genomes arising at a high rate on the one hand, and being strongly selected against on the other. The genome of Qβ phage cannot be described as a defined unique structure, but rather as a weighted average of a large number of different individual sequences" (215). These observations were linked with quasispecies theory at a meeting of the Max-Planck Society in Klosters (Switzerland), where Weissmann presented the experimental data to Eigen and colleagues (for more-detailed accounts of the initial connection between quasispecies theory and the Qβ experiments, see references 192, 206, and 216). There can be no doubt that Eigen and his colleagues regarded viral quasispecies as an experimental verification of quasispecies theory. Obviously, quasispecies cannot be taken as a synonym of variation but rather can be taken as a representation of the dynamics of genetic variation, competition, and selection (69, 192, 233, 235, 236, 238).

The studies with infectious bacteriophage Qβ were complemented with *in vitro* experiments on replication of small RNAs derived from standard Qβ RNA, carried out by Christof K. Biebricher. These kinetic analyses extended the *in vitro* Qβ RNA replication studies by Spiegelman and colleagues, described quantitatively processes of mutation, competition, and selection, and provided further experimental support to quasispecies dynamics (64, 65, 68, 70, 71).

Unless the Qβ results were exceptional, quasispecies theory signaled a major change in our views on RNA viruses as populations. The change was that what we generically term a "wild-type" virus could no longer be equated with a genome with a defined nucleotide sequence. Rather, a replicating "wild type" would consist of an ensemble of mutants, the "mutant spectrum" or "comet tail" of quasispecies theory. At the time this was an important conceptual departure, which was soon recognized as having numerous biological and medical implications ranging from a general adaptability of viral populations to consequences for viral pathogenesis and disease control strategies (188, 209, 372, 376, 800). There has been ample confirmation that the results with bacteriophage Qβ apply to RNA viruses that infect animals and plants, as evidenced by experiments carried out in cell culture and *in vivo*, in particular many fundamental studies by John J. Holland and his colleagues (reviewed in references 187, 192, and 596). Many references are included in the following sections of this article, with emphasis on animal viruses, our field of expertise. Error-prone replication is now recognized as a feature shared by all RNA viruses, as evidenced not only by rapid generation of variants but also by the fidelity properties of their RNA-dependent RNA polymerases (141, 408, 476, 832). Not only viral polymerases and reverse transcriptases (RTs) but also the reverse transcriptases of cellular retroelements are equally error prone (298, 400). Diversity and spread of mobile elements may have contributed to cellular diversification (566, 763) and also to disease (422).

It is not yet obvious to all virologists that an invariant consensus sequence reflects not an absence of mutations but rather a continuous replenishment of the mutant pool to yield the same average (Fig. 2). The consensus sequence may change either as a consequence of a random, bottleneck event or as result of modification of an equilibrium (which is generally transient) of the mutant spectrum. In viral evolution there is no such a thing as a molecular clock that dictates constancy of incorporation of mutations (discussed in "Viral Quasispecies Dynamics *In Vivo*: Long-Term Virus Evolution" below). A virus variant that emerges to slowly increase its frequency in a mutant spectrum need not be generated with a lower-than-average mutation rate. The rhythm of frequency gain depends on the fitness (or capacity to produce progeny [defined in the next section]) of the newly arising mutant and its surrounding cloud relative to those of the other components of the mutant ensemble (Fig. 3). These are key issues which are extremely relevant to understanding virus behavior.

## MAIN FEATURES OF VIRAL QUASISPECIES

### Levels and Mechanisms of Genetic Variation, Competition, and Selection

Virologists use the term viral quasispecies to mean distributions of nonidentical but related genomes subjected to a continuous pro-

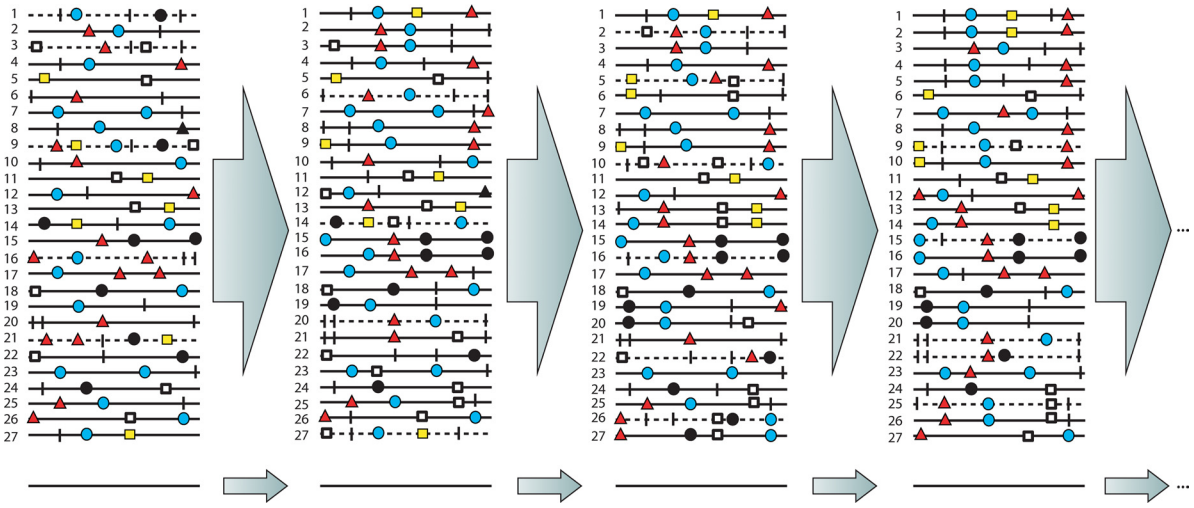

**FIG 2** Schematic representation of the evolution (change in composition) of a viral quasispecies without modification of the consensus sequence. Viral genomes are represented as horizontal lines and mutations as different colored symbols on the lines. Discontinuous lines indicate genomes that have acquired five or more mutations and that cannot survive to act as the template for the next generation of genomes (large arrows). Other genomes can incorporate mutations during replication (i.e., genome 2 in the distribution on the left generates genomes 2 and 3 in the second distribution, until an excess of mutations in genome 2 of the third distribution impedes its replication). Thus, a constant evolution in the mutant spectrum can nevertheless yield the same consensus sequence, depicted as a line devoid of mutations at the bottom. In a viral population the number of genomes in a single replicative unit in an infected cell can reach several thousand rather than 27, implying a highly dynamic and indeterminate mutant spectrum or cloud, as discussed in the text.

cess of genetic variation, competition, and selection and which act as a unit of selection (187, 652). Strictly speaking, a viral quasispecies should be considered a single replicative unit in an infected cell (177, 414, 444, 719). However, heterogeneous viral progeny from a single cell will invade neighboring cells in culture or from the same tissue or organ *in vivo*. This creates a second and successive level of competition among viral particles and viral genomes. In viremia, competition for invasion of tissues and organs is established among viruses that have originated in different replicative units. Whether at the intracellular or extracellular (whole-organism) level, the use of the term quasispecies is justified *in vivo* since it conveys the concept of ensembles of similar genomic sequences generated by a mutation-selection process (187, 215, 235, 236, 238, 466, 605, 606, 652, 861).

Although the emphasis for quasispecies has been on mutation as the source of variant genomes (mechanisms of mutation are reviewed in reference 294), the theory has been extended to include additional mechanisms of variation such as recombination, gene duplication, genome segment reassortment, and gene transfers (75, 204, 371, 398, 575, 635) (Fig. 4).

A seemingly complex and disorganized mutant spectrum may nevertheless hide subcomponents that reflect prior evolution of sets of different parental genomes. Substructuring of quasispecies may be revealed by groupings of reads of sufficient length in ultradeep sequencing analyses (889) and by nonhierarchical clustering methods such as partition analysis of quasispecies (PAQ) (39, 40). This procedure can group closely related genomes from mutant spectra (Fig. 5) and can reveal quasispecies expansions and compressions as a result of mutagenic treatments (615).

Positive selection is the process by which a genotype (or set of genotypes) becomes dominant in an evolving population as a result of positive evaluation of phenotypic traits expressed by the individuals (or set of individuals) of that population. In complex viral quasispecies, a mutant distribution whose components share the selectable trait, rather than a single individual, becomes dominant, as shown by antibody selection acting on a reconstructed foot-and-mouth disease virus (FMDV) quasispecies (653). The fixed repertoire was enriched in the individuals that displayed relatively higher fitness values (520), underscoring the influence of relative fitness (and thus of the fitness landscape of mutant clouds) on the outcome of selective processes undergone by viral quasispecies.

In contrast to positive selection, negative selection is the process by which a genotype (or set of genotypes) is eliminated in an evolving population as a result of negative evaluation of phenotypic traits expressed by individuals (or a set of individuals). A point to note is that the distinction between positive and negative selection may become fuzzy because in a competition process, negative selection of genome subsets may have the same outcome as positive selection of the remaining subsets and vice versa. Also, negative selection need not result in the elimination of subsets of genomes but may result in their maintenance at low frequencies. These low-frequency genomes may be detectable or not, depending on the analytical procedures used (e.g., minority mutations present at different frequencies revealed by ultradeep nucleotide sequencing might have been subjected to negative selection of different intensity). These arguments lead to the necessity of quantifying relative selective advantages of viral populations through fitness measurements, which is covered in the next section.

### Viral Fitness and the Effect of Population Size: Bottleneck Events

Fitness is a major parameter in genetics that was also included in the formulation of quasispecies theory and the error threshold relationship (Fig. 1). It was adapted by virologists to quantify the relative replication capacity of a virus, generally measured in

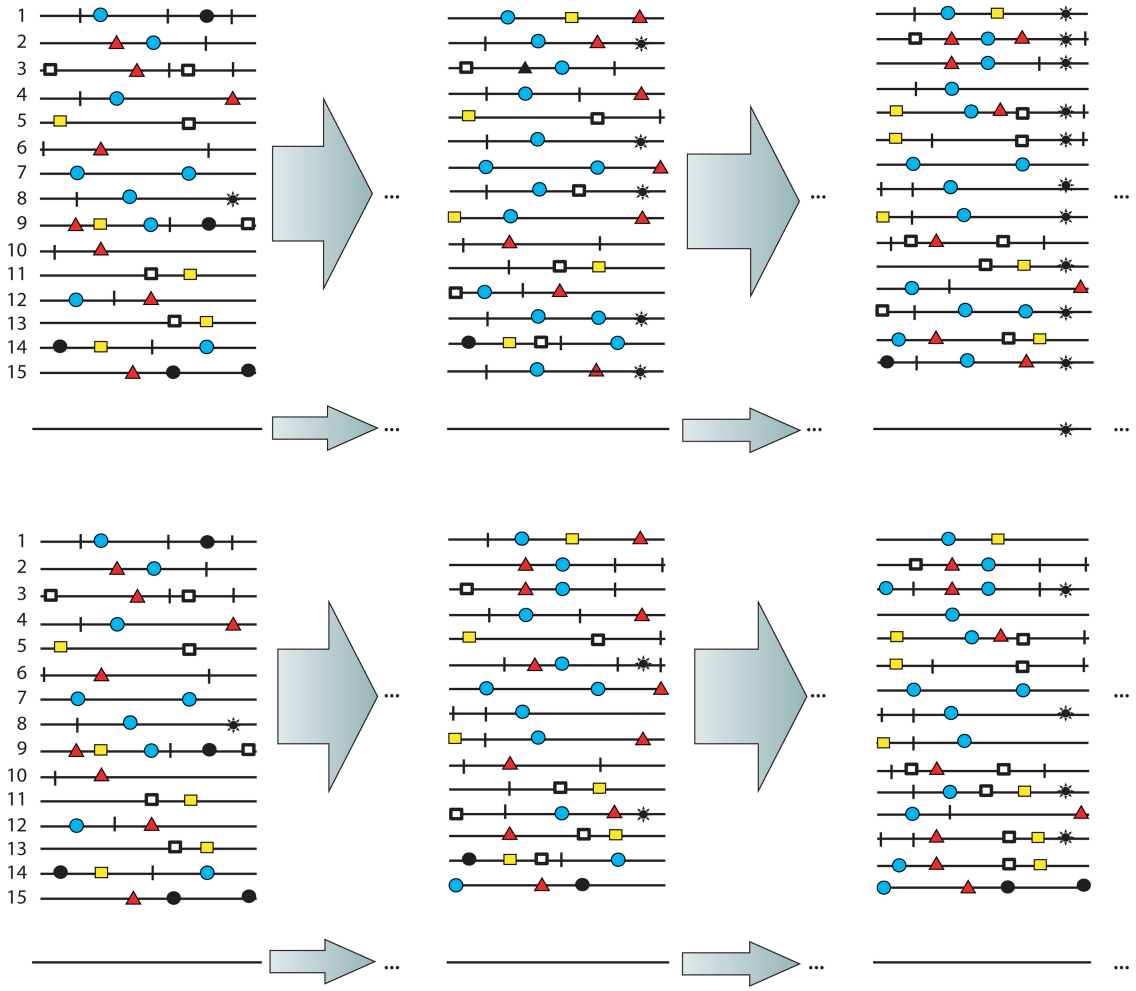


**FIG 3** The selective advantage (relative fitness) of a mutant genome (with its associated cloud) alters the rate of dominance in a population. Mutant spectra are depicted as in Fig. 2, with mutations on genomes indicated as colored symbols on the lines. In the upper mutant distribution, the mutation highlighted by a black asterisk in genome 8 of the first distribution confers a selective advantage that results in dominance of that mutation after a given number of replication rounds (several large arrows, but only two are drawn). The selective advantage results in the dominance of genomes with the mutation in the mutant spectrum (upper right distribution) and modification of the consensus sequence (below the upper right distribution). In the bottom mutant distribution, the same mutation in genome 8 confers a modest selective advantage, and despite its frequency increasing in the population, it does not become dominant (bottom right distribution) and the consensus sequence remains invariant (below the bottom right distribution). The mutations that accompanied the relevant one in the initial genome need not be maintained after multiple rounds of copying. This scheme illustrates that fitness gain may not be reflected in a modification of the consensus sequence and, consequently, that selective events can be overlooked if mutant spectra are not analyzed, as discussed in the text.

growth competition experiments with a reference virus isolate, either in cell culture or *in vivo* (215, 374, 524; reviewed in references 205, 527, and 684). Fitness measurements have been equated to the determination of a selection coefficient (514). Comparison of the consensus nucleotide sequence of a population with those of individual components of the same population indicates that quasispecies dynamics is basically reflected in variations of frequency (relative fitness) of subsets of genomes in response to environmental changes (Fig. 2 and 3).

Mutations occur unavoidably whenever an RNA virus replicates, including in the course of plaque formation on a cell monolayer. The generation of a plaque involves virus replication in an initial cell, followed by the spread of progeny virus to neighbor cells in the monolayer until resources are exhausted, virus inactivated, or plaque development interrupted. Mutations while a virus replicates within a plaque were first described by John Yin, working, interestingly, with a DNA bacteriophage (884). A passage regimen consisting of successive plaque-to-plaque transfers should result in an accumulation of mutations in the viral genome and a fitness decrease (Fig. 6). Average fitness decreases were first documented with bacteriophage ϕ6 (112), although the underlying genetic changes were not determined. The result was confirmed with several RNA viruses (133, 168, 222, 253, 258, 364, 597, 601, 602, 888).

Studies with the animal pathogen foot-and-mouth disease virus (FMDV) by Cristina Escarmís and her colleagues unveiled the molecular basis of fitness loss associated with successive bottleneck events of biological clones of the virus. A number of unusual mutations were observed, which had never been detected in field isolates of FMDV or in the same FMDV clones subjected to large-population passages. Atypical mutations included those leading to amino acid replacements at internal residues of the viral capsid

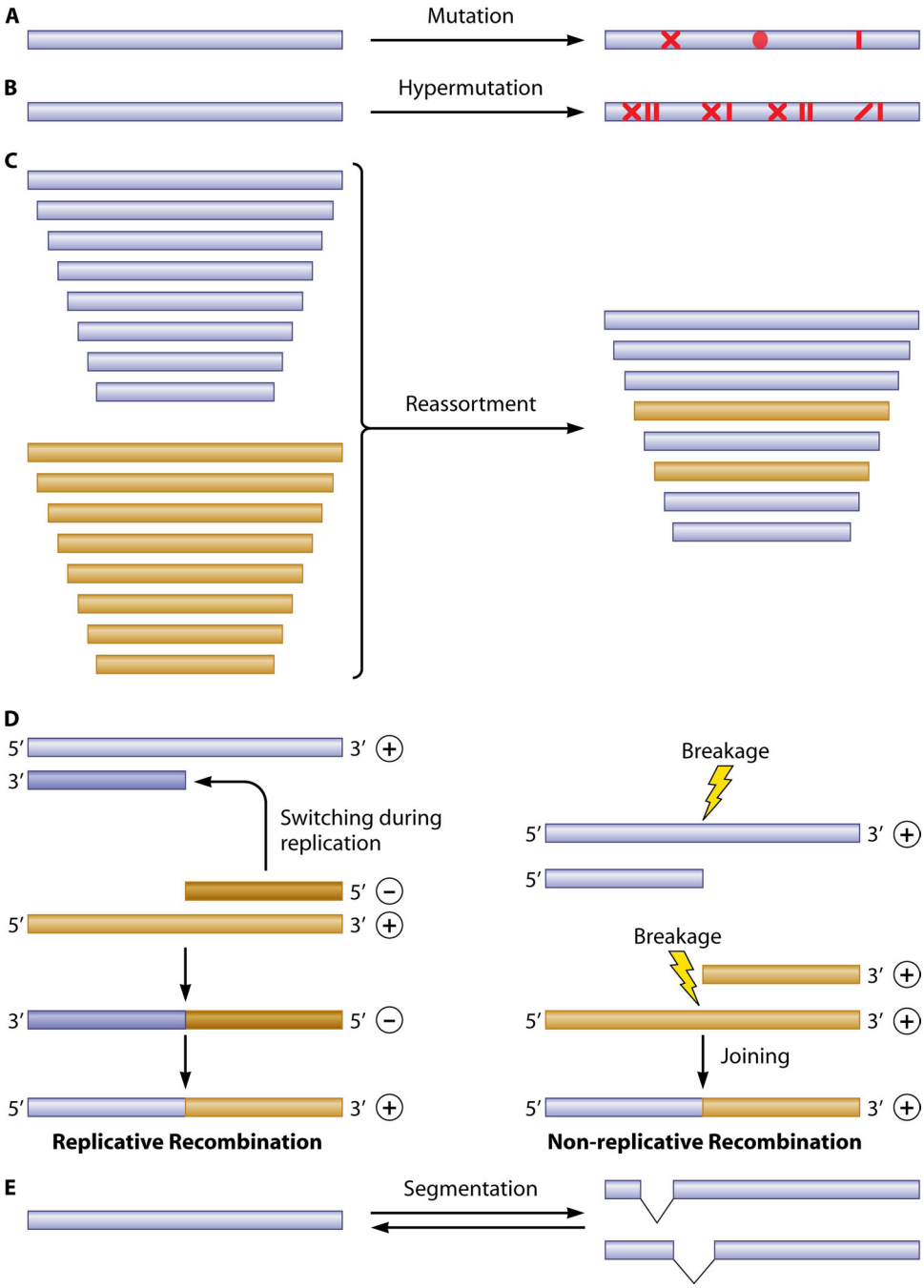


**FIG 4** Simplified representation of several types of genetic modifications that can alter the composition of viral quasispecies. (A) Mutation is a universal class of genetic variation and the basis of the original quasispecies formulation, as described in the text. (B) Hypermutation (generally biased toward some mutation types) is a consequence of cellular editing activities acting on viral genomes. (C) Genome segment reassortment occurs in viruses with segmented genomes and is responsible for the antigenic shift associated with new influenza pandemics. (D) Recombination results in formation of mosaic genomes, either by template switching (replicative recombination [left]; the negative, complementary strand is depicted with darker color) or breakage and rejoining of RNA molecules (nonreplicative recombination [right]). (E) A high multiplicity of infection passages of FMDV resulted in genome segmentation, as described in "Fitness Gain and Genetic Change: Genome Segmentation" in the text (schemes are based on references 5, 189, and 306).

(2), a point deletion within the L (leader protease)-coding region, an elongation of an internal oligonucleotide tract that precedes the second functional AUG, and even an amino acid substitution in the FMDV capsid that adversely affected the processing of the viral polyprotein despite the substitution being located far from the relevant cleavage site (253, 258). Hundreds of bottleneck passages resulted in the evolution toward noncytopathic (NC) forms of FMDV that displayed a 140-fold reduction in specific infectivity (the ratio between PFU and the amount of viral RNA present in a virus preparation) relative to that of the corresponding unpassaged parental clones (256). These remarkable NC forms of FMDV remained, however, replication competent, and could es-

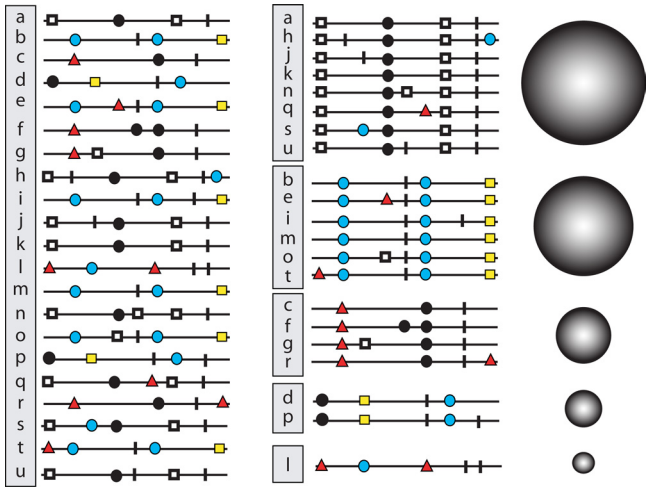


**FIG 5** A quasispecies distribution (represented as in Fig. 2 and 3, with each genome identified with a letter) may hide distinguishable virus subpopulations. Partition analysis of quasispecies (PAQ) is a nonhierarchical bioinformatics procedure that groups components of viral quasispecies (39, 40). In this example we depict mutant classes as spheres of sizes proportional to the number of genomes in each class.

tablish persistent infections in BHK-21 cells without a previous phase of cytopathology (compare references 256 and 170, 365).

The increasing mutational load due to successive bottleneck events of FMDV was associated with a biphasic and fluctuating pattern of fitness decrease. Yet, the clones that attained very low fitness displayed a remarkable resistance to extinction, with genomes rescued by compensatory mutations (255, 467, 468). Furthermore, the pattern of fitness decrease adhered to a statistical Weibull function (853), which probably reflects an intricate set of interactions that must take place for a virus to give rise to progeny and to generate a plaque on a cell monolayer (468).

The remarkable consequences of bottleneck events provide additional evidence of genetic and phenotypic heterogeneity of viral populations, as well as an experimental confirmation of "Muller's ratchet" (540, 571, 572). Hermann J. Muller proposed that asexual organisms with a small population size and a high mutation rate will tend to incorporate deleterious mutations in an irreversible, ratchet-like manner unless compensatory mechanisms such as recombination could restore the mutation-free class of genomes (571, 572). Muller's ratchet has been shown to operate not only in RNA viruses but also in DNA-based cellular organisms such as bacteria, protozoa, and plants, as well as mitochondrial DNA (15, 22, 58, 139, 248, 493, 560). Fitness decrease due to serial bottleneck passages can be compensated for by subsequent large-population passages, as anticipated by theoretical investigations (301, 342, 500, 785). However, studies with vesicular stomatitis virus (VSV) showed that huge numbers of replication rounds might be needed to overcome the effect of Muller's ratchet (223). The number of clonal pools required to maintain a fitness value of VSV is dependent on the fitness of the parental clone. For a clone displaying low fitness, pools of five clones were sufficient to maintain or increase fitness. When the initial fitness was high, pools of at least 30 clones were needed to maintain the fitness level (602). Repeated genetic bottlenecks in VSV resulted in adverse effects regarding subse-

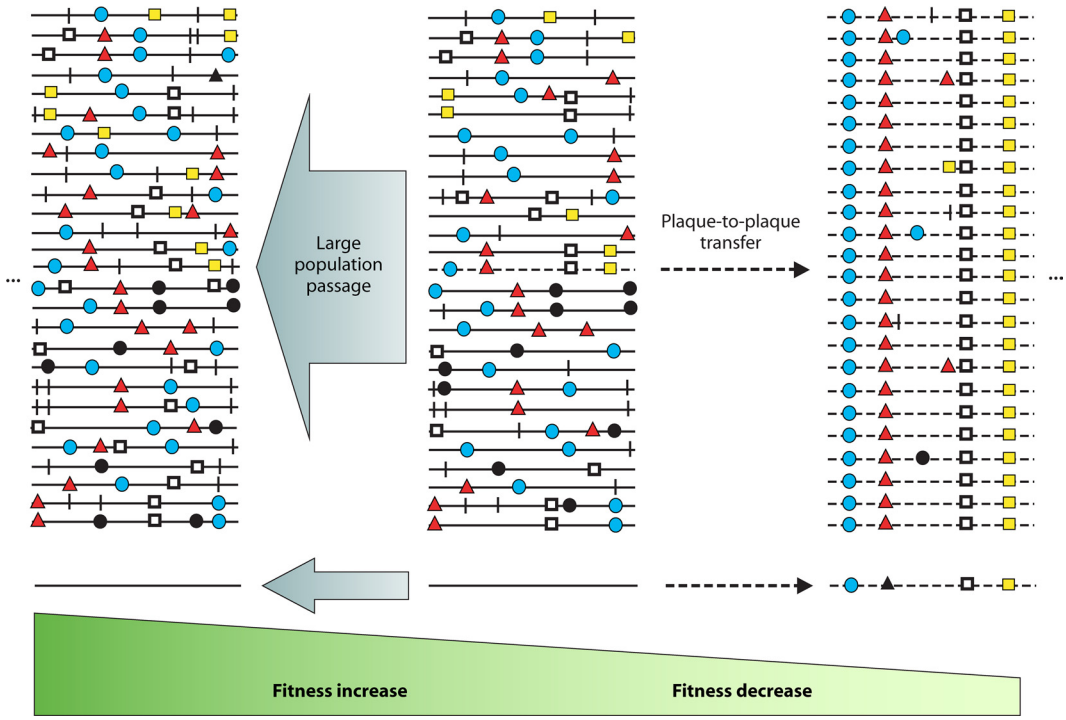


**FIG 6** Fitness evolution of RNA virus populations. Mutant distributions are represented as horizontal lines and colored symbols as in Fig. 2 and 3. Large population passages in a constant environment generally result in a fitness increase (bottom trapezoid), which may or may not result in a modification of the consensus sequence (lines below the distribution). In contrast, plaque-to-plaque transfers (discontinuous genome in the central mutant distribution and discontinuous arrow) result in an accumulation of mutations, also reflected in the consensus sequence, and fitness decline. Plaque-to-plaque transfers decrease the complexity of mutant spectra and mimic bottleneck events that occur during virus life cycles, as discussed in the text.

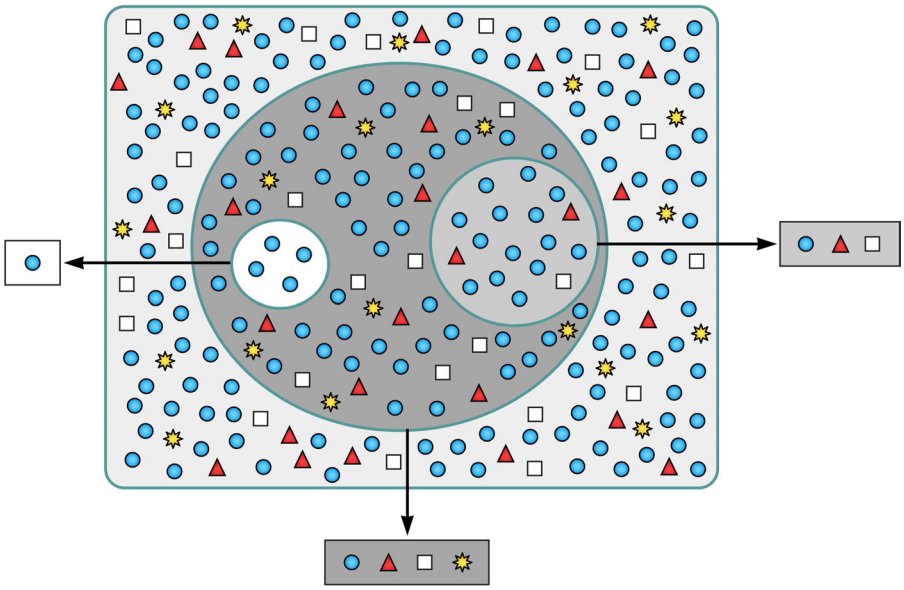

**FIG 7** Population size may affect the evolutionary outcome. The large rectangle represents a viral quasispecies. Each symbol (even of the same shape and color) portrays a slightly different sequence. Three types of particles harboring mutations that can confer resistance to a selective agent are distinguished (red triangles, white squares, and yellow stars) from other components of the mutant spectrum (blue circles). If a small population is analyzed (white inner circle on the left), no resistant variants will be found. Further viral replication of that subpopulation will be needed to generate the resistant mutants. If an intermediate-size population is analyzed (intermediate gray inner circle on the right), two of the resistant variants will be found. If a large population is analyzed (large circle), all relevant variants will be represented. This scheme illustrates how the viral population size can condition the results of intrahost or interhost virus transmission, or of experimental evolution, in relation to drug resistance or other phenotypic traits (see text).

quent adaptability of the virus (597). Compensation for the deleterious effects of Muller's ratchet is one of the arguments in favor of the evolution toward sex in ancestral asexual organisms (540, 541).

It is remarkable how profound can be the reductions in fitness and the biological alterations in viruses subjected to repeated bottleneck transfers. Such transfers mediate the surfacing of minority components present or generated in the mutant spectra of the corresponding clonal populations (reviewed in references 197, 257, and 507). From all evidence, mutant spectra are far from being distributions of neutral mutants. They can hide components that in isolation would display dissimilar biological properties (512, 552). As expressed by Schuster: "Quasispecies are the genetic reservoir of an asexually replicating species" (741).

Plaque-to-plaque transfers are an extreme case of population bottleneck because a single infectious genome initiates replication and diversification. Viruses can, however, be subjected to bottlenecks of different intensity, depending on the number of genomes from a larger ensemble that initiate replication. A small number of genomes taken from a larger ensemble will include a limited repertoire of variants for further replication or to confront a selective constraint (Fig. 7).

Current evidence is that bottleneck events are frequent in the course of the life cycles of viruses, not only in the most obvious case of host-to-host transmission, known for decades (35, 150), but also during the intrahost spread of virus (14, 63, 93, 288, 338, 480, 680, 740, 772). Quantification of intrahost bottleneck intensities would contribute to the understanding of intrahost evolutionary events. Bottlenecks introduce an additional strong element of stochasticity during short-term (intrahost) or long-term (interhost) virus evolution (Fig. 6 and 7).

In contrast to the case for bottlenecks that lead to fitness decreases, viruses passaged as large populations in a given environment tend to gain fitness in that environment (254, 496, 600). Fitness gain can be viewed as the result of competitive optimization of a viral quasispecies in its tendency toward a mutation-selection equilibrium in a given environment (861). The population size attained by a virus in an infected host can be exceedingly large, in the range of $10^9$ to $10^{12}$ infectious particles (346, 700, 746). (Additional values are given in "Human Immunodeficiency Virus Type 1," "Hepatitis B Virus," and "Hepatitis C Virus" below.) It is not obvious how to calculate the proportion of replicating genomes at any given time, which would yield the effective population size (a concept widely used in population genetics). A virus particle may not be capable of initiating an infection and producing progeny at a given time, but the same particle may immediately be exposed to a different environment that triggers its replication. Specific infectivities, similarly to efficiencies of plating, are environment dependent. It may be anticipated that the large population sizes attained after infection by a limited number of particles (the usual situation in transmission from an infected donor into a susceptible host) should result in high fitness levels (254, 600). However, an infected organism is essentially a mosaic of different environments (cells, tissues, organs, and physiological conditions), and fitness is environment dependent (205, 457, 634, 684). Viruses specialized to replicate mainly in a specific tissue (e.g., hepatitis viruses in the liver) are expected to increase their fitness in that specific tissue as the infection advances, but for viruses targeting different tissue types, compartmentalization of fitness values is to be expected, although this is an area in need of additional research.

Modification of the environment and limitations of population

**TABLE 2** Parameters in mutant spectra that are relevant to the adaptive capacity of viral quasispecies

| Parameter (values) | Major reason for relevance |
|---|---|
| Population heterogeneity (average number of mutations per genome; current estimates range from 1 to 100, depending on multiple factors[a]) | Accessibility to different regions of sequence space; heterogeneity enhances chances of intra-mutant spectrum interactions |
| Virus population size or viral load (very variable but for some viruses can reach $10^{12}$ infectious particles per organism; may refer to infectious or total [infectious and noninfectious] virus | Population size determines the numbers and types of variant genomes in a population; the specific infectivity (infectious units divided by the total amount of viral RNA) is indicative of the interfering potential of a virus |
| Genome length (most RNA viruses have a genome of between 3 and 32 kb, with little redundant information) | A small genome size permits a more effective occupation of sequence space, as discussed in the text |
| Mutations needed for a biological change (variable, but one or few mutations may suffice for important biological changes, as discussed in the text) | If important phenotypic changes were not dependent on limited numbers of mutations, quasispecies distributions with the levels of genetic heterogeneity described here would not be important for virus adaptability, and this review would not have been written |
| Virus fecundity and turnover (capacity of generating new viral particles to replace those of previous generations that are catabolized) | It influences the capacity of exploration of sequence space, with its implications for adaptability (see the text and Fig. 8) |

[a] Factors include proximity to a clonal origin, mutation rate, and environmental heterogeneity (see the text for references).

size (including bottleneck events) may perturb (delay or prevent) the process of fitness gain (223, 603, 634). Thus, viral fitness is environment and population size dependent, as well as prone to stochastic fluctuations due to finite viral population sizes and to the inherently heterogeneous and dynamic nature of viral populations (205, 506, 527, 684, 841).

Comparing the accumulation rates of mutations in FMDV subjected to plaque-to-plaque transfers (0.20 to 0.40 mutation per genome and transfer) and in FMDV subjected to large-population passages (0.10 to 0.25 mutation per passage in the consensus sequence) (Table 2) offers an interesting illustration of a counterintuitive concept that can be phrased paradoxically as "a positive outcome of negative selection." It arises when the context in which the virus is situated facilitates the survival of a minority subpopulation. Hundreds of plaque-to-plaque transfers led to replication-competent and plaque-forming clones that displayed a mutation frequency of $1.5 \times 10^{-2}$ substitution per nucleotide, measured relative to the sequence of the parental virus (256). Yet, the same FMDV populations subjected to lethal mutagenesis (deterioration of virus replication due to an excessive mutational load) were extinguished when their mutant spectra amenable to amplification reached an average mutation frequency in the range of $4 \times 10^{-4}$ to $3 \times 10^{-3}$ substitution per nucleotide (12, 634, 762) (the consensus sequence in the progression toward virus extinction by lethal mutagenesis remained invariant; see "Antiviral Approaches to Counteract Quasispecies Adaptability: Lethal Defection and Lethal Mutagenesis" below). The available evidence suggests that the critical difference is that during plaque-to-plaque transfers, selection for plaque formation rescues a tiny minority amid a majority of genomes that have lost the capacity to form a plaque. In contrast, no such opportunity for the rescuing of viable minority genomes exists during lethal mutagenesis, and the entire population collapses, probably with the contribution of multiple negative interactions established among components of the mutant spectrum (see "Complementation and Defection: Quasispecies as a Unit of Selection" below).

### Fitness Gain and Genetic Change: Genome Segmentation

In a study aimed at elucidating what could be the limits of fitness gain of FMDV replicating in BHK-21 cells under standard cell culture conditions, a biological clone of FMDV was subjected to 460 serial large-population passages. Initially, fitness gain occurred as expected, with an accumulation of point mutations in the consensus sequence of the viral genome (306, 307). However, between passages 143 and 260 the virus underwent a remarkable transition in which the standard full-length genome was replaced by genomic forms with internal deletions that were infectious and killed cells by complementation (306, 307). One of the deletions was located in the L-coding region, and other deletions affected the capsid-coding region. By passage 240 the standard virus was not detected, which means that its frequency was lower than $10^{-4}$-fold the frequency of genomes with deletions (306). Passage of the population consisting of genomes with internal deletions at a low multiplicity of infection (MOI) resulted in the rescuing of the standard-size genome, originated by recombination between genomes with deletions (307). Comparison of the kinetics in the major steps of the virus life cycle using standard and segmented FMDV genome forms suggested that capsid stability (rather than a more rapid replication of shorter RNAs or differences in viral protein synthesis) conferred the selective advantage of the FMDV genome version with internal deletions over the virus with the standard-size genome (618). The increased stability of viral particles harboring shorter RNAs was possibly due to relaxation of packaging constraints that affected the standard genome and that were relieved when a shorter genomic RNA was encapsidated (532, 618). This study with FMDV showed that conditions of replication that favor fitness increase can give rise to drastic genetic modifications, even under a constant physical and biological environment. Internal deletions within RNA genomes under widely different environments have been described, such as during replication of Venezuelan equine encephalitis virus in Vero cells (284) or during replication of coxsackie B3 virus in cardiac tissue of mice (432). Internal deletions characterize some strains of porcine reproductive and respiratory syndrome virus, although their biological relevance is not known (347, 805).

It has been suggested that genome segmentation may have been favored during evolution because it introduces a form of sex that permits counteracting the effect of deleterious mutations (111, 790). An alternative, mechanistic proposal is that segmentation can result from selection of shorter genomes whose replication is

completed in a shorter time than that for the cognate full-length genome (379, 585). A plausible mechanism is that deletions occur essentially at random (at frequencies that depend on the replication machinery, particularly polymerase processivity) and that when a combination of internal deletions compatible with complementation arises, a segmented form can overgrow the monopartite, full-length genome. This possibility is also reinforced by the fact that multiple FMDV genomes with a variety of internal deletions arose during replication, and they were maintained at low frequency in the evolving population (307). Thus, the results with FMDV point to particle stability as a mechanism that can trigger the evolutionary event toward genome segmentation (618). It will be intriguing to explore whether following this major transition, the segmented FMDV genome will maintain its bipartite genome organization or will undergo some new major transition for fitness gain. The observed evolutionary pattern includes ingredients of gradualism (accumulation of point mutations [307]) and of evolutionary shifts (or "saltations"), rendering realistic at the molecular level the occurrence of punctuated equilibria during viral genome evolution (244).

### Population Equilibrium, Apparent Stasis, and Rapid Evolution

The mutation rates for RNA viruses measured by genetic and biochemical procedures (53, 220, 726) (Table 2) imply that most template RNAs that are copied into a complementary strand must contain between 0.1 and 2 mutations. Therefore, unless the vast majority of mutations were lethal (which is obviously not the case [31, 101, 215, 272, 724, 725]), it should take only a few rounds of template copying (despite initial differences in the distribution of mutations, depending on the contributions of the stamping-machine and semiconservative replication modes [728, 730]) to produce a dynamic spectrum of mutants. In classical population genetics, variation and constancy usually refer to the consensus genomic nucleotide sequences (or deduced amino acid sequences) of the organism under study. Consensus sequences are determined by standard sequencing techniques, often referred to as Sanger sequencing. In virology, consensus sequences are those most commonly determined, and they serve as the basis for virus identification, to establish phylogenetic relationships among viral isolates, or to interpret processes of selection and random drift in virus evolution, assuming that such interpretation does not necessitate information on the underlying mutant spectra (189, 333, 378, 721). The problem arises when the presence and composition of a mutant spectrum affect relevant traits in virus biology (see reference 652 and other sections of the present article).

Ever since the quasispecies nature of RNA virus populations was unveiled, the inappropriate distinction between mutation rate (in mutations introduced per nucleotide copied, a biochemical value which is dictated largely by the template-copying fidelity of the replication machinery), mutation frequency (the proportion of mutations present in a viral population, which is the result of mutation, competition, selection, and in some cases even random events), and rate of evolution, to mean rate of evolution with a time factor in it (for example, substitutions per site and year during the evolution of a virus in the field) (Table 2) was considered a conceptual flaw (203). Mutation rate continues being used incorrectly not only in general evolution but also in evolutionary virology, thus contributing to the mistaken belief that an invariant consensus sequence implies an absence of mutations. Another misleading and amply used term is "fixation," referring either to genomes or to mutations. Given the concepts conveyed by quasispecies dynamics, it is intrinsically contradictory to use the term "fixation." The reason is that genomes and mutations can at best attain transient dominances, and they will unavoidably coexist with a mutant repertoire. Fixation was frequently used during the neutralist-selectionist controversy (now largely vanished) to distinguish "fixation" of mutations either by random drift of genomes or by positive selection of phenotypes associated with those mutations (435, 437).

Population equilibrium is sometimes used to refer to viral populations that maintain a constant consensus sequence (371, 376). Near-equilibrium conditions can be approached experimentally with large populations of simple RNA molecules replicated by Qβ replicase (64, 65, 68, 70, 71) and upon passage of large viral populations in cell culture (215, 784). In the latter studies an invariant consensus sequence was noted despite a complex, changing mutant spectrum (Fig. 2). High mutation rates permit but do not necessitate rapid evolution. The same virus can display either evolutionary stasis or rapid evolution, depending on its biological environment. A classical example is the evolutionary stasis frequently observed with avian influenza viruses (IVs) in their natural avian hosts, where they do not cause disease, and rapid evolution of the same viruses when they replicate and cause disease in alternative avian hosts or in mammalian hosts (324). Arboviruses may also display relative evolutionary stasis despite the presence of mutant spectra, probably reflecting a stabilizing selection for the capacity to replicate efficiently in vertebrate and invertebrate hosts. Although different studies on experimental evolution have provided conflicting results, evidence of a trade-off to achieve replication in two different environments has been obtained (references 142, 165, 404 to 406, 567, and 849 and references therein).

Remarkably counterintuitive outcomes may result from quasispecies dynamics. In particular, biologically significant perturbations can take place in a mutant spectrum without any modification in the consensus sequence. An initial fitness gain of HIV-1 clones in cell culture may occur with an invariant consensus sequence (77). More dramatic is the transition toward extinction due to increases in mutation rate, in which a virus undergoes a replicative collapse without any alteration of the consensus sequence (see "Antiviral Approaches to Counteract Quasispecies Adaptability: Lethal Defection and Lethal Mutagenesis" below). In other words, an ultimate transition toward the disappearance of a virus from infected cells would go unnoticed genetically if the consensus sequence was the only one monitored!

Evolution is not always understood as a modification of the consensus sequence; it can be viewed as a disequilibrium of a mutant spectrum. When positive selection or stochastic events intervene, subsets of genomes from the mutant spectrum increase in frequency and replace the previous distribution, thereby leading to a new consensus (Fig. 2). Disequilibria can come about by positive selection or by random events, and they may be durable or short-lasting, depending on the tendency of the ensemble to reequilibrate mutant distributions (which is dictated by the relative fitness or selection coefficients of parental and progeny distributions). Adaptation of a natural virus isolate to cell culture constitutes an example of modification of a viral population due to the growth of subpopulations in the new environment. This was presciently expressed by Andreas Meyerhans, Simon Wain-Hobson, and their colleagues with the statement "to culture is to disturb,"

in the first demonstration of the quasispecies nature of HIV-1 and its biological implications (552). Adaptation of a natural viral isolate to cell culture will in most cases entail a modification of the genetic composition of the viral population, even if the adaptation period is short.

When a mutant spectrum harbors an ample repository of variants, selection of genome subsets in a new environment can be very rapid. Astonishing rates of evolution of consensus sequences *in vivo*, of $10^{-2}$ substitution per site per year or even higher, can be attained (87, 136, 311, 341, 473, 698, 753). It should be appreciated that such rates of evolution are $10^6$- to $10^7$-fold higher than the average rates estimated for cellular genes during long-term evolution (the values and implications are reviewed in references 189 and 376). Thus, a dynamic mutant spectrum has a dual potential to show either stasis (rates of evolution of around $10^{-4}$ substitution per site per year or lower) or rapid evolution of its consensus sequence (even $10^{-1}$ to $10^{-2}$ substitution per site per year). Stasis does not imply mutation rates lower than those usually operating during RNA genome replication, nor does rapid evolution imply higher-than-average mutation rates.

A distinction is made between directional selection and fluctuating or cyclical selection that does not result in "fixation" of the corresponding genetic traits (259, 343). Fluctuating selection, judged by alternating nucleotide sequences, may be due to limitations in the number of acceptable nucleotide or amino acid sequences at some loci. Alternation of amino acid sequences was observed at major antigenic sites of FMDV strains of the same serotype isolated over a period of 6 decades (525).

Population size, genetic heterogeneity, adaptive capacity, and evolutionary rate are dependent on an interconnected set of parameters, some amenable to quantification and others difficult to measure. Major parameters are the rate of genome multiplication, the mutation rate, and the tolerance of genomes to accept mutations and remain functional. These parameters apply to all pathogens, be they cellular or subcellular. The mutation rate, tolerance to mutations (nonlethal mutations with a range of fitness values), and population size will determine the breadth of the mutant spectrum and the landscape of minority mutations and their frequency (Fig. 8A).

### Exploration of Sequence Space and Virus Adaptability: Fidelity Mutants

The amplitude of a mutant spectrum can also be viewed as the extent of occupation of sequence space (Fig. 8B). The term sequence space refers to a theoretical representation of all possible variants of a sequence, and it can concern nucleotide or amino acid sequences (237, 243, 542, 741). The theoretical sequence space of a virus (or any living entity) is extremely large, being equal to the number of different unit digits used to construct the relevant macromolecule to the power of the sequence length. The total theoretical sequence space of a viral genome of 10,000 nucleotides is $4^{10,000}$, a number that defies imagination (192, 238). The gigantic dimensionality of sequence space prohibits its representation in two dimensions. In Fig. 8B the theoretical sequence space is represented by a gray, punctuated, and diffuse background and the space occupied by two actual viral populations as spheres. This extreme oversimplification nevertheless allows us to make some points on evolution: a large sphere has a vastly superior number of possibilities to advance toward new regions of sequence space than a small sphere (Fig. 8B).

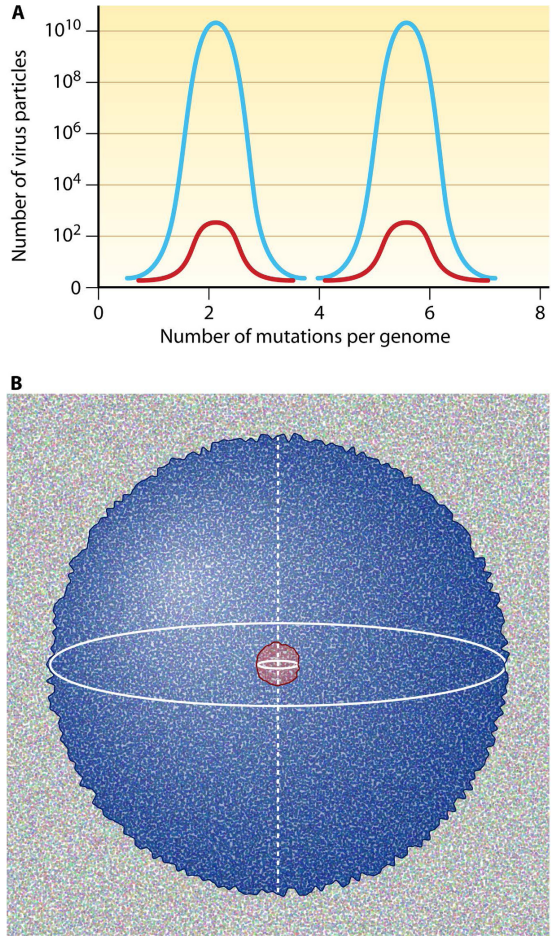


**FIG 8** Relevance of the complexity and size of the mutant spectrum in RNA virus evolution. (A) Diagram of four virus populations of different sizes and complexities. The blue and red curves represent populations that differ greatly in size (ordinate). Populations on the right contain a larger average number of mutations per genome than those on the left (abscissa). A large population size and high numbers of mutations favor adaptation unless interfering interactions or the threshold for virus viability intervenes. With an average number of about 5 mutations per genome, a population of $2 \times 10^{11}$ virus particles includes $3.3 \times 10^9$ single mutants and $2.6 \times 10^3$ genomes with 20 mutations (calculation based on the Poisson distribution, ignoring fitness effects of mutations). A population of $1 \times 10^8$ particles includes $3.3 \times 10^6$ single mutants and only 26 genomes with 20 mutations. (B) An imaginary representation in only three dimensions of a different occupation of sequence space. In the background of a huge theoretical multidimensional sequence space (square) (see text), viruses occupy a tiny minority that may require expansion to neighbor points in an adaptation process. The two spheres with a 10-fold difference in radius represent two viral populations that differ in sequence space occupancy. In this drastic simplification to three dimensions, viruses in the large sphere have a 100-fold-larger number of potential direct contacts in neighboring, unoccupied positions of sequence space than viruses in the small sphere (based on the sphere surface). Such neighboring positions can be reached by a limited number of mutational steps. Several examples of adaptability mediated by an increase of mutation rate or population size are described in different sections of the text.

The sequence space that can be actually occupied by a virus is a very minor portion of the theoretical space for obvious biological reasons: the viral genome must encode sequence-dependent regulatory signals, several secondary and tertiary structures in RNA perform functional roles, and open reading frames must encode

functional proteins which interact with other viral or host proteins, among many other limitations.

The comparison between a viral genome and a mammalian genome serves to illustrate distinctive implications of quasispecies depending on genome size. A viral genome of 10,000 nucleotides has a maximum of $3 \times 10^4$ possible single mutants (a theoretical figure that disregards fitness effects). This number is below the population size of many natural viral populations, even within a single infected organism (see "Viral Fitness and the Effect of Population Size: Bottleneck Events" above). In sharp contrast to the case for a typical RNA virus, the total number of possible single mutants in a mammalian genome is about $10^{10}$, a value which is well above the population size (total or effective) estimated for mammalian species. That is, the capacity to explore sequence space (and therefore the adaptive capacity) within reach of viruses is far greater than the capacity available to cellular organisms. Other parameters that also favor the adaptive capacity of RNA viruses are the number of mutations needed for relevant phenotypic traits and virus fecundity and turnover (Table 2) (see also next section). These quantitative arguments reinforce the concept that quasispecies dynamics is much more profitable to viruses than to cellular organisms as an engine for adaptation. This does not deny that important features of quasispecies dynamics, particularly those related to group selection, are displayed by subcellular and cellular collectivities and even by some "nonreplicative" biological macromolecules such as prions (65, 184, 483, 530, 619, 857) (see also Conclusions, Connections, and Prospects below).

At any given time, a mutant spectrum can be considered a cloud in sequence space (sphere in Fig. 8B). The cloud shifts its position (and, of course, variant composition) in response to selective forces, or a new cloud is started as a result of a bottleneck or founder event. An essential feature of sequence space is its high connectivity. Any two genotypes are separated by a number of point mutations that never exceeds the genome length. In practical terms, this means that points separated by a few mutations can be rapidly reached when movements are guided by a fitness gradient (238). Even in the case of a single mutation (or a small number of mutations, depending on the mutation rate exhibited by the replication machinery), neighboring points of sequence space can be reached through mere mutational pressure without a guiding fitness gradient. An example is the occurrence of drug resistance mutations that can be reached in the viral populations that replicate in infected patients. Drug resistance mutations are often selected in the presence of the drug, and in this case the drug is the selective agent that drives the fitness gradient. However, drug resistance mutations can be found in the mutant spectra of viruses whose populations had never been exposed to the drug (see "Selective Forces and Escape Mutants" and Specific Viral Systems below).

When a fitness value is assigned to each position of a portion of sequence space, a fitness landscape is obtained, following the classical metaphoric visualization of evolutionary events depicted by Sewall Wright (875, 876; see also reference 741 for limitations of this display). Two points (that are not easy to capture in theoretical models) must be emphasized with regard to fitness landscapes in viral quasispecies: they are extremely rugged and transient. These features are basically due to the rare occurrence of truly neutral mutations in compact genomes and to the fact that variable environments are the rule, and more so considering that the viral population structure itself is part of the environment (192).

The extension and portion of sequence space occupied by a virus during its replication may be relevant to its behavior, and both depend on parameters of the virus (see the previous section) and of the environment in which the virus replicates (738). The host factors that may contribute to modify the amplitude of a mutant spectrum have not been identified, but they may belong to two broad categories, one based on constraints and the other on participation. Stronger constraints in one host type versus another may render a larger proportion of viral mutations lethal (i.e., they occur, but they cannot be part of the mutant spectrum). Alternatively, the participation of host proteins in complexes with the viral polymerase may result in modifications of the template-copying fidelity. At any given time, the multiple genetic and biochemical factors involved in fidelity render the complexity of a mutant spectrum largely unpredictable. Its quantification requires empirical characterization through sampling of genomes and calculation of genetic distances, mutation frequencies, and Shannon entropies (see references 12, 31, and 289, among many other analyses) or characterization of mutant spectra by ultradeep sequencing (see references 56, 94, 175, 252, 281, 359, 454, 515, 556, 780, 788, 846, 858, 874, and 889), among other analyses).

A broad mutant spectrum means higher accessibility to multiple points in sequence space and, therefore, higher adaptability reflected in fitness gain and its biological derivations, including clinical outcome (130, 265, 404, 496, 705). Occupation of sequence space is multifactorial, but an important parameter is the error rate exhibited by the viral replication machinery. Studies with picornaviruses have contributed substantially to understanding of the genetics and biochemistry of RNA genome replication and to elucidation of the biological consequences of modifications of template-copying fidelity (reviewed in reference 232). A poliovirus (PV) mutant encoding amino acid substitution G64S in its RNA-dependent RNA polymerase (termed 3D) was selected by its decreased sensitivity to the purine nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1-*H*-1,2,4-triazole-3-carboxamide) (662). The same G64S mutant was obtained independently in the Andino's and Kirkegaard's laboratories. The mechanism by which substitution G64S confers resistance to ribavirin is an estimated 3- to 5-fold increase of the general template-copying fidelity of the enzyme that in this manner restricts the incorporation of ribavirin triphosphate (RTP) into RNA during RNA synthesis (33, 108, 660, 833). The limitation of ribavirin incorporation had as a trade-off the generation of a narrower mutant spectrum, and the narrowness restricted the tissue trophism of the virus, as evidenced by the inability of the virus to cause neuropathology in a strain of mice (660, 833). Neuropathology was restored when the mutant spectrum was expanded by mutagenesis (832, 833).

The studies with the G64S PV mutant and subsequent work with other picornavirus fidelity mutants (476) have revealed a number of implications of quasispecies dynamics (reviewed in reference 832). They validated the concepts that the error rate of a viral polymerase plays a key role in the observed heterogeneity of the corresponding mutant spectrum, that a limited heterogeneity can result in a selective disadvantage for the virus, that the amplitude of the mutant spectrum can be a stable trait maintained after several passages in cell culture, and that intramutant spectrum complementation can occur *in vivo*. Resistance to ribavirin mediated by substitution G64S in 3D had two related consequences: a limited incorporation of ribavirin into viral RNA and a greater tolerance to new mutational events (which could still occur as a

result of ribavirin incorporation) by virtue of the higher-than-average mutant fidelity and decreased average mutation frequency (i.e., its replicating further away from the error threshold than the wild type) (153, 832).

It must be indicated that the adaptabilities of the high-fidelity G64S polymerase mutant and wild-type PV might have been indistinguishable if the selective constraints had been less demanding. For example, for a given virus population size, the mutant and wild-type viruses could be equally adaptable regarding generation of antibody or drug escape mutants or replication in highly susceptible animal hosts, with no noticeable differences in the ensuing pathogenesis (426). In contrast, when either constellations of mutations or complementation between different mutant genomes is required for adaptation to a complex environment, a broad mutant spectrum may prove to be advantageous or even essential. New fidelity mutants of RNA viruses are becoming available (48, 141, 180). Further biological studies with fidelity mutants should provide a more quantitative picture of the selective advantage assignable to the complexity of the mutant spectrum. It has been suggested that the long-term adjustment of high mutation rates during RNA virus evolution might have been commensurate with the advantage derived from generating an ample mutant spectrum following a population bottleneck (466, 832, 833). As reviewed in "Viral Fitness and the Effect of Population Size: Bottleneck Events" above, bottlenecks are abundant in the course of the natural life cycles of viruses, and mutants capable of adapting to a complex environment following a population bottleneck should overgrow those mutants which can spread to only a limited subset of surrounding cells. This is an important point that deserves additional experimental and theoretical studies.

High- and low-fidelity retroviral reverse transcriptases have been described and characterized. Their study at the enzymological level is an active field of research, but the biological effects of fidelity on retrovirus adaptability have been only partially investigated (546, 547). Some of the amino acid substitutions involved in template-copying fidelity are related to resistance of HIV-1 to antiretroviral agents (221, 412, 545, 548, 629, 690, 752). Increased fidelity often resulted in decreased HIV-1 fitness (842, 860), and the relevant mutants offer valuable materials to evaluate the biological impact of retroviral mutant spectra displaying differences in complexity (see also "Human Immunodeficiency Virus Type 1," "Hepatitis B Virus," and "Hepatitis C Virus" below).

The term barrier is used to describe the limitations that a virus must overcome to achieve a required phenotype. Genetic barrier refers to the number of mutations needed for adaptation. A single amino acid replacement can have either a low genetic barrier if its occurrence requires a single transition mutation or a high barrier if it requires two mutations (more so if transversions are needed). Phenotypic barrier refers to the fitness cost that the amino acid substitution imposes upon the virus. The concept of barrier is further treated in connection with modifications of host cell tropism and escape mutants in later sections, but here we want to emphasize that a broad mutant spectrum can contribute to overcome genetic barriers during selection of mutant viruses (Fig. 7 and 8).

The discovery of a functional 3′-to-5′ exonuclease domain in coronavirus nonstructural protein nsp14 (228, 555) is highly informative with regard to the operation of the error threshold relationship in RNA viruses. Coronaviruses are the largest RNA genomes characterized to date, with the number of genomic nucleotides being between 28,000 and 32,000. No proofreading function has been identified in the polymerases of other RNA viruses with shorter genomes, although some indirect mechanisms of RNA repair have been described (580). Significantly, other *Nidovirales* which have a genome half the size of the coronavirus genome lack the exonuclease function (776). Coronaviruses with mutations in nsp14 display either severe defects in RNA synthesis (555) or a dramatic (12- to 15-fold) decrease in template copying fidelity, as judged from the complexity of the spectra of the mutant viruses in relation to the wild-type virus (180, 227, 228). Interestingly, nsp14-defective mutants are viable, and their capacity to enhance the mutant spectrum complexity appears to be a stable trait, at least in the course of several cell culture passages (180, 227). A coronavirus with a functional 3′-to-5′ exonuclease and the same virus with an inactive exonuclease constitute an attractive system to probe the biological consequences of mutant spectra that differ more than 10-fold in mutational input. Important questions such as adaptability *in vivo* or the effects of proximity to an error threshold for two RNA genomes that differ only in the proofreading activity are now amenable to investigation. However, since most viral proteins are multifunctional, when a fitness modification (typically a fitness decrease) is measured with an exonuclease mutant, it must be ascertained that the trait can be attributed to the modified fidelity and not to other roles of the protein harboring the exonuclease function.

Replicative RNA-dependent RNA polymerases and DNA-dependent DNA polymerases display multiple mechanisms to modulate their template-copying fidelity. Amino acid replacements may modify proofreading-repair activities or the intrinsic capacity of the polymerases to discriminate among nucleotide substrates during nucleic acid polymerases (363). The adaptive capacity of viruses and the availability of molecular mechanisms to modify polymerase fidelity suggest that high mutation rates have been positively selected during RNA virus evolution.

The studies reviewed in the previous paragraphs argue in favor of RNA viruses generally benefiting from an ample mutant spectrum. However, as predicted by the error threshold relationship (Fig. 1) and further documented in later sections of this article, too broad a spectrum may be deleterious to a virus. Mutant spectrum complexity cannot be considered a neutral trait. Amid the intricate network of cellular functions that contribute to (or oppose) viral multiplication, quasispecies dynamics provides a general adaptive strategy to respond to environmental requirements, including the array of innate and adaptive immune responses. A point of caution is in order here. When a complex mutant spectrum displays a selective advantage over a narrow one of the same virus, it may be because of increased complementation among components of a wider repertoire of mutants or because subsets of specific variants endowed with adaptive potential (independent of complementation) are represented in the complex (but not the narrow) mutant spectrum. If, in contrast, a narrow mutant spectrum is found to be more adaptable to a given environment than a broad one, it may be due to exclusion of interfering genomes or to enrichment in specific adaptable subpopulations in the narrower repertoire.

### Positive Selection and Molecular Memory: Deterministic Features of Viral Quasispecies

Since positive selection (defined in "Levels and Mechanisms of Genetic Variation, Competition, and Selection" above) involves

replication of a viral subpopulation that expresses the selectable trait and since replication generally entails fitness gain (254, 496, 600), selection permits an increase in the frequency of subsets of genomes. The increase of fitness resulting from replication during selection allows the subsequent maintenance of the selected subpopulation as memory genomes (707). They can remain present in the mutant spectrum at frequencies higher than those dictated by basal mutation rates (85, 707). The search for quasispecies memory was inspired in the fact that mobilization of minority biological elements in response to a stimulus is typical of complex adaptive systems such as the immune system. In response to a foreign antigen, the genomes of cells from the immune system undergo recombination and localized hypermutation, and those cells that manifest the highest affinity for the antigen are clonally expanded. This course of events, which has parallels with a selection of viral subpopulations, generates long-lived memory T cells that confer long-term immunity against the triggering antigen (11, 268, 291, 397, 403, 640, 647). We postulated that genomes present in the mutant spectra of viral quasispecies may include genomes that represented a record of those that were dominant at earlier phases of the same evolutionary lineage. Experiments with FMDV documented that when subsets of genomes that had become dominant in a viral lineage as a result of positive selection were outcompeted by other genomes of the same population, they did not reach their basal, standard mutation frequency. Rather, they attained a frequency higher than the basal one. This higher frequency was termed memory level, and it reflects the previous dominance of the same or similar genomes in the population (32, 85, 707, 710).

Molecular memory in viral quasispecies is a consequence of quasispecies dynamics, as suggested by the following observations. (i) Population bottlenecks eliminate memory, consistent with the fact that memory is a feature of the quasispecies as a whole (entire mutant distribution) and not of the individual genomes independently of the ensemble (190, 707). (ii) The frequency attained by a memory genome in a viral population is dependent on the relative fitness of the virus destined to become memory. In comparing memory levels of FMDV mutants, a 7.6-fold-higher initial fitness of a mutant resulted in 30- to 100-fold-higher frequency when the mutant became a memory genome (708). (iii) When present in a replicating viral population, memory genomes gained fitness in parallel with the most frequent genomes of the same quasispecies (32), in agreement with the Red Queen hypothesis (825). This hypothesis proposes that in competing populations there is a continuous selection of the most fit genomes in both populations, and this hypothesis has found experimental confirmation in studies with several RNA viruses (26, 132, 419, 487; reviewed in reference 652). (iv) The memory level can be durable, but it is gradually lost in replicating populations. Memory decay followed strikingly identical courses in parallel lineages, a fact that suggested a deterministic behavior of the virus population (710).

An alternative mechanism proposed for maintenance of quasispecies memory is complementation and phenotypic mixing-hiding (862). For reasons previously discussed (710), we think that complementation and phenotypic mixing-hiding did not participate in memory of the two genetic markers investigated with FMDV, although their participation in other cases cannot be excluded.

The demonstration of the presence of memory genomes was extended to HIV-1 *in vivo* (86). Two types of memory were distinguished and documented experimentally in HIV-1: a replicative memory similar to that described for FMDV and a cellular or reservoir memory derived from DNA integration into cellular DNA, inherent to the retroviral life cycle (86). Memory genomes influenced the evolution of HIV-1 in infected patients (84) and may have favored the reemergence of ancestral genomes documented in several HIV-1 populations (151, 166, 318, 390, 418). The hepadnavirus HBV, by virtue of it including a stable closed circular DNA in its life cycle, can also display a memory reservoir that has a number of implications for the intrahost evolution of this important human pathogen (see "Hepatitis B Virus" below).

The presence of replicative and reservoir memories in viral populations as the result of quasispecies dynamics has been supported not only by experimental studies but also by theoretical models on the behavior of evolving quasispecies (84, 86). It may be a widespread feature of living systems, since the concept of a genetic memory described for viruses was later extended to *Drosophila melanogaster* (802). Memory genomes confer on viral populations preparedness to respond to selective constraints already experienced by the same evolutionary lineage (reviewed in references 85, 190, and 652). The dynamics of memory acquisition is expected to operate *in vivo* when an antiviral agent is used to treat an infection and an antiviral-resistant mutant is selected and then outcompeted by other components of the mutant spectrum. Conversion into memory may occur as a result of treatment interruption or implementation of an alternative treatment regimen. This has been evidenced in the case of HIV-1 (84), and it is expected to operate in the case of new antiviral agents to treat chronic viral infections. It is presently a debated issue whether it will be cost-effective to subject viral populations to microarray screening or to ultradeep sequencing analysis for personalized treatment planning. The administration of drugs whose resistance mutations are present in the mutant spectrum could be avoided, thereby delaying selection of resistant mutants and extending the benefits of a treatment. The debate is reminiscent of the one that took place 3 decades ago regarding the benefits of standard sequencing information to manage HIV-1 infections, which was clearly resolved in favor of using such information.

It was particularly interesting that memory decay followed strikingly identical kinetics in four parallel lineages despite finite population sizes and populations being far from equilibrium (710). Two phenomena were proposed to underlie the deterministic kinetics of memory decay. One is an averaging effect of different mutations on fitness of the memory genomes relative to other genomes of the same population. Similar effects of mutations on fitness in independent lineages were also noted in biological clones of FMDV subjected to plaque-to-plaque transfers (255, 467, 468, 509) (see "Viral Fitness and the Effect of Population Size: Bottleneck Events" above). The second phenomenon that could contribute to the deterministic behavior is the limited tolerance to mutations of a compact picornavirus genome that would limit the pathways toward discordant diversification (710).

Memory decay is not the only process for which a deterministic behavior has been observed in RNA viruses. Two competing populations of VSV gained fitness in parallel, in agreement with the Red Queen hypothesis, and only occasionally a superior mutant arose and excluded other mutants from the competition (132), in agreement with the competitive exclusion principle of population genetics (308, 350). Whether this principle is really of general va-

lidity was a strongly debated issue decades ago. For example, Ayala showed that two species of *Drosophila* coexisted for many generations despite competing for limited resources (38). The different behaviors of *Drosophila* and VSV may reflect one of the salient features of RNA genetics: the highly dynamic nature and large population size of VSV—with opportunity for the generation of perturbing high fitness variants—versus the genotypic and phenotypic inflexibility of the *Drosophila* population within the time frame of the experiment.

This difference renders even more surprising a deterministic feature that was identified in competitions between a VSV and a neutral mutant derivative that was phenotypically marked (682). At nearly constant periods of time (competition passages), a critical point was reached at which the wild type dominated over the neutral mutant but never the converse. It was proposed that this nearly deterministic behavior was mediated by an averaging of mutational noise signals that occurred during the competition process (682). This interpretation is related to the one proposed for deterministic memory loss (710). Additional experiments showed that environmental perturbations introduced during the competitions between VSV and its neutral derivative (for example, an increase of temperature, 5-fluorouracil mutagenesis, or the presence of defective interfering [DI] particles) favored the dominance of the wild-type virus (681). The consensus sequence of the two competing viruses differed at six positions. It was proposed that the six mutations that occurred when generating the marked neutral mutant, and which moved the virus to a different position in sequence space, rendered the mutant less adaptable. While the mutant maintained the same fitness as the wild type under normal growth conditions, under environmental demands it could not attain beneficial mutations as rapidly as the wild-type virus (681). This led to a contingent neutrality that could explain the deterministic behavior observed. These results are in agreement with *in silico* competitions carried out with simple replicons (864).

The molecular basis for deterministic behavior of finite populations of RNA viruses will generally be difficult to resolve. What the experimental results suggest is that stochastic or deterministic features may dominate virus evolution and that it will be extremely interesting to define which parameters (tolerance to mutations, virus population size, environmental homogeneity versus heterogeneity, etc.) may favor one or the other (468, 704, 710).

### Complementation and Defection: Quasispecies as a Unit of Selection

Complementation is a process by which a genome expressing a functional protein can promote replication of another closely related genome whose corresponding protein is defective (or suboptimal). Interference is the mirror image of complementation in the sense that the defective protein impedes the functionality of the competent partner.

The occurrence of complementation among components of a mutant spectrum was suggested in the early studies that showed that individual biological clones isolated from a virus population displayed lower average fitness values than the entire population from which they had been isolated (215, 224) (Fig. 9). Since then, extensive evidence in favor of intraquasispecies complementation has accumulated. The PV fidelity mutant G64S (see "Exploration of Sequence Space and Virus Adaptability: Fidelity Mutants" above) did not replicate efficiently in the brain of susceptible mice. However, when it was coinoculated with wild-type PV or with a

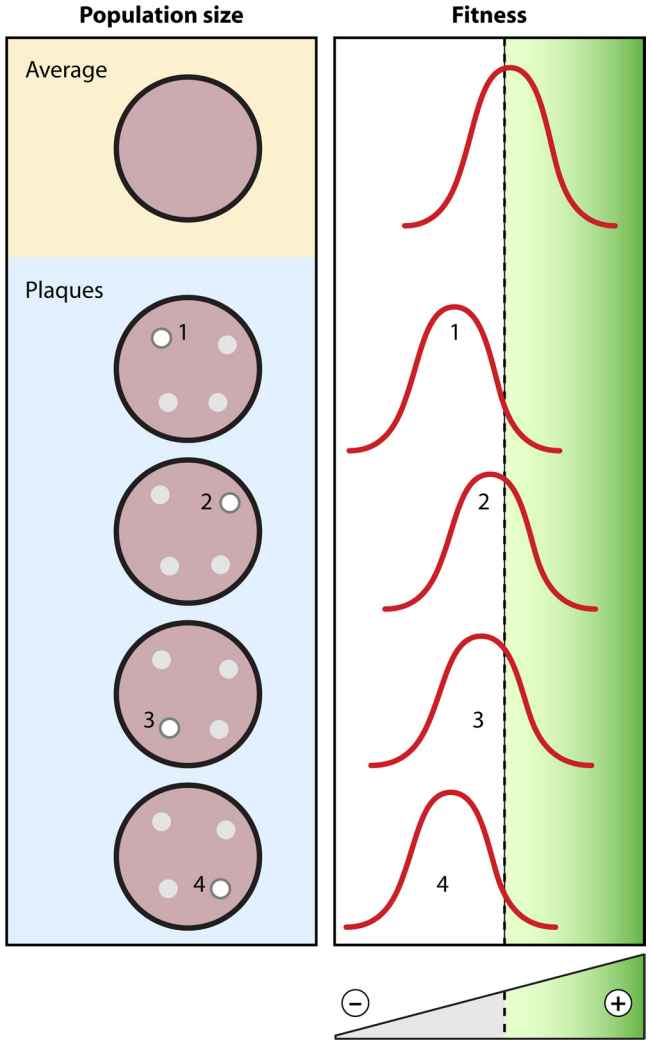


**FIG 9** Early evidence of complementation within a viral quasispecies. The average fitness of an uncloned population is higher than the fitness of viral populations grown from individual viral plaques 1, 2, 3, and 4 (215, 224) (fitness values are depicted as a triangle at the bottom).

G64S mutant with an expanded mutant spectrum, the G64S mutant entered the central nervous system (CNS) and replicated (832, 833). This observation suggests that members of the more diverse PV mutant spectrum complemented the more restricted population derived from the G64S mutant, allowing the latter to access the brain. It is not clear whether complementation was due to coinfection of the same cells or to other, indirect mechanisms (66, 832).

Complementation between viral mutants has been extensively described, and it underlies maintenance of defective genomes (defective interfering particles or other defective types) in viral populations (1, 312, 563, 618, 703, 867). The attractive extension to quasispecies dynamics is that complementation may be exerted not only among dominant genomes (as in the experiments to define complementation groups and cistrons of classical genetics or in an evolved bisegmented form of FMDV [618] [discussed in "Fitness Gain and Genetic Change: Genome Segmentation" above]) but also among components of a quasispecies, the result of which is fitness enhancement (Fig. 10).

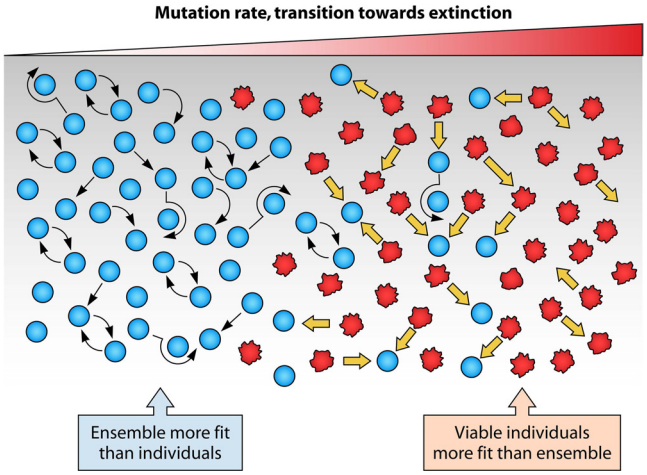


**FIG 10** A schematic representation of interactions of complementation and interference (or defection) within mutant spectra. Standard genomes are depicted as smooth blue spheres and interfering genomes as rough red spheres. Each symbol (even of the same shape) portrays a slightly different genomic sequence. Complementing interactions (thin arrows, left side) dominate when an RNA virus replicates with standard mutation rates. Under such conditions, the fitness of the population ensemble is higher than that of the individuals that compose the population. When mutation rates increase, interfering interactions (thick arrows, right side) dominate and viable individuals are more fit than the ensemble. (Adapted from reference 212.)

Related in origin but converse in its result on fitness, interference is exerted by RNA genomes or their expression products on replication of other components of the viral population. This interference phenomenon is distinct from RNA interference by small RNAs or from the high negative interference that concerns the unexpected multiple template switches that may occur during recombination (114, 384). De la Torre and Holland showed that a VSV quasispecies could suppress VSV genomes that in isolation displayed higher fitness than the suppressing population (171). This result represented the first experimental observation that a mutant spectrum could guide the behavior of specific mutants included in the spectrum. That a lower-fitness genome can acquire a selective advantage over a more fit genome by virtue of a favorable mutant spectrum was predicted by an early computer simulation based on quasispecies theory (238, 789). Suppression of high-fitness antigenic variants by low-fitness antibody escape populations was described for FMDV replicating in cell culture (78). PV mutants delayed replication of drug-resistant mutants (154).

As a counterpart to the complementation required for replication of the high-fidelity PV (832, 833), attenuated PV suppressed the neurological disease in monkeys associated with virulent PV (126). Also, the growth hormone deficiency syndrome in mice, which is associated with replication of pathogenic lymphocytic choriomeningitis virus (LCMV) strains in the animals, was suppressed by nonpathogenic LCMV variants (801). Thus, a variety of cell culture and *in vivo* virus-host systems have provided evidence of suppressive and interfering effects within quasispecies. An important challenge is to elucidate the molecular interactions that lead to the interference. They may involve the same mechanism displayed by dominant negative interactions of a virus mutant on the corresponding wild type exerted by one of several proposed mechanisms (886). Some may be parallel with interference exerted by defective interfering (DI) particles (703), although when viable mutants are involved, subtle interactions among viral gene products might play a role. Coelectroporation of BHK-21 cells with wild-type RNA and an excess of specific capsid or polymerase mutants resulted in suppression of infectivity by mutants that were competent in RNA replication (654). A single amino acid substitution could convert an interfering mutant into a noninterfering mutant and vice versa. Thus, small movements in sequence space can transform a complementing functional protein into a defective, interfering counterpart so that mutational events can modulate complementation and interference in quasispecies.

An excess of a mixture of interfering FMDV mutants synergistically suppressed the infectivity of standard, infectious FMDV (654). This result is in agreement with suppression of FMDV infectivity by preextinction, heavily mutagenized FMDV RNA populations (320). Indeed, sequence analyses show that preextinction viral populations are enriched with multiple mutants that, although present at low frequency, could give rise to interference as a collective manifestation of low-frequency, functionally deficient mutants (331, 392). Defective genomes in natural viral populations appear to be abundant, as judged by the identification of stop codons within open reading frames. However, their biological roles are largely unknown. APOBEC3 decreased the specific infectivity of Friend retrovirus, and noninfectious particles stimulated the neutralizing antibody response (773).

A number of theoretical models have introduced competition, cooperation, and complementation in the description of quasispecies and the error threshold (27, 729). These models offer realistic elements that may provide them with a predictive value (562). The collective behavior of viral quasispecies suggests that at least at times a mutant ensemble rather than an individual mutant can be the target of selection. This has been and still is a hotly debated topic in general genetics. Nevertheless, the evidence that a viral quasispecies can be the target of selection provides an important distinction from the classical Wright-Fisher formulations of evolutionary dynamics, and it has been one of the reasons to recognize that quasispecies can embody evolutionary mechanisms not always reconcilable with those of classical population genetics (for papers and reviews on this topic, see references 95, 140, 530, 578, 619, and 861). The intraquasispecies interactions may be exerted by mutants generated intracellularly within the same replicative ensemble or by exogenous mutants that penetrate into a cell together with the standard virus (and both will generate a corresponding mutant cloud). Some virus-host systems display superinfection exclusion, and therefore, potentially interfering and standard genomes should coinfect cells simultaneously (562). Polyploidy (the presence of several genome molecules in the same virus particle) has been documented for some viruses (59, 383, 498). Therefore, interactions among viral genomes and their expression products within a cell can occur with participation of mutants generated within the cell or with mutants introduced either by coinfection, by reinfection (when superinfection is not excluded), or through polyploid particles.

The collective properties of mutant spectra represent an effect of mutations present in different genomes of a given population. This level of interaction in viruses has been explored to a lesser extent than interactions among mutations present in the same genomic viral RNA molecule. Such interactions are referred to as positive epistasis, in which fitness of a multiple-mutant genome is higher than those of the individual mutants. Conversely, negative

epistasis refers to lower fitness of the multiple mutant than of the individual mutants (245, 446, 626, 695). Epistatic effects in viruses may be obscured by the presence of a mutant spectrum, since the genome under analysis is in reality a mutant cloud in which subsets of genomes may increase or decrease their frequencies dynamically. The sequence indetermination may blur interactions between two mutations unless epistasis in an individual genome is strong (523). Compensatory mutations are an example of positive epistasis that permit survival of escape mutants when the mutation relevant for escape inflicts a fitness cost upon the virus (references 591 and 632 and references therein). Compensatory mutations are key to the maintenance of drug resistance mutations in viral populations, as further discussed in the next sections.

## BIOLOGICAL IMPLICATIONS OF QUASISPECIES DYNAMICS

### Cell Tropism and Host Range Mutants: Biological Alterations and Viral Emergence

The main features of viral quasispecies described in the previous sections have several biological implications that affect the virus-host relationship. The dynamics of selection of minority components can play a role in the modifications of cell tropism and host range, as documented with designed experiments and during virus evolution *in vivo*. The number of amino acid substitutions needed and the genetic and phenotypic barriers involved are determinant parameters of how likely a modification of cell tropism or of host preference will be. The understanding of the dynamics of modification of cell recognition is still fragmentary compared with, for example, that of the dynamics of drug resistance or drug dependence, although the underlying mechanisms involving selection of viral subpopulations are likely to be parallel.

The capacity of a virus to productively infect a cell does not depend only on recognition of a cellular receptor on the cell surface but also on intracellular host factors that render a cell either permissive or refractory to virus multiplication and to formation and release of virions. Host cell specificity is multifactorial. That is, amino acid substitutions either in the viral capsid or surface proteins (which interact with receptors or potential receptors) or in nonstructural viral proteins (which interact with cellular components and influence permissivity to virus multiplication) can result in modifications of cell tropism or host range.

The receptors used by viruses to recognize and penetrate into cells belong to different families of macromolecules (proteins, lipoproteins, glycoproteins, glycolipids, glycosoaminoglycans, etc.), which are often organized as complex structures on the cell surface (46, 61, 155, 517, 739, 866). These diverse molecules perform disparate cellular functions (adhesion, signaling, etc.) and are often present in large numbers ($10^3$ to $10^4$ per cell) on the cell surface (155). The presence of the receptor is a necessary but may not be a sufficient condition for infection. Molecules such as sialic acid or heparan sulfate (HS) may function as bona fide viral receptors or may act as macromolecules that recruit viruses on the cell surface to facilitate their reaching a true receptor which mediates penetration into the cell. Other molecules, often termed coreceptors, might be needed for efficient entry (61). The absence or blockage of any required receptor or coreceptor may impede virus entry into a cell.

When a new virus emerges or is discovered in the field, it is very difficult to predict the type of receptor molecule it might use. One of the reasons for such unpredictability is that receptor usage does not follow any obvious correlation with the phylogenetic position of a virus, as determined by nucleotide and amino acid sequence comparisons (436). The current system of virus classification, despite serving as a fundamental guide to virologists, suffers from serious limitations derived in part from the variable nature of most viruses it intends to classify. Historically, virus classifications were guided by physical properties, antigenic relationships, and disease manifestations. More recently, comparisons of genomic nucleotide sequences and amino acid sequences of viral proteins have served as the basis for classification (references 464, 465, and 760 and references therein). With a phylogenetic relationship based on comparing several genes of viruses (or ideally the entire genomic sequence), a single nucleotide or amino acid change in one of the viruses has no effect on its phylogenetic position. Yet, a single or few amino acid changes can nevertheless profoundly alter host cell tropism and other important phenotypic traits of viruses, as covered in several sections of this article. A virus may include in its mutant spectrum genomes that despite belonging to the same phylogenetic group defined by the consensus sequence may differ in salient biological properties. The ambiguous situation created is not easy to solve, but it has been suggested that sequence data banks could be expanded to include characterized minority sequences present in the mutant spectrum (see references 193 and 208 for discussions of the limitations of the current system of virus classification).

Related viruses (according to current classifications) may use different receptors, unrelated viruses may share a receptor, and viruses may display the same tissue tropism but using different receptors. The majority of human rhinovirus (HRV) serotypes bind to intracellular adhesion molecule 1 (ICAM-1), while a few HRV serotypes bind members of the low-density lipoprotein receptor (LDLR) family. Subgroup C adenoviruses and group B coxsackieviruses share the appropriately termed coxsackievirus-adenovirus receptor (CAR) (reviewed in reference 61). Binding to decay-accelerating factor (DAF) is shared by phylogenetically divergent clusters of enteroviruses (672). Although not all receptors and coreceptors recognized by hepatitis viruses have been identified, the receptor for hepatitis A virus (HAV) (HAV receptor 1) and the candidate receptors and coreceptors for HCV (the tetraspanin CD81, scavenger receptor type B1 [SR-B1], epidermal growth factor receptor [EGFR], ephrin receptor A2 [EphA2], and the tight junction components claudin-1 and occludin) belong to different protein families (49, 261, 417, 665, 668, 683) despite these viruses sharing hepatotropism. The hemagglutinin esterases (HEs) are glycoproteins present in coronaviruses, toroviruses, and influenza virus (IV) type C. These proteins probably have a common origin and have evolved to confer on the viruses different sialic acid recognition specificities (references 461 and 890 and references therein).

Either mutations that lead to amino acid substitutions at external residues of surface proteins (arrived at by either diversification or convergence) or recombination events that result in an exchange of modules (encoding relevant protein motifs) may result in modification of the host cell tropism. When mutations alone can mediate a shift in receptor recognition, the mutant spectrum (with minority genomes encoding altered proteins that have the potential to use alternative receptors) can play a role. Passage of FMDV in cell culture selected virus mutants that included amino acid substitutions in the capsid surface that permitted infection through the binding to HS at the cell surface (45–47, 61, 395, 715). HS-binding variants of FMDV are present as minority compo-

nents of FMDV that replicate in cattle tissues *in vivo* (874). A severe acute respiratory syndrome (SARS) coronavirus with increased replication in human proximal tubular epithelial cells (PTEC) of the kidney was selected after a few passages in these cells (624). The availability of a given receptor molecule (be it HS in cells in culture, DAF in epithelial cells lining the intestine, coreceptor CCR4 or CXCR5 in cells of the immune system, or others) may favor selection of those components from mutant spectra that can derive a productive infection from using an available (and functional) receptor (46, 687). That new receptor might be different from the one used by the majority of components of the mutant spectrum (45, 46, 61). Selection dynamics of minority quasispecies components was evidenced during IV adaptation to different cell lines (699). Again, there is a biological implication of the presence of minority subpopulations that can be selected in response to a matching receptor molecule.

The concept of barrier, explained in "Exploration of Sequence Space and Virus Adaptability: Fidelity Mutants" above, applies also to modifications of cell recognition. A new functional receptor-virus interaction may require the replacement of multiple amino acids on the virus surface, thus rendering their occurrence and the ensuing selection unlikely (836). While a single amino acid substitution to eliminate receptor recognition is expected to be a frequent event (not noticeable because the corresponding virus will be eliminated by negative selection), generation of a new receptor recognition site through multiple amino acid substitutions is an event with a high genetic barrier (reference 688 and references therein). The low probability of occurrence can be very roughly estimated from the actual number of amino acid replacements needed and the number and type of mutations involved. The newly generated receptor recognition site should obviously have a positive effect on fitness because it permits the virus to penetrate in a new cell type. However, the required amino acid substitutions may negatively affect some other trait such as capsid or surface protein stability. In this case a large phenotypic barrier may impede selection, and the order of appearance of each mutation would be of importance for fitness at specific stages.

Minimal genetic change has proven sufficient to modify the cell tropism and pathogenic potential of a virus (several examples were reviewed in references 45, 46, 82, 149, 539, 678, 820, and 879). This is the case for the modification of CCR5 or CXCR4 coreceptor usage by HIV-1 (82, 167, 355) or changes in the sialic acid-binding preference of the hemagglutinin of IV (reference 822 and references therein). Mutations that abolish sugar-binding activities of the coronavirus transmissible gastroenteritis virus (TGEV) eliminate the enterotropism of the virus (448). The respiratory tropism and absence of enteric tropism of porcine respiratory coronavirus are associated with a deletion at the N-terminal domain of the coronavirus spike protein (see reviews and additional references on coronavirus receptor usage and its variations in references 328 and 646). Structural studies have documented at least three different mechanisms by which the surface glycoprotein of paramyxoviruses can establish functional interactions with protein and glycan receptors, suggesting a great potential for the emergence of new paramyxoviral pathogens (81).

HRV mutants adapted to using HS as receptor must either be acid labile or acquire acid lability through mutation to compensate for the absence of the uncoating activity of ICAM-1 (428, 837), which constitutes an example of a phenotypic barrier. FMDV uses integrins to infect cells *in vivo* (54, 394, 396, 558, 586). The integrin recognition amino acid triplet RGD is located on a mobile loop of capsid protein VP1, protruding from the capsid surface (2). Because the RGD is highly conserved among field isolates, this triplet was considered universally essential for FMDV infection. However, FMDV that was passaged multiple times in BHK-21 cells did not require the RGD to infect cells, and instead it used HS or a third, unidentified receptor (45–47).

The canyon hypothesis proposed that a receptor recognition site should be shielded from the variation that occurs at antigenic sites (702). Contrary to the canyon hypothesis, the VP1 loop in which the integrin recognition site of FMDV resides is also a major antigenic determinant of the virus that includes multiple overlapping epitopes (2, 533, 536–538, 829, 830). These studies showed an intricate connection among the different epitopes and documented that some of the amino acids that determine integrin recognition are also involved in binding to neutralizing monoclonal antibodies (MAbs) (533, 534, 538, 829). Thus, receptor recognition sites need not be separate from antibody recognition sites. Any mutation that abolishes integrin recognition, for example, mutations affecting the RGD, will be subjected to negative selection (like those affecting any other amino acid critical for a viral function), and the corresponding virus will not survive unless it can be rescued by complementation. In this view, the selective advantage derived from hiding a receptor recognition site from antibody recognition is largely irrelevant. Not only can receptor and antibody recognition sites overlap, but some antibodies penetrate deep into the picornavirus canyon (774).

Overlap of receptor and antibody recognition sites has been documented with many other RNA and DNA viruses, and it now appears to be the rule rather than the exception (46, 61, 391, 892). One of its consequences is the potential of coevolution of host cell tropism and antigenicity, two important traits in the life cycle of viruses. Coevolution is defined as an interaction between two (or more) biological entities by which they exert evolutionary effects on one another (21, 297, 419, 873), as clearly documented with viruses and cells during persistent infections in cell culture (see, e.g., references 10, 172, 701, and 893). An alteration of receptor specificity that entails a change in binding to antibodies or vice versa can qualify as a coevolutionary event. An antigenic domain that shares amino acid residues with a receptor-binding site will lose part of its constraints for variation when the receptor ceases to be functional, for example, because an alternative receptor is used instead. When evolution of FMDV in cell culture rendered the RGD dispensable, the repertoire of MAb escape mutants expanded relative to the repertoire obtained with the wild-type virus. This was shown with a major neutralization epitope located at amino acid residues 138 to 147 of capsid protein VP1 of FMDV (Fig. 11). None of a total of 84 independently isolated MAb SD6-resistant mutations of the parental FMDV C-S8c1 affected the RGD sequence, because the sequence is needed for integrin receptor recognition. In contrast, 26 out of a total of 46 SD6 escape mutations obtained using C-S8c1p100 (the passaged C-S8c1 population that evolved to recognize alternative receptors) mapped within the RGDL (47, 196, 526, 711) (Fig. 11). This specific example illustrates how escape mutant repertoires present in mutant spectra can vary depending on biological features that in this case were recognized but that in other cases can go unnoticed.

A remarkable case of coevolution *in vivo* was revealed by the analysis of the FMDV that replicated in cattle previously immunized with a peptidic vaccine. Such vaccines generally confer par-

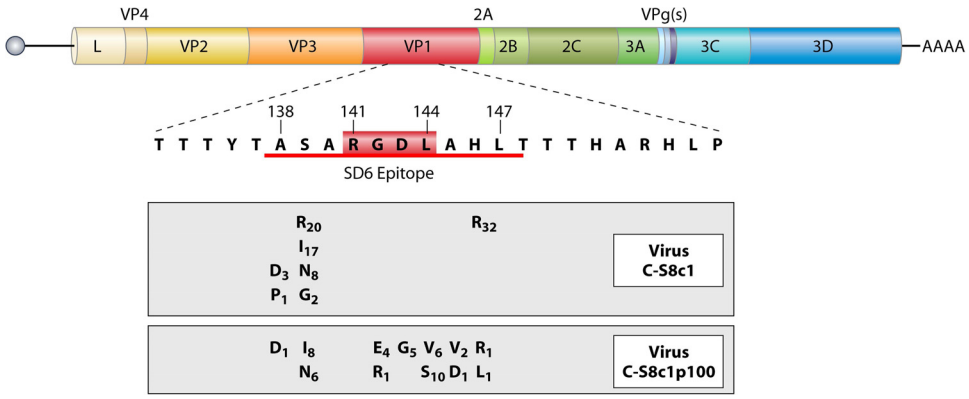


**FIG 11** Modification of the repertoire of antibody escape mutants of a virus associated with dispensability of a host receptor for infectivity. The picornavirus FMDV genome (displayed at the top) includes a major antigenic determinant (amino acid sequence written with the single-letter code), with an epitope recognized by neutralizing antibody SD6 (underlined sequence) which includes the sequence RGDL (boxed) also involved in recognition of integrin receptors. FMDV C-S8c1 SD6 escape mutations map around but not within the RGDL (top gray box). Subscripts indicate the number of times that a given escape mutant was obtained in independent selection events. The repertoire of SD6 escape mutants of FMDV C-S8c1 passaged 100 times in cell culture (C-S8c1p100) expanded to include substitutions at residues G142, D143, and L144 (bottom gray box). The expansion of the escape mutant repertoire was due to integrin receptors being dispensable for the multiply passaged FMDV. (Adapted from reference 653 with permission.)

tial immunity because they present the host immune system with a limited repertoire of B- and T-cell epitopes. Exposure of a limited antigenic repertoire by a vaccine is against one of the principles of vaccinology, which asserts that for a vaccine to be effective it should evoke an immune response which is similar to that evoked by the authentic pathogen (72, 202, 260). Upon challenge with virulent FMDV, some of the peptide-vaccinated animals developed viremia and vesicular lesions. Virus from several lesions included amino acid substitutions at the RGD or neighboring residues that resulted in altered host cell specificity (791, 795). Modifications such as those described for FMDV require very few mutations, and therefore, there is a tangible probability that these classes of mutants are present in the viral quasispecies, either because the mutations do not inflict a significant fitness cost or because mutants harboring them can be maintained by complementation.

Other cases of modification of host cell tropism emphasize the relevance of quasispecies dynamics in presenting collections of mutants or recombinant genomes with the potential to interact with alternative receptors (reviewed in references 45, 46, 61, and 192). Modification of antigenic sites can also affect the antibody-dependent enhancement of viral infection, a mechanism exploited by several viruses (793), including FMDV (336, 531, 696).

Analysis of mutant spectrum composition, together with structural studies, may provide some hints about possible evolutionary outcomes in relation to receptor recognition. Specifically, three-dimensional structures of complexes between viral attachment proteins and their cognate (standard) cellular receptors may be informative in regard to how related viral proteins can adapt to using alternative receptors (159, 783).

Host cell tropism and pathogenicity can depend on substitutions at structural or nonstructural viral proteins. Amino acid substitutions in the IV polymerase subunit PB2 can promote adaptation to alternative hosts, and one of the mechanisms probably involves enhancement of viral replication and increases in viral load through interaction with host proteins (299, 300, 357, 786, 881). In a laboratory study designed to investigate the events that led to adaptation of a swine FMDV to the guinea pig, it was shown that upon serial passage of the virus in guinea pigs, variants with amino acid substitutions I248T in protein 2C and Q44R in protein 3A were selected (609). Only upon further passage was replacement L147P in the antigenic loop of capsid protein VP1 selected. The three amino acid substitutions were maintained upon replication of the virus in swine and suckling mice. Amino acid substitution T248N in nonstructural protein 2C, not detectable in the parental virus, was present as a minority genome in the guinea pig-adapted virus and became dominant upon infection of pigs. A minority variant arising in an unnatural host may become dominant on reinfection of the original host species (610).

Cross-species transmission can be influenced by the ability of the virus to select mutants that overcome host restriction elements (438). An LCMV variant differing in two amino acids from the wild type displayed altered cell tropism in spleen, induced a generalized immunosuppression, and established a persistent infection in mice (79). An amino acid substitution in encephalomyocarditis virus (EMCV) can render the virus diabetogenic (42). Cardiovirulence of coxsackievirus B3 can be determined by a single site at the 5′ untranslated region (UTR) (815). (For additional studies on coxsackievirus B pathogenesis and evolution and the relevance of quasispecies for enteroviruses in general, see several chapters of references 232 and 812).

Phenotypic plasticity and, in particular, coevolution of antigenicity and cell tropism can promote host range alterations of viruses and therefore contribute to viral disease emergence. New human viral diseases have emerged at the rate of about one per year over the last decades and in some cases with a monumental impact on public health, such as with the current AIDS pandemic. Several studies have dissected a number of contributing sociopolitical and environmental influences, including the zoonotic origin of many human viral diseases (reviewed in references 24, 41, 185, 337, 387, 447, 503, 565, 638, 658, and 775). Pangenome analyses using ultradeep sequencing are currently unveiling the presence of multiple virus types in a number of biological habitats (583). The results have confirmed previous evidence that rodents are a source of multiple viruses, some of which are yet to be classified (reference 663 and refer-

ences therein). Viruses that replicate in rodents will generate mutant spectra that may reach new hosts as a result of animal movements and increases of rodent populations due to agricultural practices. Rodent populations offer a typical example of the evolutionary and environmental influences on viral disease emergence, but many additional animals (notably bats and primates) can be a potential source of multiple variant forms of many viruses (185, 447, 587). The detection of a viral sequence, however, does not imply that an infectious virus is present.

### Diversification and Self-Organization of Viral Quasispecies

In previous sections of this article we have provided evidence of virus behavior being the result of interactive genome ensembles, as in the presence of a genetic memory that is erased by bottleneck events. Here we describe the evolution of quasispecies toward ensembles that display new phenotypic traits. A significant example of how viral quasispecies can self-organize to modulate cell killing was observed with FMDV replicating in BHK-21 cells, a stable biological environment (616). A clone of FMDV (C-S8c1, the progeny from a single genome) was passaged at a high MOI to ensure that multiple viral particles infected the same BHK-21 cell. At passage 143, a MAb-resistant variant termed MARLS was selected (113). This variant displayed high fitness and killed BHK-21 cells efficiently (here called "high virulence" despite referring to a trait in cell culture) (364). Surprisingly, the sequences that were the signature of MARLS (32 mutations relative to the parental genome C-S8c1) did not become dominant even after 460 passages. When viral samples from several passages were subjected to three low-MOI passages, MARLS-like populations were rescued. Moreover, biological clones isolated from those populations segregated into two classes: those similar to the uncloned populations (termed p200 clones) and those that were MARLS-like. This evolutionary pattern suggested that the high-fitness, high-virulence MARLS subpopulation was suppressed by subpopulation p200. Cell killing measurements and cell killing interference assays indeed distinguished between the MARLS-like clones, which were termed the colonizers, and the p200 clones, which were termed the competitors. Both subpopulations exhibited a competition-colonization dynamics similar to that previously recognized in classical ecological systems (806). A theoretical model of coinfection dynamics was developed, and it predicted a density-dependent (MOI-dependent) selection of either colonizers or competitors, a point that was confirmed experimentally (616). The experiments indicated that a single clone of FMDV can diversify into genetically distinct subpopulations that exhibit a competition-colonization dynamics in which the killer colonizers are suppressed by the competitors that act as modulators of virulence. The study with FMDV has stimulated theoretical simulations of virulence modulation through the interaction of viral subpopulations in coinfected cells (174).

Coinfections of cells by multiple viruses, perhaps preferentially in coinfection-prone cell subsets (122, 129, 177), must be frequent as judged by the generation of recombinant viruses *in vivo* (1, 4, 5, 157, 414, 766, 769, 804). Thus, coinfections may play a role in modulation of virulence in addition to being a source of recombinant viruses. Specifically, in experiments with serial passages of FMDV in mice, attenuated strains were isolated in organs in which coinfection was more likely (compare references 616 and 727).

Thus, molecular memory, positive interactions of complementation to facilitate survival of mutant subsets, negative interactions that favor virus extinction, diversity that modulates virulence, and density-dependent selection (90, 171, 604, 750, 816, 817) are features than depend on mutant spectra and that can critically influence virus behavior.

### Selective Forces and Escape Mutants

Escape mutants are the subset of mutants present in a viral population that can replicate despite the action of a selective constraint that impedes replication of the genomes that represent the majority of the population. The selective constraints can be antiviral drugs, components of the innate or the adaptive immune response, or interfering RNAs, among others. The first descriptions of escape mutants to antiviral inhibitors were by Tamm, Barrera-Oro and their colleagues (230, 544). The ample occurrence of escape mutants of different viruses under a variety of environmental conditions *in vivo* and in cell culture is yet another manifestation of the presence of relevant variants in the mutant spectra of viral populations. Here we summarize only some representative quantifications and the implications of escape mutants for viral persistence.

The frequency of inhibitor-resistant mutants of viruses is generally in the range of $10^{-3}$ to $10^{-5}$ (reviewed in references 188, 202, 212, and 693). Relevant considerations that determine the actual frequency are the number of amino acid substitutions required for resistance, the genetic barrier (number and type of mutations needed for the amino acid substitutions), and the fitness cost that the mutations entail. Resistance to inhibitors often requires one or a few mutations, and there are multiple, alternative mutations that can confer resistance to a drug. Those mutations that confer resistance with a limited fitness cost are likely to be represented in mutant spectra. If 20 or more defined mutations were needed simultaneously to escape a selective constraint, a quasispecies with the range of complexities of mutant spectra encountered in infected organisms would be largely irrelevant. No escape mutants would be selected, but the survival of viruses would be incompatible with the environmental challenges posed by our biosphere. A demonstration of the adaptive capacity of a virus is not only that escape mutants are readily selected but also that virus replication may even become drug dependent (44, 173, 229).

All genetically variable viruses display similar positive selection events conducive to single-drug and multidrug resistance associated with one or several point mutations and, less frequently, with insertion-deletion or recombination events. Resistance within infected individuals or at the epidemiological level has been described for each of the drugs that have been used to treat human influenza infections. Multiple amino acid substitutions in the neuraminidase (NA) have been associated with resistance to the NA inhibitors used in anti-influenza virus type A drug therapy (I117V, E119V, D198N, I222V, H274Y, R292K, N294S, and I314V for oseltamivir and V116A, R118K, E119G/A/D, Q136K, D151E, R152K, R224K, E276D, R292K, and R371K for zanamivir), and resistance mutations have been detected in multiple avian, swine, and environmental isolates of the virus (622). Substitution H275Y in the NA of the 2009 pandemic IV confers resistance to oseltamivir; ultradeep sequencing revealed the presence of this mutation in 5% of clinical samples, the majority from patients subjected to oseltamivir treatment (179). Substitutions E119G and E119V conferred multidrug resistance to several NA inhibitors at a considerable fitness cost for the virus. I222V had a synergistic effect on

the oseltamivir and permivir resistance conferred by H274Y and compensated for the reduction of viral fitness (667).

Several families of inhibitors of picornaviruses are available, and drug resistance and sometimes drug dependence have been reported (173, 181, 210, 212, 229). Tamm and Eggers were pioneers in showing that combination therapy had an advantage over monotherapy (796).

New, effective inhibitors are often claimed to offer great promise because they can inhibit mutant viruses which are resistant to other drugs. This transient optimism generally ends with the observation that the new drug can itself select for additional resistant mutants and that multidrug resistance becomes increasingly frequent when several drugs are used in clinical practice. Drug resistance for HIV-1, HBV, and HCV is reviewed in Specific Viral Systems below. The course of events is similar for all pathogenic viruses and can be summarized as follows. (i) Drug resistance is a general phenomenon, and selection of a drug-resistant mutant is taken as evidence of specificity of an antiviral drug (188, 366, 666, 693). (ii) Multiple different mutations or combinations of mutations and, thus, mutational pathways can lead to drug resistance. There are several reasons for this, one being that multiple short-range and long-range interactions occur within a protein molecule (782, 818). As a consequence, a decrease of affinity for a ligand drug can come about through multiple amino acid replacements at different sites in the protein that binds the drug. A protein that forms a complex with the one targeted by the drug can also affect drug binding. (iii) High-level resistance to a drug may entail mutations different from those involved in low-level resistance, and thus, genomes displaying low and high resistance to the same drug occur at different frequencies (8, 360, 361, 828). (iv) As a general rule, substitutions that confer drug resistance increase viral fitness when fitness is measured in the presence of the drug and decrease fitness measured in the absence of the drug. (v) When multiple substitutions are involved in drug resistance in a given mutational pathway, some mutations may be selected for their fitness-enhancing effect, but they may not contribute directly to decreasing the affinity for the drug. These features should become more defined at the molecular level with the advent of ultradeep sequencing techniques, combined with additional fitness measurements *in vivo*.

Parallel arguments of Darwinian selection of mutant viruses apply to the immune response. The innate immune response against viruses has several components, some established on a permanent basis and others recruited as a consequence of virus infection. Permanent components include proteins of the complement system and cytokines and chemokines that gather cells (neutrophils, monocytes, macrophage, dendritic cells, natural killer cells, etc.) at the sites of infection. Systems recruited as part of the immune response against viruses are editing activities that perform important cellular functions. Among them, the adenosine deaminase acting on RNA (ADAR) proteins are a family of adenosine-to-inosine editing enzymes that act on double-stranded RNA (594). One of the isoforms of ADAR 1 is a restriction factor for several (but not all) RNA viruses (848). Little is known about viral antagonists of ADAR restriction, but hypermutated viral genomes generated by ADAR can contribute to antibody escape (521, 522) or to the generation of pathogenic hypermutated genomes (such as hypermutated measles virus genomes associated with subacute sclerosing panencephalitis [109]). Other cellular functions can modulate susceptibility to viral infections. This is the case of murine proteins Fv1 and Fv4, which are the products of expression of endogenous retroviruses; Fv1 blocks the entry of preintegration complexes into the nucleus, and Fv4 acts as a competitor of env for receptor binding. Other examples for retroviruses are the tripartite motif protein 5α (TRIM5α), zinc finger antiviral protein, or the APOBEC family of editing proteins (see "Human Immunodeficiency Virus Type 1" below for mechanisms used by HIV-1 to counteract immune responses and reference 186 for a discussion on how cellular functions can either contribute to or interfere with viral replication).

The adaptive immune response poses multiple selective constraints to virus replication, including neutralizing antibodies, several subsets of virus-specific T cells that can eliminate infected cells either by direct contact or through soluble cytokines (gamma interferon [IFN-γ] and tumor necrosis factor alpha [TNF-α]). The latter effectors also participate in the recruitment of innate immune components, thus contributing to the clearing of the infection. Viral escape mutants, which permit virus survival and replication in the face of the host immune response, can play an important role in either the establishment or maintenance of viral persistence (3, 131, 311, 388, 479, 643, 748, 749) (see also the discussions of the immune responses for HIV-1, HBV, and HCV in Specific Viral Systems below).

Vaccination may be an important evolutionary force for viruses (124). Vaccine escape mutants have been reported for HAV, a fact which is particularly relevant because a single serotype has been described for this virus (271, 440). HAV needs to maintain rare codons in the capsid-coding region, probably to regulate ribosome traffic to achieve an adequate folding of the capsid proteins (26). The requirement of rare codons might prevent the generation of antigenic variants that are sufficiently distant to qualify as a new serotype (25). Despite such likely restrictions, antigenic variants of HAV that probably escaped the immune response evoked by the available vaccines were isolated among immunocompromised individuals (656). This study reinforces the concept that partial immunization due either to low immunogenicity of a vaccine or to limited capacity of the vaccinees to evoke an immune response may allow continued viral replication and sufficient viral loads to promote the emergence of antigenic and cell tropism variants (46, 185) (see "Cell Tropism and Host Range Mutants: Biological Alterations and Viral Emergence" above). The observations on vaccine escape mutants of HAV agree with the presence of complex mutant spectra despite limited variation of the consensus sequence of HAV populations (152, 723).

It is tempting to invest in immunotherapeutic approaches based on the induction of immune responses against highly conserved epitopes or on administration of neutralizing antibodies directed to conserved regions of viral proteins (23, 598). However, it is unlikely that a virus will yield to such pressures. A conserved viral domain may have a cellular counterpart for which immune tolerance has developed (e.g., the RGD motif present in the capsids of some picornaviruses, which is an integrin recognition site for cell adhesion [598]). Even if a cellular counterpart is not present, once subjected to selection, a hitherto-invariant viral site may find escape routes through substitutions that affect long-term interactions within the target protein and compensatory mutations. Despite this, new drugs designed to target highly conserved sites within mutant spectra (revealed by application of ultradeep sequencing) may be included as part of antiviral treatments that take

into consideration quasispecies dynamics, as discussed in the next section.

An independent line of research that bears on evolutionary constraints of viruses has been the preparation of poliovirus with deoptimized codon usage and codon pair frequencies (96, 146, 569). Codon-deoptimized viruses are attenuated, with an extremely low probability of reversion due to the number of mutations needed to restore part of the codons to those of the original virus. The authors refer to this situation as "attenuation by a thousand cuts." If we turn the phrase into "fitness decrease by a thousand cuts," it becomes a fascinating evolutionary question how such a codon-deoptimized virus will find fitness recovery pathways. These multiply modified viral genomes constitute a means to study routes of virus escape from deep fitness valleys that cannot consist of the mere reversion of the original lesion or limited numbers of compensatory mutations.

### Antiviral Approaches to Counteract Quasispecies Adaptability: Lethal Defection and Lethal Mutagenesis

The need for new paradigms to approach infectious diseases has been expressed from different points of view, including the application of Darwinian concepts of evolutionary ecology (105, 865). The problems that quasispecies dynamics pose for viral disease prevention and control have been amply recognized (for overviews, see references 134, 135, 187, 188, 201, 202, 279, and 599 and several chapters in reference 211). The current aim of antiviral interventions is to achieve a low viral load, which often results in decreased pathogenicity and can provide the immune system with an opportunity to clear the virus or to prevent disease manifestations. A low viral load is linked to low replicative fitness, and new antiviral strategies can be directed to viral fitness, reducing the chances of fitness recovery during viral replication in the host organisms (134, 135).

Five main strategies have been implemented or proposed to try to minimize or delay the selection of antiviral-resistant mutants: (i) combination therapy, successfully achieved with the highly active antiretroviral therapy (HAART) for HIV-1-infected patients; (ii) splitting of the treatment between an induction regimen and a maintenance regimen; (iii) the use of drugs directed to cellular functions, alone or in combination with drugs that target viral functions; (iv) the combined use of immunotherapy and chemotherapy; and (v) lethal mutagenesis or virus extinction mediated by accumulation of mutations in the viral genome.

The advantage of combination therapy over monotherapy is a consequence of quasispecies dynamics, due to the decreased probability of generating a viral variant with two or more mutations required to escape to a drug combination (76, 188, 192, 201, 202, 368, 475, 573, 592, 670, 692, 824). Although prolonged, low level replication will often lead to selection of multidrug-resistant mutants and treatment failure, combination therapy remains the standard of care to treat diseases associated with highly variable viruses. A recent model study in cell culture of the interplay between a mutagenic agent and an inhibitor of viral replication suggested that a sufficiently low viral load may impede the selection of inhibitor-resistant viral mutants (649). Despite several factors contributing to the probability of selection of drug-resistant mutants (mutation rates, genetic and phenotypic barriers, etc.), a low viral load is at present a major aim of a combination therapy.

A more recent proposal is a proactive treatment, divided in two steps: an induction treatment aimed at reducing the viral load and the number of viral mutants, followed by a maintenance regime to sustain a low viral load (840). This strategy has been proposed on the basis of theoretical considerations of viral dynamics, but to our knowledge it has not yet been tested in experiments.

Many cellular functions are essential to sustain the multiplication of virus in the infected cell. Therefore, targeting of cellular proteins with drugs can result in inhibition of viral multiplication (305, 313, 381, 451). Two potential problems may arise: (i) toxicity for the cell resulting from the inhibition of a cellular function and (ii) selection of viral mutants either that can use a host protein-drug complex or that render ineffective the inhibition of the cellular target. The latter possibility may seem unlikely, but it is due to the fact that some cellular proteins participate in the replication cycle of viruses through complexes with viral proteins. As an example, amino acid substitutions T17A, E295K, and V44A in protein NS5A and T77K, and I432V in NS5B of HCV confer resistance to the cyclophilin inhibitor SCY-635 because cyclophilins interact with NS5A in the course of the virus replication cycle (reviewed in references 115, 169, 456, 733, and 831).

A low viral load could be also achieved though a combined strategy that involves administration of neutralizing antibodies (or other means of adaptive immune response stimulation) together with antiviral agents (484, 745, 850). Despite being strategies that should help in suppressing mutant spectra, their exploration still needs *in vivo* experiments and clinical trials.

Lethal mutagenesis is based on the concepts of violation of error threshold (Fig. 1) (see Theoretical and Experimental Origins of Quasispecies above) and of negative interactions among components of a mutant spectrum (Fig. 10) (see "Complementation and Defection: Quasispecies as a Unit of Selection" above). This new antiviral strategy aims at extinguishing viruses through an excess of mutations introduced in viral genomes upon replication in the presence of mutagenic agents, notably nucleotide analogues (reviewed in references 20, 191, 287, and 325) (Fig. 12). The first experiments that demonstrated a negative consequence of mutagenesis for virus adaptability and survival were performed by John Holland and colleagues, who examined the effects of several mutagenic agents on PV and VSV (375, 472). The term lethal mutagenesis was coined by Lawrence A. Loeb, James I. Mullins, and colleagues to describe the loss of replicative potential of HIV-1 in cell culture caused by the pyrimidine nucleoside analogue 5-hydroxydeoxycytidine (490), and they proposed that mutagenic ribonucleotide analogues could be investigated as anti-HIV-1 agents (491). The exploration of base or nucleoside analogues as antiviral mutagenic or nonmutagenic agents is currently an active field of research that has linked antimicrobial therapy with anticancer therapy (57, 99, 226, 315, 316, 323, 330–332, 356, 375, 495, 576, 633, 634, 637, 676, 869). It involves both the design of new compounds and the search for an application to viral mutagenesis of previously described analogues (125, 326, 327, 351–354).

Several studies with cell culture have documented the extinction of RNA viruses that employ different replication strategies upon infection in the presence of base or nucleoside analogues (12, 128, 148, 153, 162, 164, 293, 330–332, 459, 501, 561, 633, 634, 747, 761, 762, 799, 838; see also several chapters in references 187, 192, 211, and 232). This is the case for the pyrimidine base analogue 5-fluorouracil, which is used in anticancer therapy (6, 7, 57, 99, 226, 315, 316, 323, 330–332, 356, 375, 495, 576, 633, 634, 637, 676, 869).

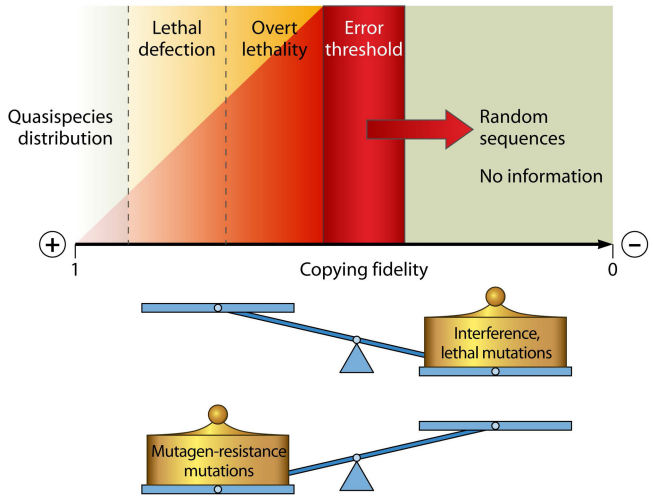


**FIG 12** Schematic representation of our present assessment of the events involved in virus extinction by increased mutagenesis. The picture is based on the concept of error threshold of quasispecies theory (Fig. 1), modified by experimental findings with RNA viruses. The error threshold is depicted here as a region of template-copying fidelity where the transition from a quasispecies distribution to random sequences (loss of information) occurs. Before entering the threshold domain, at least two transitions occur: lethal defection followed by overt lethality. Interference and lethal mutations drive viral populations toward extinction, while selection of mutations that confer resistance to the mutagenic agent used preserves quasispecies identity. See the text for experimental evidence and references.

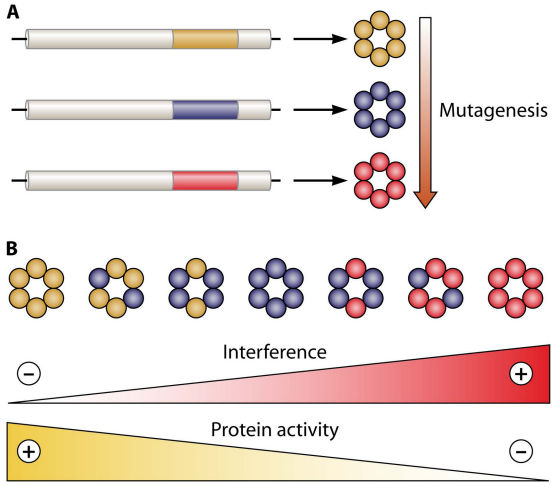


**FIG 13** Schematic representation of interference exerted by a *trans*-acting viral protein. (A) A viral genome and two mutants (with tan, blue, and red genomic regions) encode a wild-type (tan, functional) and two mutant (blue and red, increasingly defective and interfering, respectively) proteins as a consequence of mutagenesis acting on a viral genome. (B) The relevant protein acts as a hexamer in the virus life cycle. An increasing proportion of mutant proteins will accentuate interference (defection) by decreasing the activity of the protein. It is worth noting that the same mutant protein can act to interfere or to complement, depending on the biological context. A blue protein may rescue some activity when the red protein is dominant but may decrease activity when the wild-type (tan) protein is dominant. This is one of the mechanisms postulated to modulate viral fitness in mutagenized populations (compare with Fig. 10; see text for references).

The transition into error catastrophe can be viewed in the case of viruses as the loss of the superiority of the master sequence and a drift in sequence space that would lead to nonfunctional viral genomes (Fig. 1 and 12). Experimentally, the events in viruses that are a parallel to violation of the error threshold and entry into error catastrophe have been investigated using a number of virus-host systems (reviewed in several chapters of references 187 and 232). From these studies, the current picture of the molecular basis of lethal mutagenesis is that an initial increase in mutation rate generates defective but RNA replication-competent viral genomes, termed defectors, which interfere with the replication of the standard virus, thus contributing to the suppression of replication of the entire ensemble (Fig. 13). This model is termed the lethal defection model of virus extinction, and it is supported by both experimental results and *in silico* simulations (212, 320, 331, 392, 506, 654). Following this initial phase of the action of defectors, if mutagenesis continues or its intensity increases, lethal mutations can become prominent and participate in virus extinction (29, 331) (Fig. 12 and 14). Although the kinetics of accumulation of interfering and lethal mutations is largely unknown and participation of other viral and host mechanisms in extinction is likely, the current lethal defection model is coherent with well-established features of viral quasispecies (187, 211, 232, 651).

In addition to experimental evidence, the loss of replicative competence of viruses associated with an excess of mutations is supported by several theoretical models (17, 67, 91, 233, 506, 593, 611, 742, 794). A point to underline regarding models that deemphasize a role of mutagenesis is that extinction could not be achieved by the decreases in viral load due to nonmutagenic inhibitors, while extinction occurred by the same decreases in viral load due to a mutagen (633, 634, 649). Thus, the decrease in viral load that impedes progression of the infection (a reproductive ratio Ro of $<1$ at the level of cell-to-cell spread) is a consequence of mutagenesis, not the primary mechanism of extinction. The transition of viruses into error catastrophe or lethal mutagenesis is a new paradigm in antiviral therapy that has as its main features (i) the requirement of mutagenesis, (ii) the presence of an invariant consensus nucleotide sequence during the transition (321, 330), and (iii) at least in some cases a movement in sequence space with the early occurrence of hypermutated genomes (330, 651) (Fig. 14) (see also the next section).

The efficacy of lethal mutagenesis may be favored by the multifunctional nature of the great majority of viral proteins (207) and domains within proteins (51, 829). The same amino acid substitution may have multiple negative effects on different steps of the virus life cycle, thereby accelerating its replicative collapse. Evidence that lethal mutagenesis can be effective *in vivo* (709) and a promising first clinical trial in which a mutagenic pyrimidine analogue affected the mutational pattern of HIV-1 in AIDS patients (574) offer interesting prospects. However, as summarized in the next section, protocols involving nonantagonic mutagenic agents and nonmutagenic antiviral inhibitors should be explored.

An encouraging aspect of lethal mutagenesis is that its principles operate in nature as a mechanism to combat viruses and other molecular parasites. Notably, some members of the APOBEC (apolipoprotein B mRNA editing complex) family of editing proteins are diverted from their physiological editing roles in the cell to become part of antiviral functions, in particular antiretroviral activities exerted via hypermutagenesis, as discussed in "Human Immunodeficiency Virus Type 1" below. The RIP (repeat-induced point mutations) system in some filamentous fungi is a

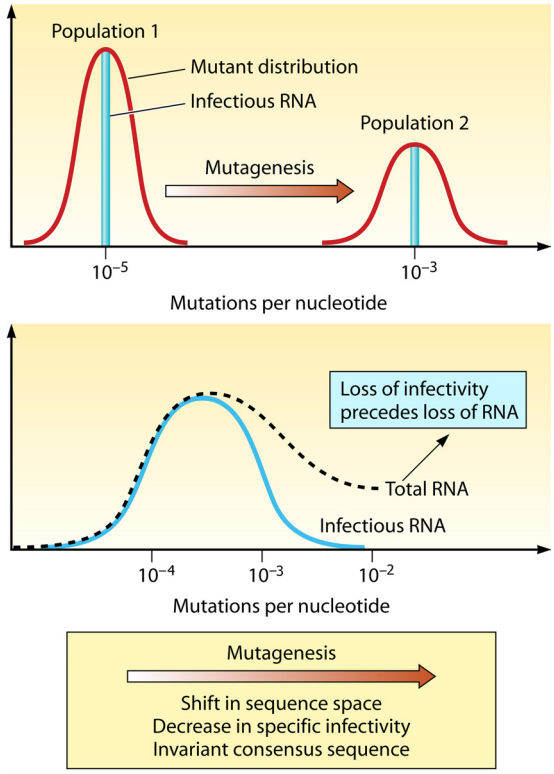


**FIG 14** Effect of increased mutagenesis in a viral population. In the top panel, mutagenesis increases 100-fold the average mutation frequency of a mutant spectrum, resulting in a decrease of the amount of infectious RNA. The second panel indicates that high mutation frequencies result in a decrease of the proportion of infectious viral RNA in a population, with loss of infectivity preceding loss of viral RNA replication. The bottom box stresses three major events associated with enhanced mutagenesis, as discussed in the text.

defense mechanism consisting of the introduction of mutations in repeated DNA sequences of genetic parasites (138, 302). Editing is part of the replication cycles of several viruses, including *Paramyxovirinae*. The polymerases of these viruses stutter in a controlled fashion to edit the viral phosphoprotein mRNA. It has been proposed that these viruses have evolved to possess a genome of polyhexameric length (a fact known as "the rule of six") to prevent the deleterious effects of hypermutagenesis that may result from uncontrolled editing (443). The observation that several biological systems exploit mechanisms to produce or prevent "natural error catastrophe" events suggests that enhanced mutagenesis has the correct conceptual foundations to become an effective antiviral strategy.

An increase in mutation rate caused by nucleoside analogues may not be disadvantageous to a virus. The mutagenic nucleoside 5-azacytidine (AZC) enhanced the replicative ability of bacteriophage Qβ during plaque development in the presence of AZC (107). In contrast, Qβ populations were driven to extinction by AZC during growth in liquid medium, unless mutagen-resistant phage was selected (34, 107). Evidence suggests that the determinant element of adverse versus beneficial effects of increased mutagenesis may lie in the proximity to an error threshold for maintenance of genetic information, and this argument may be valid for viral and cellular systems (160, 779, 781).

### Mutagen-Resistant Mutants and the Molecular Basis of Virus Extinction

Ribavirin-resistant mutants have been described for several viruses (269, 661, 662, 734, 885). However, in many cases it is not clear whether ribavirin acts as a mutagenic agent, as a nonmutagenic inhibitor, or as both. Consequently, it is not known whether resistance refers to its mutagenic activity or inhibitory activity or both. It has not been established either whether resistance to a mutagenic agent will be as easily selectable as resistance to nonmutagenic inhibitors. Since the same ribavirin-resistant PV polymerase mutant G64S (described in "Exploration of Sequence Space and Virus Adaptability: Fidelity Mutants" above) was obtained independently in two laboratories (108, 660, 662), it was thought that picornaviruses may have available very few alternative mechanisms to achieve ribavirin resistance. However, an FMDV passaged in the presence of ribavirin did not select substitution G62S (the one equivalent to G64S in PV), and it selected 3D substitution M296I instead (761). Substitution M296I increased viral fitness in the presence of ribavirin and decreased the incorporation of RTP into viral RNA, without a significant alteration of the general template-copying fidelity of the enzyme (30, 761). FMDV with a G62S substitution in its 3D was constructed by site-directed mutagenesis. It displayed a strong selective disadvantage relative to FMDV encoding the wild-type polymerase; passage of the mutant virus in cell culture resulted in reversion to the wild-type polymerase, contrary to the case for the M296I mutant, which was stable (277). Thus, a substitution that was well accepted and conferred PV resistance to ribavirin was highly detrimental to a closely related virus encoding a similar polymerase molecule such as FMDV (276, 277). M296 is located on a loop close to the active site of the polymerase, while G62 is in the fingers subdomain of the enzyme. Comparison of the three-dimensional structures of the polymerases including either substitution G62S, M296I, or both documented that despite the two residues being 13.1 Å apart, they are connected through a network of interactions that reach the polymerase active site (277). An effect of substitution G62S was to restrict the flexibility of the loop where M296 lies and the ability of the enzyme to bind to the RNA template. Thus, despite the potential of G62S in the FMDV polymerase to reduce incorporation of ribavirin into RNA, the altered enzyme inflicted upon FMDV such a selective disadvantage that substitution G62S could have never been selected unless compensatory substitutions (which have not been found) were introduced in the polymerase (277).

The polymerase mutants described for PV and FMDV display two distinct mechanisms of ribavirin resistance with different implications regarding the adaptive potential of the viruses: while G64S in PV resulted in a general increase of template-copying fidelity that impaired virus adaptation to complex environments (660, 833), M296I in FMDV resulted in the specific restriction of ribavirin incorporation. The mutant virus could incorporate additional replacements at the standard rate to achieve high-level resistance to ribavirin (8, 30, 761). The alternative mechanisms of picornavirus resistance to ribavirin suggest again that due to the many subtle interactions among distant sites of proteins in general (782, 818), multiple pathways for drug resistance are probably possible in most cases (as discussed in "Selective Forces and Escape Mutants" above), but only a subset of them are compatible with the virus maintaining sufficient fitness. The results under-

score the interest of targeting viral fitness as a general approach to antiviral interventions (134, 135).

Mutant clouds need not be symmetrical in an imaginary (multidimensional) representation of sequence space (Fig. 8B). FMDV mutants displaying high levels of ribavirin resistance offer a relevant example. When FMDV with M296I in 3D was passaged in the presence of increasingly larger concentrations of ribavirin, two additional replacements, P44S and P169S, were sequentially added to yield the triple polymerase mutant termed SSI (8). These three substitutions inflicted a modest decrease in FMDV fitness quantified in BHK-21 cells in the absence of ribavirin and a remarkable fitness gain in the presence of ribavirin. The mutant polymerases were compared with the wild-type enzyme functionally and structurally. The most salient biochemical feature found was that substitution P44S restricted ribavirin incorporation more strongly opposite C than opposite U in the template-primer combinations tested *in vitro* (8). A consequence of this misincorporation bias was that during replication in the presence of ribavirin, the triple mutant could maintain a balance among the four transition mutation types [ratio of (G→A) + (C→U) to (A→G) + (U→G)] which was very similar to the balance attained by the wild-type enzyme in the absence of ribavirin. The excess of G→A and C→U mutations associated with ribavirin mutagenesis proved deleterious for FMDV, and the mutational bias was prevented in the case of the triple SSI mutant, resulting in escape of extinction by ribavirin (8). Examination of the mutant spectra of wild-type and SSI mutant viruses indeed documented a higher frequency of deleterious mutations due to G→A and C→U transitions, supporting a selective advantage of a virus capable of avoiding a mutational bias. Yet, the wild-type and mutant SSI FMDVs produced mutant spectra of similar complexities. Modulation of transition types represents a third mechanism of ribavirin resistance that did not involve obvious modifications of general copying fidelity.

The critical amino acid substitution to maintain the FMDV mutational balance in the presence of ribavirin was P44S, a residue conserved among picornaviruses that lies in a loop that connects a β-strand and a α-helix in the fingers domain (8, 276). A local reorganization of amino acid residues at the amino-terminal region of the enzyme was noted. This region, together with amino acids of motifs G and F of 3D, binds the 5′ region of the template, driving the RNA toward the active site. Substitution P44S triggered the conformation change at the amino-terminal region of the enzyme, reoriented template nucleotides, and most probably led to a template residue-dependent alteration of ribavirin recognition (8, 275). Ribavirin resistance in FMDV has been described here in detail to illustrate the capacity of amino acid substitutions near or far from a polymerase active site to modulate replicative parameters for the benefit of a virus. Our current knowledge with polymerases in general suggests that any virus has the potential to invent an array of molecular "tricks" for survival. In the words of Joshua Lederberg: "Abundant sources of genetic variation exist for viruses to learn new tricks, not necessarily confined to what happens routinely or even frequently" (471).

From the point of view of occupation of sequence space, what the triple SSI FMDV mutant achieved was to limit the occupation of regions with genomes with high frequencies of A and U. FMDV populations passaged in the absence or presence of ribavirin were analyzed using differential DNA denaturation PCR (3D-PCR), a procedure that selectively amplifies A- and U-rich genomes from a DNA population (787). Interestingly, FMDV genomes with high frequencies of A and U (termed hypermutated genomes) were present in both ribavirin-treated and ribavirin-untreated populations, again documenting the presence of a deep mutant spectrum in nonmutagenized, multiply passaged populations of FMDV (651). A major effect of ribavirin was to generate an A- and U-rich repertoire of genomes in a single passage. In the absence of ribavirin, a statistically significant increase of genomes enriched in A and U was not obtained until passage four (651). The high frequency of hypermutated genomes after one passage in the presence of ribavirin was not maintained at subsequent passages, a conclusion that was also reached in a previous analysis using phylogenetic procedures and partition analysis of quasispecies (616). We interpret this quasispecies compaction as a consequence of negative selection that operates on genomes rich in A and U, following their abundant but transient presence as a result of ribavirin mutagenesis (8, 615, 651).

FMDV polymerase substitution P44S, the one responsible for high-level ribavirin resistance (8), was present in the mutant spectra of FMDV populations passaged in the absence of ribavirin (651). This is probably a consequence of the modest effect of P44S on viral fitness (8). The P44S frequency increased as a result of ribavirin treatment, as expected (651). Its presence in FMDV populations never exposed to ribavirin is related to a wealth of observations on the presence of inhibitor-resistant viral mutants in natural isolates from patients who have not been exposed to the relevant inhibitors (156, 358, 409, 452, 470, 581, 582, 697, 733).

The 3D-PCR study with FMDV was also revealing from the point of view of the molecular basis of lethal mutagenesis. The accelerated presence of hypermutated genomes associated with ribavirin treatment represents a shift in sequence space which is compatible with the main observations previously described as a hallmark of lethal mutagenesis: increased frequency of defector genomes and an invariant consensus sequence (321, 330, 392). Movements in sequence space may prove relevant to lethal mutagenesis, and the new-generation sequencing techniques may contribute in the planning of new antiviral protocols based on administration of mutagenic agents.

The investigations summarized in this section suggest that selection of viruses resistant to mutagenic agents is not a rare occurrence. To what extent such mutagen-resistant mutants may jeopardize antiviral treatments based on lethal mutagenesis remains an open question.

### The Interplay between Mutagenesis and Inhibition in the Design of Antiviral Protocols

Ever since quasispecies dynamics was recognized as a key feature of pathogenic RNA viruses, the need to use combinations of compatible inhibitors to suppress viral replication was acknowledged (see "Antiviral Approaches to Counteract Quasispecies Adaptability: Lethal Defection and Lethal Mutagenesis" above). The widely accepted and amply supported advantage of combination therapy may, however, not be always applicable when a mutagenic agent enters the picture, as in the case of lethal mutagenesis-based antiviral designs. Although several mechanisms of mutagenesis-based extinction have been proposed (89, 91, 158, 233, 331, 506, 794, 861), very few experimental and theoretical studies have approached combination therapies involving mutagenic agents alone or mutagenic agents and nonmutagenic inhibitors. The few

studies that have been carried out suggest that this is likely to become a fertile domain of antiviral research.

The mutations that conferred resistance of FMDV to ribavirin prevented the extinction of the virus by high doses of ribavirin (see the previous section). However, the ribavirin-resistant virus was extinguished by a combination of the mutagenic base analogue 5-fluorouracil and the inhibitor of RNA replication guanidine (648). This observation suggests that the availability of two or more mutagenic agents with different mechanisms of action (i.e., purine versus pyrimidine analogues, resembling one or other purine or pyrimidine type) can provide expanded antiviral possibilities provided the analogues are virus specific and toxicity for the host is limited. The use of two mutagenic agents (sequentially or in combination) is conceptually parallel to the administration of two inhibitors. Investigations on the use of multiple mutagenic agents are needed.

An alternative approach is the use of combinations of mutagenic agents and antiviral inhibitors. Since the early studies on lethal mutagenesis, it was found that a low viral load and low viral fitness favor extinction (762), as expected from the parameters that enter into the error threshold relationship (233, 243) (Fig. 1). High-fitness HIV-1 was not extinguished by the mutagenic analogue 5-hydroxydeoxycytidine, but systematic extinction was achieved by a combination of 5-hydroxydeoxycytidine and the antiretroviral inhibitor 3′-azido-3′-deoxythymidine (AZT) (799). This result would seem a reasonable extension of the preferred combination therapy over monotherapy, involving a mutagenic agent and a nonmutagenic inhibitor. The extension, however, proved to be far less straightforward than anticipated.

The following considerations are relevant to the use of mutagens in combination with inhibitors. First, a mutagen may display a mutagenic activity and an inhibitory activity that may be related or unrelated. 5-Fluorouridine triphosphate (FUTP) inhibits the uridylylation of FMDV primer protein VPg, a critical step in the initiation of picornaviral RNA synthesis (867), and 5-fluorouridine monophosphate is incorporated into RNA, causing misincorporations (7). In this case, the inhibitory and mutagenic activities of FUTP appear to be unrelated. Ribavirin was considered to be an inhibitor of arenavirus replication (250, 399, 424, 430, 497, 709, 736, 770). When this issue was reexamined using LCMV, it was found that concentrations of ribavirin that allowed viral replication were mutagenic, while no evidence of mutagenesis was obtained with ribavirin concentrations that suppressed viral replication (561). This result is not unexpected, because expression of a mutagenic activity associated with incorporation of an analogue into RNA will be dependent upon RNA replication. It cautions, however, against considering nucleoside analogues inhibitors of viral genome replication without testing a possible mutagenic activity by lower concentrations of the analogue. It is not known whether the inhibitory activity of ribavirin on LMCV is independent of or related to its mutagenic activity (see further discussion in reference 561). There is also evidence that some antiretroviral treatments that have been used in clinical practice can be mutagenic (510).

Whether a base or nucleoside analogue acts as an inhibitor or as a mutagen during therapy is relevant because interactions between a mutagen and an inhibitor can influence the effectiveness of an antiviral treatment, in particular sequential versus combination inhibitor-mutagen administration. The activity of a mutagen, present simultaneously with an inhibitor, can increase the frequency of inhibitor-resistant mutants, resulting in higher viral production. A mutagen can also help in finding a position in sequence space in which multiple mutations can provide resistance to the inhibitor and sustain high viral fitness (compensatory mutations) (349). Increased mutagenesis can have beneficial effects through enhancement of genotypic and phenotypic diversity but deleterious effects when excess mutations jeopardize viral functions. Furthermore, the viral load can be a determinant factor of the effect of mutagenic agents and antiviral inhibitors. Despite a history of enhanced mutagenesis of FMDV, a sufficiently low viral load allowed continued viral replication, without selection of guanidine-resistant mutants (649). Thus, multitudes of environmental factors (viral load, population bottlenecks, environmental heterogeneities, compartmentalization of infection, etc.) can all affect the consequences of mutagen-inhibitor interactions for viral survival. In general, the combined action of any two drugs (be they mutagenic or not) cannot be reduced to the sum of their independent effects (282, 393, 809).

Extinction of FMDV by a combination of a mutagenic agent and an antiviral inhibitor was more effective than extinction by the mutagenic agent alone (633, 634). When a sequential protocol involving first inhibition by guanidine and then mutagenesis by ribavirin was compared with the corresponding combination protocol (simultaneous administration of guanidine and ribavirin), the former proved to be more effective to achieve the extinction of FMDV in BHK-21 cells (650) (Fig. 15). Two main factors were identified as being responsible for the advantage of the sequential protocol. One is the enhancement of generation of guanidine-resistant mutants by ribavirin when present together, and this was supported by a theoretical model in which the mutagenic activity is a key parameter. In the absence of mutagenic activity, the model predicts an advantage of combination (over sequential) therapy (650), in agreement with previous models of virus dynamics (see "Antiviral Approaches to Counteract Quasispecies Adaptability: Lethal Defection and Lethal Mutagenesis" above). A second factor that could contribute to the superiority of the sequential treatment is that the defectors that are generated by ribavirin mutagenesis, and that contribute to viral extinction, may have their interfering action inhibited by the presence of guanidine. This was demonstrated experimentally by coelectroporation of BHK-21 cells with an interfering mixture of FMDV mutants and with standard RNA. Interference of the expected intensity was exerted either in the absence of drugs or in the presence of ribavirin, but it was strongly diminished in the presence of guanidine (650). Therefore, at least two different manifestations of the simultaneous presence of a mutagen and an inhibitor may render suboptimal the transition toward extinction.

The results with FMDV raised the important prospect that new antiviral protocols involving a mutagenic agent may not necessarily follow the classical combination-therapy paradigm. The theoretical generalization of the FMDV findings came with a model of viral dynamics that described the viral response (progeny infectivity and frequency of guanidine-resistant mutants) in the presence of different doses of ribavirin and guanidine (393). One of the main predictions of the model is that an advantage of the sequential versus the combination protocol is manifested at a high concentration of the inhibitor, a point that was confirmed experimentally (393). The general model furnishes information with which to reduce the number of preliminary experiments to be carried out

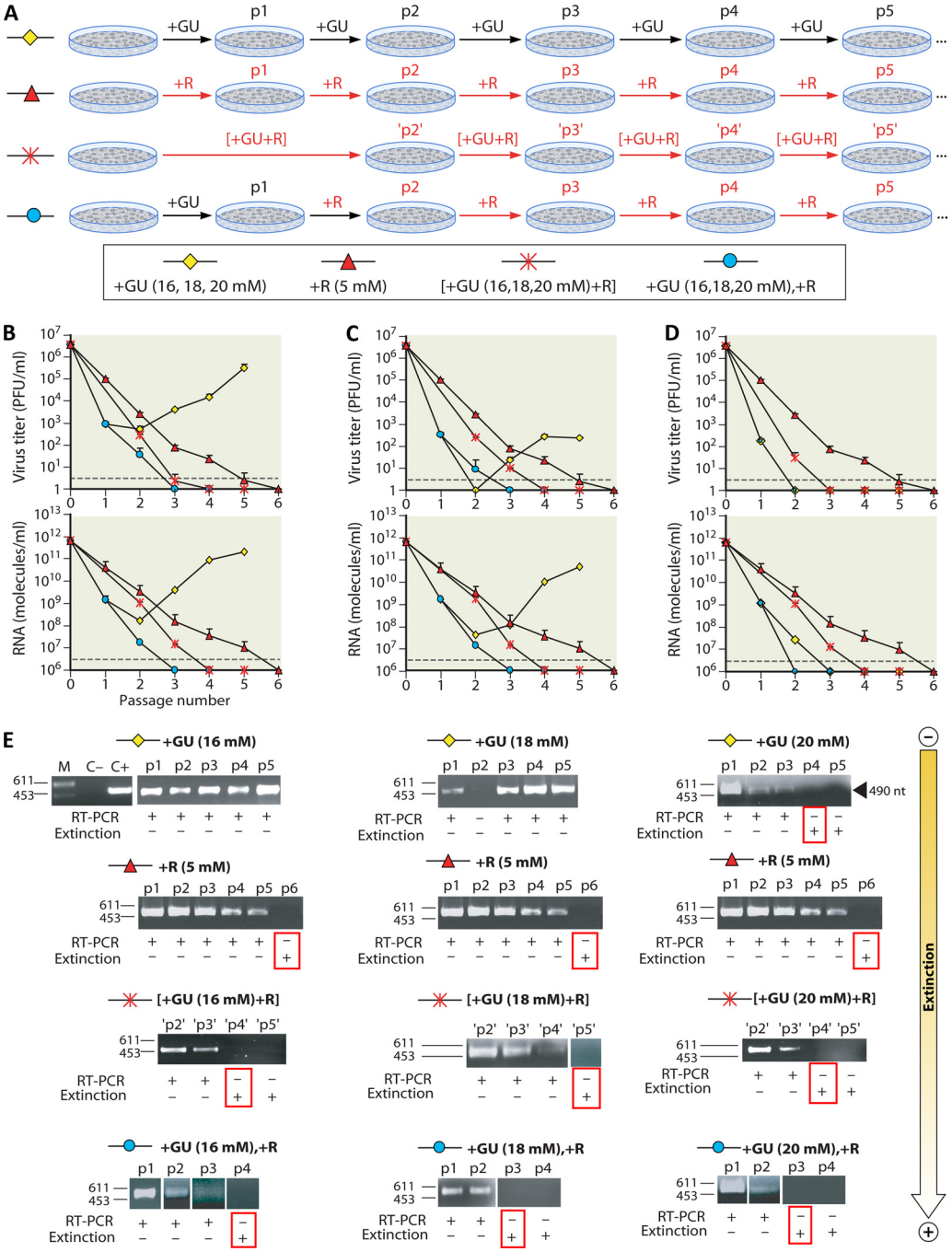


**FIG 15** A sequential administration of first an inhibitor and then a mutagenic agent can be more effective than the corresponding combination of drugs. In this model experiment with FMDV in cell culture, guanidine hydrochloride (GU) was used as the inhibitor and ribavirin (R) as the mutagen. (A) Scheme of four drug administration protocols involving five virus passages in BHK-21 cells: only GU, only R, a combination of GU and R (GU+R), or first one passage with GU and then four passages with R (GU, +R). (B, C, and D) Virus titer and viral RNA molecules at different passages as a function of treatment regimen and drug concentrations (given in the box above the panels and below the abscissas). Note that the lowest titers and viral RNA levels are attained with the sequential protocols. (E) Reverse transcription-PCR (RT-PCR) amplifications used to detect viral RNA in the corresponding cell culture supernatants to monitor viral extinction. The upper part of each panel identifies the drug treatment and passage number. + or − below the panels indicates positive or negative RT-PCR amplification and extinction. M, molecular size markers; C+ and C−, positive and negative controls, respectively. Note the earlier extinction with sequential treatment at the two highest GU concentrations. (Adapted from reference 650, in which experimental details are described.)

prior to the design of an antiviral protocol. The obvious challenge now is to explore the applicability of the results to other virus-host systems in cell culture and *in vivo*, to open the way to a possible clinical application.

We wish to emphasize the consequences that an understanding of quasispecies dynamics can have in the design of antiviral interventions. In this case, theory is closely interconnected with practice.

## Viral Quasispecies Dynamics *In Vivo*: Long-Term Virus Evolution

In previous sections, concepts related to quasispecies dynamics have been elaborated based both on designed protocols of experimental evolution (using cell cultures, the natural host organism, or model host organisms) and on observations of viral population dynamics in the field. Two questions arise: (i) to what extent the conclusions of the studies on experimental evolution reflect the population dynamics of viruses in their natural environments and (ii) what are the consequences of quasispecies dynamics for the understanding of long-term virus evolution. Concerning the first question, an overall comparative assessment suggests that no fundamental contradiction has been found between the behavior of viruses in model experiments and in natural infections with regard to genetic variation, competition and interference among components of a mutant spectrum, and selection of genome subsets. Concerning the second question, increasing evidence suggests that quasispecies dynamics must be taken into consideration to interpret long-term virus evolution. It is not possible to list all results in support of such assertions, but we list a few.

The presence of mutant spectra of FMDV in virus-infected cattle was one of the early observations on quasispecies *in vivo* (194), and it has been amply supported by many subsequent studies (102, 103, 311, 609, 778, 791, 795, 874). The FMDV population heterogeneity and dynamics—including fitness variations and biological effects of bottleneck events—revealed by these studies are perfectly coherent with the behavior of the virus in cell culture (777; also see many subsequent studies reviewed in references 195 to 197, 212, and 214). Likewise, several observations on VSV population heterogeneity and dynamics (reviewed in references 376 and 596) had counterparts *in vivo* (512).

Quasispecies memory was described in a designed experiment using FMDV (707), and it was shown to occur also with HIV-1 *in vivo* (reviewed in reference 85). Memory probably underlies many reemergences of ancestral sequences recorded in several studies (see "Positive Selection and Molecular Memory: Deterministic Features of Viral Quasispecies" above). The frequency of drug, antibody, or cytotoxic T-lymphocyte (CTL) escape mutants and the effects of population size on the repertoire of virus variants can best be quantified in experiments designed in cell culture. Virtually all major conclusions with important pathogens in cell culture had a counterpart *in vivo* (see references 80, 136, 252, 281, 454, and 494, among many other studies). Parallel quasispecies behavior in cell culture and *in vivo* is expected because its roots lie in error-prone replication, which is increasingly recognized as a shared feature of several viruses and retroelements both in cell culture and *in vivo* (192, 400, 408).

Shared features also make it likely that antiviral approaches (such as the ones based on lethal mutagenesis described in the previous section) can be of general applicability (see Conclusions, Connections, and Prospects below). However, the possible advantage of sequential versus combination therapies that require introduction of specific parameters (discussed in the previous section) has been predicted for nonretroviral RNA viruses. Extensions to retroviruses or DNA viruses that have a latency step will require analogous models and parameters adapted to the corresponding replication strategy (393). Additional examples of the behavior of viruses *in vivo* that fit the expectations from model studies are given in the next section of this article, which is concerned with the impact of quasispecies dynamics for HIV-1, HBV, and HCV.

Quasispecies dynamics relates to long-term virus evolution because mutant spectra provide the types of genomes that will be transmitted from infected into susceptible hosts. For some viruses, concepts derived from molecular quasispecies have been integrated to the epidemiological level, notably in the case of PV by Vadim Agol and colleagues (309). Multitudes of formation cycles of quasispecies swarms and transmission, together with changes in the composition of mutant spectra, are determinants of long-term evolution patterns. Rapid evolution of some viral lineages in the field has been related to pathogenic potential relative to that of slower-evolving related lineages (891). Genetic diversity derived from a higher polymerase error rate has been shown to underlie norovirus pandemic potential (92). Enhanced viral fitness may also contribute to dengue disease severity, an increasingly important, still unsettled issue (614).

Viruses diversify into genotypes and subtypes, and it is the general course of events that as increasing numbers of isolates are analyzed, the number of subtypes becomes too large for subtyping to be justified (for example, subtyping of FMDV was stopped during the second half of the 20th century because refined tools such as monoclonal antibody-based subtyping showed that virtually each new isolate was a new subtype [195, 535]). A genotype of a virus can be viewed as a set of related genomes that have found a high fitness domain and acquired epidemiological relevance associated with replicative or nonreplicative traits. Initially, a genotype tends to abound in a given geographical area, although for socioeconomic reasons (increasing migration, traveling, and trade), geographical clusters tend to be blurred. For viruses with active recombination, mosaic genomes from different parental genotypes or subtypes may be an additional important source of genomic variation, adaptability, and diversification.

A corollary of the mechanisms involved in quasispecies dynamics is that RNA viruses do not evolve as a result of a linear (or nearly linear) accumulation of mutations but rather evolve as a result of disequilibria of mutant distributions. The uncertainty of how divergent the transmitted genome will be relative to the one that initiated the infection, together with the alternation of periods with and without active viral replication subjected to positive and negative selection, renders unlikely the operation of a molecular clock in the case of virus evolution. John Coffin expressed skepticism about extrapolating from phylogenetic analyses the date of the common ancestor of retroviruses or the year of divergence of HIV-1 and HIV-2 (144). Additional extrapolations to date ancestral viruses have been made based on strict or relaxed clock models, often resulting in conflicting results. In particular, despite rates of evolution for viruses being in the range of $1 \times 10^{-1}$ to $3 \times 10^{-5}$ substitution per site per year (with some exceptions such as the simian foamy viruses) (reviewed in reference 189), for the same virus a higher rate of evolution is obtained when the compared sequences correspond to viruses isolated within a short time span than when they correspond to isolates separated by a long time interval (189, 778). For closely related isolates from the same disease episode, it was suggested that a steady accumulation of mutations might result from the random sampling of sequences from the quasispecies followed by amplification in successive susceptible hosts (834). The operation of a molecular clock follows the tenet of the neutral theory of molecular evolution, implying that rates of evolution reflect mutation rates. This is untenable considering our current understanding of quasispecies dynamics. Several contradictions have now become apparent, including not

only the dating of viral ancestors, but also rates of codivergence of viruses and their hosts (reviewed in references 754 and 768). The reasons for such gross discrepancies are not known, but they might relate to the inadequacy of extrapolating short-term quasispecies evolution based on population disequilibria into long-term evolution under the assumption of a linear accumulation of mutations.

The term epidemiological fitness has been proposed to describe the relative selective advantage that some natural viral isolates manifest over other related (but distinguishable) isolates in the field (189, 559). The epidemiological advantage of a given virus has many replicative and nonreplicative components, including infection dynamics in any individual host, transmissibility, virion stability, and others. Such variable parameters render even less plausible the operation of a molecular clock.

Another potentially misleading calculation is the ratio of nonsynonymous to synonymous mutations (dN/dS) as an indicator of positive selection versus negative selection and random drift in virus evolution. Despite being an extensively used parameter, its implications regarding evolutionary forces are questionable on at least two grounds. First, the dN/dS ratio was initially developed to be applied to distantly related genomes, not to closely related sequences as is the case in quasispecies evolution (450). When a selective constraint is applied to a quasispecies swarm, many genomic sites unrelated to the selective constraint may be subjected to negative selection, while only one or a few sites may respond with dominance of an amino acid substitution that reflects a positive selection event. Thus, a minority of amino acid replacements accompanied by an excess of synonymous substitution that will produce a low dN/dS ratio may nevertheless drive virus evolution.

Thus, evolution through disequilibria of mutant swarms has important immediate effects for short-term, intrahost evolution, but the present evidence has started to indicate that it may also condition long-term events. Classical tenets of population biology may have to be reconsidered for rapidly replicating, error-prone, collectively integrated biological entities.

## SPECIFIC VIRAL SYSTEMS

Several viruses contributed decisively to establish the quasispecies nature of viral populations and to unveil its biological implications, and they have provided the current picture of viral quasispecies dynamics summarized in previous sections. However, accelerated Darwinian evolution found maximal clinical impact in persistence and treatment strategies for three major human pathogens, HIV-1, HBV, and HCV, for which the presence of complex mutant spectra as the fuel for adaptability was soon recognized (references 100, 416, 518, and 552, followed by many other studies). The lack of vaccine efficacy and failure to maintain a low viral load by antiviral drugs were understood as a consequence of quasispecies dynamics. In the next sections we review some of the major implications of quasispecies for these three human pathogens.

Infections by HIV-1, HBV, and HCV share a number of features. They affect millions of humans worldwide, with estimates of nearly 40 million people infected with HIV-1, 2,000 million seropositive for HBV (with 350 million chronic carriers), and around 210 million people infected with HCV. These infections constitute a true insidious pandemic, with remarkable numbers of coinfections, in particular with HIV-1 and HCV, that reach about 7 million people. The viruses replicate continuously in their hosts, albeit at different rates, from fulminant outbursts to a seemingly controlled, slow pace. Bottleneck events are frequent during transmission of these viruses, which occurs mainly through the parenteral route (blood-to-blood contact). The viruses share error-prone replication, attributed partly to the absence of a proofreading-repair activity in the corresponding polymerases, and remarkable genetic heterogeneity. They have diversified into multiple genotypes and subtypes that sometimes represent a genetic signature that affects virus behavior. Some biologically relevant features are common to different types and subtypes, and these general features are addressed here. Also, they have in common very high rate of virion production, with a number that can reach $10^{11}$ to $10^{12}$ per infected individual per day (590, 607, 852). Despite these shared traits, HIV-1, HBV, and HCV differ in fundamental biological aspects of their replication and interaction with their host organisms, sometimes affecting the interpretation of the observed population heterogeneities.

### Human Immunodeficiency Virus Type 1

**Viral dynamics.** The mutation and recombination rates and frequencies, the virion production rate, and a short generation time all contribute to HIV-1 exhibiting quasispecies dynamics (references 145, 369, 385, 407, 478, 511, 552, 588, 589, 639, 655, 674, 675, 735, 800, 804, 844, and 852, among numerous other studies). The interpretation of the origin of HIV-1 heterogeneity and diversification of viral sequences is complicated by the compartmentalization of the infection, the presence of viral reservoirs (and therefore replicative and nonreplicative quasispecies memory), and reactivation of latent provirus (85, 110, 127, 176, 296, 390, 458, 488, 706, 835, 872). Retroviral sequences integrated into cellular DNA will be subjected to the relative evolutionary stasis typical of cellular genes (except in the case of potential integration at sites undergoing cellular hypermutational events). Proviral activation will contribute new replicating genomes that correspond to virus that was actively replicating at an earlier phase of the infection. Thus, the generation of millions of mutant and recombinant HIV-1 variants and competition among them in the compartmentalized and continuously changing host environment create a dynamic diversity that defies imagination. HIV-1 population complexity is increasingly documented and quantified by application of massive sequencing techniques (370, 513, 516, 570, 846), as summarized in the next paragraphs.

Major questions related to HIV-1 quasispecies evolution that remain only partially understood are (i) the effect of mutations on the response to antiretroviral treatment, with an ever-increasing catalogue of mutations and combination of mutations that confer different degrees of resistance to one or several antiretroviral agents, (ii) the interaction of the virus with the different branches of the adaptive immune response, with its many implications for vaccine design, and (iii) dynamics of coreceptor switching in relation to viral pathogenesis and disease progression. These fundamental questions cannot be approached without considering viral parameters interconnected with host factors. One of the key parameters that influences HIV-1 behavior and response to selective constraints is fitness (684), a measure of replication capacity of general relevance for RNA viruses (see "Viral Fitness and the Effect of Population Size: Bottleneck Events" above). HIV-1 fitness is closely related to viral load, which is an important element in virus adaptability and in turn is connected with viral pathogenesis.

Current strategies to control HIV-1 infection either pharmacologically or by immune stimulation (or combinations of the two strategies) should aim at decreasing viral loads and sinking diminished HIV-1 populations into deep fitness valleys without escape, at least with the genetic variation and exploration of sequence space accessible to the virus. The question is whether such valleys can be reached through antiviral interventions, given the provirus stage in the replication cycle.

**Drug resistance.** Multiple mechanisms of HIV-1 drug and RNA interference resistance have been described (545, 843, 859). The dynamics of the generation and increase in frequency of HIV-1 drug-resistant mutants adheres to the basic principles established experimentally for a variety of RNA viruses, which have the support of theoretical models (188, 692, 693) (see "Selective Forces and Escape Mutants" above). Due to the continuous input of new genomic sequences during HIV-1 replication, mutations that diminish sensitivity to antiretroviral agents often preexist in infected humans, even if they have never been treated with the relevant drugs (358, 409, 470, 581, 582, 808, 814). This phenomenon must be distinguished from primary resistances due to infection by HIV-1 harboring resistance mutations selected in the infected, previously treated donor individual. Nonprimary resistance mutations are a consequence of the elevated mutation frequencies, since mutations are expected to affect codons related to drug resistance as much as any other sites in the genome (581).

No list of antiretroviral resistance mutations will be presented here, since periodic updates can be found in the literature and on websites (137, 411). HIV-1 treatment has mobilized unprecedented resources to develop new antiretroviral agents, with (at the time of this writing) 25 inhibitors available that target reverse transcriptase, protease, fusion, virus entry, or proviral integration. For the majority of them, multiple mutational pathways leading to resistance have been described. The general picture emerging from drug and multidrug resistance in HIV-1 is very similar to that described for RNA viruses in general, with the added ingredient of the presence of the proviral reservoir. Even if a drug resistance mutation or set of multidrug resistance mutations inflicts a fitness reduction on the virus, compensatory mutations are frequently selected to restore fitness (231, 349, 386, 527, 529, 549, 591). Moreover, and pertinent given the potential impact of defective genomes on the replicative capacity of a virus, multidrug-resistant variants can exist in cells as defective quasispecies and may be rescued by recombination to yield infectious progeny (679).

Recombination can contribute to generate multidrug-resistant strains by bringing together genomic sequences that each confers resistance to an inhibitor (292, 423, 442, 445, 568, 570, 887). It is a debated issue whether the presence of minority subpopulations with mutations that decrease the sensitivity to antiretroviral agents, independently of their origin (transmitted, memory, or mutational pressure), should help to determine the treatment regimen or not. According to some studies, low-frequency baseline drug resistance is associated with a higher risk of treatment failure (43, 314, 322, 409, 550, 630, 764). Other studies have not found an influence of minority resistance mutations on the treatment response (551, 659). Different views on the usefulness of ultradeep sequence analysis of HIV-1 from treatment-experienced patients have been also expressed (163, 469, 826). Previous experience suggests that the more detailed the molecular information on the virus populations present in a patient, the better for treatment management.

Development of drug resistance requires a minimum replicating load to explore sequence space in search of the relevant mutations (649). It is a common experience that to succeed in selecting a drug-resistant viral mutant, it is best first to multiply the virus in the presence of limited drug concentrations to ensure a sufficient replicative load and then to increase the drug concentration gradually (8, 55, 662, 761). This probably reflects gradual selection, first of mutations with a low barrier and that display a limited degree of resistance, followed by other mutations that express more firmly the resistance trait. In agreement with these observations in cell culture, drug resistance is less frequent in patients who adhere to HAART and maintain a low viral load than in those who sustain high-level virus replication (368). Resistance may develop very quickly when treatment is suboptimal and viral replication is only partially suppressed.

When fitness recovered through compensatory mutations is only fragmentary, resistant virus mutants will be replaced by wild-type genomes upon treatment interruption. These rebound viruses may originate from virus that had been archived in latently infected cells before the start of therapy (413) or from the competitive advantage of true revertants that arise during replication (439, 631).

It is difficult to anticipate whether treatments with only limited side effects will be found to decrease HIV-1 fitness without possibility of fitness and viral load recovery. The treatment options presently accessible are those that have been discussed in "Antiviral Approaches to Counteract Quasispecies Adaptability: Lethal Defection and Lethal Mutagenesis" above (313, 393, 574, 819, 840). A major challenge is to target the pool of resting memory $CD4^+$ T lymphocytes and other cells that act as a latent reservoir of HIV-1 genomes and that do not respond to antiretroviral treatment. Reservoir cells have a long half-life, estimated at an average of 14 days for macrophages and 4 years for memory $CD4^+$ T cells. The administration of mutagenic agents specific for the HIV-1 RT, sequentially or in combination with antiretroviral inhibitors (354, 574, 799) is worth exploring. Perhaps a combined pharmacological and immunotherapeutic approach could offer some encouraging prospects, provided that the effects of the immune response against HIV-1 come to be better understood.

**Coping with the immune response.** In contrast to antiretroviral agents that generally target a single viral protein (reverse transcriptase, protease, integrase, etc.), the host immune response generates multiple and diverse constraints that act simultaneously upon the virus. This is true of the multiple branches of the innate immune response (52, 645) and acquires an additional dynamic component in the adaptive immune response (multiple B-cell and T-cell epitopes located at different sites in viral proteins subjected to variation). The immune response imposes several selective constraints to all viral infections, but in the case of HIV-1 there are the added complications that subsets of cells that take part in the immune response can also be infected and that infection-mediated immunosenescence (273) modifies the immunological environment over time.

HIV-1 has evolved both interaction and evasion strategies to cope with the innate immune response. One of the functions of Vif is to counteract the antiretroviral activity of some members of the APOBEC family of cytidine deaminases (362, 504). APOBEC3G and APOBEC3F hypermutate negative-strand re-

verse transcripts, which results in multiple G→A mutations in positive-sense cDNA. This mechanism can be regarded as a natural counterpart of lethal mutagenesis as an antiviral strategy based on compromising viral survival through an excessive mutational load (see "Antiviral Approaches to Counteract Quasispecies Adaptability: Lethal Defection and Lethal Mutagenesis" above). Editing activities can contribute to HIV-1 variation (716). Several mechanisms for HIV-1 to evade activated natural killer cells have been described (16, 267, 389).

HIV-1 escape mutants contribute exuberantly to virus survival in the response to neutralizing antibodies and to cytotoxic T cells. Current evidence suggests that HIV-1 is continuously evading the neutralizing response of antibodies to the point that the effect of virus neutralization in plasma viremia is minimal (694, 851). In this antibody-virus arms race, the virus seems destined to be the winner unless massive amounts of polyclonal neutralizing antibodies could be delivered (410). Escape from neutralizing antibodies may be facilitated by the limited fitness cost inflicted by the amino acid substitutions needed to elude neutralization (823) (see "Selective Forces and Escape Mutants" above). Fitness effects are expected to be more pronounced in the case of cytotoxic T lymphocyte (CTL) escape mutants because T-cell epitopes can be located at conserved regions of nonstructural and structural proteins. CTL escape HIV-1 mutants play a key role in viral persistence (80, 106, 218, 664). As in the case of escape from neutralization, the relevance of quasispecies dynamics is evidenced by multiple escape routes tested in a continuous process of variant generation and selection with viral fitness as a contributing factor (281).

The rate of viral escape from a CTL response depends on the magnitude of the response (which itself is dependent on associated HLA alleles), the diversity of the CTL response, the viral load to which the response is directed, and the fitness cost associated with the amino acid substitutions needed for escape (304, 415, 528). The rate of CTL escape may slow down in the chronic phase of infection. Escape mutants can be transmitted, and the mutants may revert depending on their fitness level in the new host (295). Fitness is an environment-dependent parameter (205), and the rate of epitope evolution is expected to vary in different individual hosts. A rapid intrahost evolution need not be reflected in rapid evolution at the population level (the one resulting from multiple interhost transmissions [295]). (This is analogous to high mutation rates not necessitating high rates of evolution [compare with "Population Equilibrium, Apparent Stasis, and Rapid Evolution" and "Viral Quasispecies Dynamics *In Vivo*: Long-Term Virus Evolution" above].)

In contrast to the neutralizing antibodies evoked during infection, the cellular immune response can partially control the initial HIV-1 replication and viral load, whose decrease may depend on the fitness decrease associated with CTL escape (528, 673, 737, 895). In some elite controllers, (a small proportion of HIV-1-infected individuals who maintain a low viral load in the absence of antiretroviral treatment), a broad CTL response and maintenance of low-fitness CTL escape mutants may contribute to the control of the infection (612). A reason why the HIV-1 infection is often not controlled by the cellular immune response may be the functional impairment of HIV-1-specific $CD8^+$ T cells, and continued viral replication may lead to their exhaustion. In this view, HIV-1 often emerges as the winner in the arms race between the viral quasispecies and the diverse array of immune cells that in itself is highly heterogeneous (183, 623). From the point of view of adaptability of a mutant spectrum and its ensuing diversification, heterogeneity of the major target host cells marks a difference between HIV-1 and the hepatotropic HBV and HCV, to be discussed in the next two sections.

A fitness increase of retroviral immune escape variants need not affect directly the genomic regions related to immune escape (B-cell or T-cell epitopes or their neighborhood in the relevant viral proteins). Rather, fitness may be compensated for by enhancement of other, unrelated traits such as cell-to-cell spread (878). Exploitation of different pathways for fitness recovery has been observed with other viral systems (254), it is expected from quasispecies dynamics and from the connectivity of sequence space (see "Exploration of Sequence Space and Virus Adaptability: Fidelity Mutants" above), and it is highly relevant to vaccine design and to the mechanisms underlying progression of HIV-1 infection toward AIDS.

The capacity of HIV-1 to overcome the host immune response and persist for extensive time periods constitutes a major difficulty for the design of effective vaccines. The well-established concept that multiple B-cell and T-cell epitopes should be targeted using sequences that can cover a broad spectrum of circulating viruses should be followed for the design of anti-HIV-1 vaccines (120, 188, 202, 425, 441, 579). From the results summarized in previous paragraphs, it appears that the focus should be on targeting the immune response toward those epitopes whose variation inflicts a high fitness cost upon the virus (134, 135, 161). Again, a combination of immunological and pharmacological approaches could be considered in attempts to submerge HIV-1 and other highly variable viruses into deep fitness valleys.

The rate of progression to AIDS appears to result from a complex interplay of viral and host factors (references 19, 474, 557, and 753, among many other studies). Concerning the influence of HIV-1 dynamics, it has been difficult to associate global features of the viral population (genetic diversity, number of positively selected sites, and recombination rate) with disease progression (104). Patterns of HIV-1 evolutionary change, defined as the rate of divergence of the viral population relative to the founder virus, and the degree of heterogeneity of the evolving population have produced conflicting results and multiple interpretations. During the asymptomatic period in moderate progressors, Shankarappa and colleagues distinguished three phases. An early phase involved a rapid linear increase of divergence and heterogeneity of the C2-V5 region of env. At the time of emergence of X4 variants, a second phase was characterized by a continuing increase of divergence but not of heterogeneity. Coincident with a decline of $CD4^+$ T cells, a third phase was marked by an invariance or decline of both divergence and heterogeneity (753). This consistent pattern contrasts with those from other studies, which found positive and negative relationships between viral diversification and disease progression (quoted and discussed in reference 753).

A procedure to infer the HIV-1 genomic sequence of transmitted/founder virus has been developed (720). In the cases studied, the transmitted viruses were CCR5-tropic. Characterization of the viruses in ensuing months revealed a few adaptive mutations that were explained mainly, but not exclusively, by CTL responses. An analysis of the $CD8^+$ T-cell response during early stages of the acute infection suggested that the total T-cell response was associated with selection of escape mutants (274). This study also showed that the capacity of T-cell epitopes to tolerate amino acid

substitutions correlated with the emergence of escape mutants, in agreement with fitness cost being an important element in immune escape, as discussed in previous paragraphs.

Ultradeep sequencing is currently providing new information on the dynamics of modification of coreceptor usage (94, 788, 814). In one of the studies, V3 env sequences predicted to be X4-tropic were identified at least 3 months prior to such sequences being detected in a phenotypic assay (94). The transition between CCR5- and CXCR4-tropic variants followed stepwise mutational pathways involving multiple, low-frequency intermediate mutants. Individual patients varied regarding the number and diversity of variants with any given predicted phenotype. One of the problems in trying to anticipate disease progression from general evolutionary parameters of the relevant viral populations is that disease progression may relate specifically to some genetic changes (for example, the determinants of host cell tropism) and not to global features of a viral population. This argument was already expressed regarding the difficulty of associating phylogenetic groupings with biological features that depend on limited genetic change (see "Cell Tropism and Host Range Mutants: Biological Alterations and Viral Emergence" above). Despite the contribution of multiple host factors, HIV-1 evolution within infected patients recapitulates most features of viral quasispecies in relation to pathogenicity and the challenge of finding means to limit the progress of the infection.

### Hepatitis B Virus

**Viral dynamics.** Quasispecies dynamics of HBV has been profusely documented, with processes of mutation, competition, and selection occurring as part of the natural life cycle of the virus, with evidence of past recombination events (769). Multiple types of HBV variants are selected by antiviral treatments or by the host immune response, which arises more slowly than in the case of HIV-1 (for a review, see references 73, 178, 334, 402, 421, 489, 683, and 894). Despite operation of universal Darwinian principles, the HBV genome and those of other, related animal hepadnaviruses have unique features that modify some of the consequences of the acquisition of mutations.

HBV shares with HIV-1 the presence of a genetic reservoir that in the case of HBV is a covalently closed circular DNA (cccDNA) found in the nuclei of infected hepatocytes. During infection, the relaxed circular DNA (RC-DNA) present in virions is converted by cellular enzymes into the cccDNA, which is the template for the synthesis of pregenomic RNA (pgRNA) and viral mRNAs (reviewed in references 421, 477, and 744). The cccDNA constitutes a genetic archive of the virus in the same cells where active viral replication takes place. This has evolutionary implications similar to those of proviral DNA in retroviruses. Due to its high stability (long half-life), cccDNA serves as a repository of ancestral HBV genomic sequences in liver tissue. In addition, viral DNA can integrate into host DNA by an unknown mechanism in what appears to be an unregulated process. Because of the necessary presence of cccDNA, HBV can display both a replicative memory and a cellular (or reservoir) memory. Sequences that had been dominant at an earlier phase of the evolution of the same HBV lineage can be reintroduced in the pool of actively replicating HBV (85, 463) (see "Positive Selection and Molecular Memory: Deterministic Features of Viral Quasispecies" above).

Multiple copies of cccDNA organized as minichromosomes can be found in the nuclei of a single infected hepatocyte. The capacity of cccDNA to contribute to actively replicating HBV depends on the "replication space" available, which means the number of cells that can be productively infected in the liver (not to be confused with sequence space of a virus [compare with "Exploration of Sequence Space and Virus Adaptability: Fidelity Mutants" above] [683, 743, 894]). HBV cccDNA is responsible for the occult HBV infection (OBI) in patients with low or negative surface antigen (HBsAg) and anticore antibodies and who may have a low level of or undetectable HBV DNA in serum (118, 717, 758). Persistence of HBV DNA and possibly its integration into cellular DNA are probably involved in the development of hepatocellular carcinoma (HCC) (751, 797, 871). An alternative model of HBV carcinogenesis involves a virus-mediated increase in the cellular mutation rate. According to this model, the HBV X protein acts as a potent transcriptional activator and stimulates generation of reactive oxygen species that enhance the cellular mutation rate, evoking malignant transformation (813).

HBV has some features that distinguish it from HIV-1 regarding short-term, intrahost evolution. One feature concerns the heterogeneity of the cells that support viral infection, due to susceptible cells belonging to different lineages or sublineages. Despite hepatocyte heterogeneity within a liver (or liver "zonation" associated mainly with differences in metabolic activity [810]), HBV encounters a more homogeneous intracellular biological environment than HIV-1 (183, 623). The intracellular environment and the receptors expressed on the host cell constitute powerful constraints for the selection of viral subpopulations, such as those associated with coreceptor usage switch in HIV-1 (see the previous section). The dynamics due to coreceptor shift that operates in HIV-1 is unlikely to have a parallel in the case of HBV. Despite this, extrahepatic replication of HBV cannot be excluded. Distinct HBV populations can be found in the liver, peripheral blood mononuclear cells (PBMC), and plasma up to 15 years post-liver transplant. Antiviral drug-resistant variants were more frequent in liver than in PBMC, and both compartments were potential sources for reinfection (143). Thus, some HBV compartmentalization, with implications for selection of viral subpopulations, may occur.

Another distinct characteristic of HBV is the remarkable number of overlapping reading frames in its genome. The pre-S1- and pre-S2-coding regions, which precede the surface antigen (HBsAg)-coding region, overlap the spacer domain of the polymerase (P) gene, while the region encoding HBsAg overlaps with the polymerase domain involved in nucleic acid synthesis (421, 894). Gene overlaps can amplify the phenotypic consequences of mutations. In particular, some drug-resistant mutations that map in the HBV polymerase can give rise to amino acid substitutions in the surface antigen that can decrease the affinity of the virus for antibodies. It is generally accepted that the presence of overlapping open reading frames restricts virus variation, because any single mutation has some probability of adversely affecting two proteins and, consequently, viral fitness. This is certainly true, but nevertheless, HBV can manage quite successfully to overcome selective constraints, be they of immunological or pharmacological nature. An argument against overlapping reading frames being responsible for a substantial decrease of virus adaptability is that current virology is teaching us that most (if not all) viral proteins perform multiple functions in the life cycle of viruses. Thus, any amino acid substitution will in effect be responsible for the alter-

ation of multiple functions even if the viral genome lacks overlapping genes.

Despite important differences between HIV-1 and HBV, the basic Darwinian principles of genetic variation, competition among variants displaying different phenotypic traits, and dominance (often transient) of the most fit variants in a given environment obviously apply to HBV, as extensively documented with clinical studies involving each of the viral genotypes (143, 178, 339, 421, 434, 683, 685, 894). The dynamics of mutant generation, competition, and selection is noted *in vivo* even in the absence of external selection pressures such as drug administration or immunotherapy (see "Coping with the immune response" below).

**Drug resistance.** After the implementation of lamivudine (LMV) for treatment of HBV infections, it became apparent that the virus had the capacity to become resistant to antiviral agents, following basically the same course of events described for HIV-1. Resistance of HBV to small interfering RNAs in cell culture and in mice has been described (877). Resistance to nucleotide analogues occurs in a considerable proportion of treated patients, albeit with different kinetics of dominance (reviewed in references [178 and 894]). Antiviral drug-resistant mutants emerge as a function of host determinants and at least six viral factors: the viral mutation frequency, the capacity to tolerate mutations of the antiviral target site, the selective pressure exerted by the drug (effective drug concentration at the viral replication site), the magnitude and rate of virus replication, the overall replication fitness of the resistant mutant relative to that of the wild type, and the availability of replication space (50, 225). Some drug resistance mutations confer cross-resistance to other drugs or prepare the virus for acquisition of mutations that confer resistance to other drugs, compensatory mutations permit fitness recovery following a fitness decrease associated with acquisition of a primary resistance mutation, mutations that confer resistance can preexist in viral quasispecies, and the cccDNA is responsible for the reactivation of viral replication upon therapy interruption (178, 401, 434, 489, 894). This course of events is similar to that documented for HIV-1.

HBV virological breakthrough to lamivudine (LMV) is preceded by the emergence of quasispecies variants bearing amino acid substitutions at polymerase (RT) position 204, i.e., within the YMDD catalytic motif (abbreviated rtM204V/I). Patients tend to have a gradual switch from a YMDD wild-type population at baseline to a 100% lamivudine-resistant population. Careful analysis of amino acid substitutions located outside domain C of HBV RT, including those known to partially restore replication capacities *in vitro*, show that the *in vivo* replication of HBV variants is driven by multiple forces, including replicative advantages conferred by mutations accumulating outside domain C and the changing environment in which these variants replicate (628). In patients who interrupt lamivudine treatment, reversion (partial or complete) to wild-type virus is often observed after only a few months (97).

The distribution of genetic variability of HBV shows different patterns in antiviral-treated and untreated subjects, and the quasispecies divergence of different regions of HBV may vary substantially even within a single host. Antiviral-treated subjects show amino acid changes within the known T-cell or B-cell epitopes in the surface or core antigen, most of which are accompanied by mutations in the RT region. In patients who received lamivudine as initial antiviral therapy, nucleotide polymorphism and nonsynonymous divergence decrease at lamivudine breakthrough but increase after rescue therapies. Patients treated with adefovir-telbivudine sequential therapies show distinct changes in divergence. When untreated subjects have an alanine aminotransferase (ALT) flare, they exhibit an increase in heterogeneity and divergence in the precore-core region (798).

The quasispecies complexity and diversity do not appear to differ between responders and nonresponders to lamivudine at baseline, but both parameters are significantly lower in responders than in nonresponders after 4 weeks of therapy. The dynamic changes of quasispecies complexity and diversity during the first 4 weeks correlate with lamivudine antiviral efficacy and antiviral resistance (119).

Edited HBV sequences with G→A hypermutations are present in infected individuals at different stages of liver disease (787, 827). This innate immune mechanism, initially identified as a defense against retroviruses, may also promote HBV variation. Using ultradeep sequencing technology and a classification model that measures excess G→A mutations, host-mediated G→A hypermutations have been identified. Interestingly, 2.9% of sequence reads from 45 untreated chronically HBV-infected patients were classified as hypermutated, and of those, the majority included two of the most common drug resistance mutations, A181T and/or M204I in HBV RT, that arise from G→A changes (515, 689).

It is well established that viral load is a key parameter to allow the virus to explore sequence space to find pathways for drug or multidrug resistance, while maintaining adequate fitness levels. Insufficient viral suppression, such as that associated with sequential monotherapy with nonmutagenic inhibitors, can promote selection of drug-resistant variants (883). It is anticipated that combination treatments with three or more inhibitors directed to different steps of the virus life cycle may achieve viral suppression, which is essential to prevent disease progression (485). If mutagenic agents specific for HBV RT were developed, current models suggest that a strong reduction of viral load followed immediately by mutagenesis should be an effective protocol to prevent selection of drug-resistant mutants (393). However, these strategies necessitate the development of new anti-HBV inhibitors and specific mutagenic agents. Furthermore, they will be faced with the cccDNA reservoir unless means to deplete this DNA pool or render it inactive are found.

Liver transplantation offers a situation in which residual virus of the acceptor or donor individual finds a new environment (the grafted liver under immunosuppression) to multiply and generate fit quasispecies. A challenge is to suppress HBV viral loads to prevent recurrence of the infection after liver transplantation. Combinations of nucleoside analogues with specific anti-HBV immunoglobulins are currently used with some success (123).

**Coping with the immune response.** HBV can escape immune pressure, as evidenced by selection of HBs antigen mutants in the presence of actively generated or passively administered anti-HBs antibodies (641). As a consequence of the production of antibodies against the HBeAg, anti-HBe escape mutants are selected. Since HBeAg expression is not essential for HBV replication, the virus can evade the anti-HBe response by reducing or turning off HBeAg expression. Precore point mutations, insertions, and deletions occur with high frequency. The introduction of stop codons (such as the frequent G→A transition at nucleotide 1896) may prevent HBeAg production. This mutation also affects the structure of a highly conserved encapsidation signal (termed epsilon),

since G1896 base pairs with nucleotide 1858 at the stem-loop of an RNA secondary structure. Mutation G1896→A is favored in HBV of genotypes B, D, E, and G and some representatives of genotype C, as in these viruses nucleotide 1858 is thymidylic acid and the stop codon stabilizes the RNA structure involved in encapsidation (reviewed in reference 755).

Other common HBeAg variants are core promoter mutants, characterized by point mutations in the promoter for both HBeAg mRNA and core protein mRNA (807). The core promoter mutants decrease HBeAg expression through transcriptional downregulation. The frequent double A1762T and G1764A nucleotide exchange results in a substantial decrease in HBeAg expression but enhanced viral genome replication, again mediated by reduced precore RNA transcription. These virological properties lead to enhanced pathogenicity of core promoter mutants *in vivo*. Increased replication capacity and reduced virion secretion may raise the viral load in the liver, which triggers liver damage directly or indirectly through the immune response (485). Massive liver damage during acute infection leads to fulminant hepatitis. Damage during chronic infection increases hepatocyte turnover, induces fibrosis, and increases the chance of hepatocellular transformation and malignancy (807). As opposed to precore variants, core promoter variants can be detected in patients who are either HBeAg positive or negative. The prevalence of core promoter variants is about 40%, and they are evenly distributed among the major HBV genotypes.

The term HBeAg seroconversion refers to HBeAg becoming undetectable while anti-HBe antibodies are present. When accompanied by low viral DNA levels in serum, undetectable HBeAg is indicative of a reduced risk of liver disease. HBsAg positivity and high DNA serum levels, however, denote viral persistence and elevated alanine aminotransferase (ALT) (a marker of liver disease). Thus, HBeAg negativity can be associated with different disease outcomes, the most negative of them signaled by elevated viral loads (340). The core antigen-coding region appears to be stable prior to seroconversion, but mutations are rapidly selected as a consequence of the anticore immune response (13, 334). Fitness enhancement of HBV can follow after mutations in the basal core promoter that increase binding of liver-specific transcription factors are selected, leading to enhanced viral replication (335, 792).

The S protein contains an exposed major hydrophilic region (residues 110 to 155) which encompasses the antigenic "a" determinant that is important for inducing immunity. The "a" determinant of HBV is a loop with immunological and evolutionary implications similar to those of the RGD-containing loop of FMDV (discussed in "Cell Tropism and Host Range Mutants: Biological Alterations and Viral Emergence" above). Amino acid substitutions at this site can lead to conformational alterations that may affect the binding of neutralizing antibodies or mediate failure to detect hepatitis B virus surface antigen (HBsAg) in diagnostic assays. The most frequent amino acid substitutions in S are G145R and D144A (74, 377). HBV "a" mutants have been shown to escape vaccine-induced or passively transferred neutralizing responses, leading to the development of a true, HBs antigen-positive HBV infection in the presence of high titers of anti-HBs antibodies.

The overlap between the S and polymerase genes has as a unique consequence for HBV that drug resistance mutations may result in a modification of the antigenic behavior of the virus and vice versa. Polymerase (RT) substitutions associated with lamivudine resistance produce changes in the overlapping S gene that result in reduced antigenicity of the HBsAg protein. Escape mutations, present as a result of vaccination or immunoglobulin treatment, map in the fingers subdomain of the polymerase. When combined with lamivudine resistance mutations in the YMDD domain, the S escape mutations have the incredible ability to behave as compensatory mutations that can restore the replication of LMV-resistant HBV (74, 755). The situation faced is that of a man-made coevolution established between treatment response and antigenic variation, which may be accentuated as new immunological or chemotherapeutic interventions are implemented. The availability of variant databases for HBV should contribute to monitoring of the prevalence of mutations and perhaps anticipating their effect on HBV adaptability (691).

In conclusion, quasispecies dynamics is deeply rooted in the natural life cycle of HBV, even in the absence of external interventions. When immunological or pharmacological pressures occur, an extremely complex interplay of mutual influences between viral proteins is triggered. The system offers a fascinating field of research to understand the intricate set of molecular interactions that ensure fitness gain. Again, the challenge is to find means to sink the virus in deep fitness valleys and to deplete or render nonfunctional the cccDNA reservoir.

### Hepatitis C Virus

**Viral dynamics.** Quasispecies dynamics of HCV, first proposed by the Vall d'Hebron group in Barcelona (518), is now a well-established feature of its biology. In infected patients, continuous evolution of HCV takes place, and viral persistence, disease progression, or response to treatment appears to be influenced by HCV population heterogeneity (88, 116, 200, 319, 643, 683). Patrizia Farci has thoroughly reviewed the implications of the quasispecies nature of HCV for evasion of the adaptive immune response and viral persistence, disease progression, liver transplantation, and response to therapy (264). Therefore, here we only underline some aspects of HCV quasispecies, mainly to compare this virus with HIV-1 and HBV and to illustrate some concepts covered in previous sections.

The vast heterogeneity of the HCV that replicates in an infected patient has been amply documented by molecular cloning and Sanger sequencing and recently by application of ultradeep sequencing (543, 584). HCV evolves at rates that are in the range of $10^{-2}$ to $10^{-3}$ substitution per site per year (499). These evolutionary rates rank among the highest reported for RNA viruses (189). In interpreting data on the mutant spectrum diversity and consensus sequence evolution of HCV, some differences between its replication cycle and those of HIV-1 and HBV are worth emphasizing. One is the absence, from all evidence, of a DNA reservoir that could play a role as a repository of ancestral genomic sequences, similar to proviral DNA in HIV-1 or cccDNA in HBV. This implies that a replicative memory, but not a reservoir memory, can operate during HCV evolution subjected to sequential selective constraints (see "Positive Selection and Molecular Memory: Deterministic Features of Viral Quasispecies" above and the previous sections on HIV-1 and HBV, for which a reservoir memory plays a role). Ancestral sequences might be introduced into replicating HCV if there are subsets of infected cells in which HCV replicates at a much lower rate than in the bulk of infected cells. A mechanism of delayed replication relative to the major liver qua-

sispecies would be through extrahepatic replication in organs such as blood or brain. Despite some evidence for extrahepatic replication, compartmentalization of HCV quasispecies is not a settled issue (reviewed in reference 264). If a slow, extrahepatic replication occurred, a replicative memory could in effect act as a reservoir memory, thus introducing a new element of complexity in the understanding of HCV quasispecies evolution. Features that are common to HCV and HBV are a strong preference for replication in liver cells and a delayed adaptive immune response. Hepatotropism should, in principle, provide for HCV a relatively constant biological environment compared with HIV-1 (see discussion of this point in "Hepatitis B Virus" above). Again, extrahepatic HCV replication would introduce compartmentalization of viral subpopulations and environmental heterogeneity (286, 502, 627).

The dynamics of a continuous generation of mutant swarms, competition among variants, and selection of the most fit in each of the environments follow the same tenets expounded throughout this article (264). The availability of cell culture systems that sustain extended multiplication of the entire HCV or genomic replicons has mirrored selective events documented *in vivo*, with clear evidence of adaptability under experimentally controlled conditions. Evidence includes introduction of fitness-enhancing mutations, selection of drug- and interferon-resistant mutants (264, 344, 420, 669, 845), and selection of more stable virus particles (431). A virus-host coevolution in the course of a persistent infection in cell culture (893) reinforces the Darwinian behavior of HCV in interaction with its host cells, as documented with other viruses (see "Cell Tropism and Host Range Mutants: Biological Alterations and Viral Emergence" above). Bottleneck events contribute to diversification of HCV (93, 462, 680). HCV genotype 4a dominates in Egypt, presumably due to poorly sterilized needles in a nationwide parenteral treatment of schistosomiasis in the middle of the 20th century, implying transmission with bottlenecks of different intensity (290). The observed genetic variation along the HCV genome is not distributed in a uniform manner. There are relatively conserved regions such as the 5′ and 3′ untranslated regions (UTRs), where specific sequences and RNA secondary structures are required for replication and translation functions. The E1 and E2 glycoproteins and the nonstructural protein NS5A show more variability, in particular some hypervariable regions that also display rapid amino acid sequence change over time. Part of this variability arises through selection events associated with immune escape (264, 765).

Most acute HCV infections evolve to chronicity, and a significant proportion of chronically infected patients will develop severe liver disease (380, 756). Once liver function is compromised, the only reliable therapeutic intervention is liver transplantation. However, HCV recurrently infects the liver graft in almost all HCV-infected patients following orthotopic liver transplantation (OLT), and the progression of HCV disease posttransplantation is generally accelerated compared with that in nontransplanted patients (60). Although HCV quasispecies diversity in most of the patients decreased in the first week after OLT, it was more pronounced in the patients with a lower fibrosis 1 year after OLT (28). In patients with severe HCV recurrence, evolution of HCV quasispecies was also more pronounced, and HVR1 divergence occurred earlier following transplantation (657). Neutralization escape mutants that display an efficient entry into liver cells may play an essential role in reinfection of the graft following liver transplantation (262). HCV quasispecies have been extensively studied both before and after OLT. During the first days following transplantation, there is generally a rapid decrease in the HCV quasispecies diversity, resulting in a more homogenous viral population. These very early changes in the quasispecies composition strongly suggest that attachment and entry of HCV into hepatocytes constitute a bottleneck contributed by selection and random sampling events from the viral population (519). The homogeneity of the HCV quasispecies continued to increase, being at its highest value 4 weeks after OLT, coincident with high levels of immunosuppression; therefore, relative homogeneity might be explained by a deficient immune response against HCV (270).

As the demand for liver transplants exceeds the organ supply, one approach to expand the pool of organs for transplantation is to use grafts from extended-criterion donors, such as HCV-positive donors. Superinfection with different HCV strains within a single patient is common under these circumstances. Competition between different strains can lead to the exclusion of one strain by the other as soon as the first day after OLT, with no preference as to whether the donor or recipient HCV prevails (263, 686). These observations are analogous to the competitive exclusion principle described for cell culture studies with other RNA viruses (see "Positive Selection and Molecular Memory: Deterministic Features of Viral Quasispecies" above). The level of HCV genetic heterogeneity in pretransplant patients predicted disease severity after transplant (481).

Regarding the connection between the pathogenic potential of a virus and quasispecies complexity, it is worth stressing again that the advantage of a broad mutant spectrum lies in the increased capacity of the viral population to find portions of sequence space in which to increase its fitness. Once a genome with a good fitness level is found, a relative homogeneity can be associated with the replication-competent, disease-provoking subpopulation. In other words, enhanced pathogenesis can be associated with high heterogeneity in the process of searching for the adequate subpopulation or with relative homogeneity when selection has occurred. The time at which population complexity is examined can be critical.

**Treatment response and drug resistance.** Both host genetic determinants and properties of the viral population determine the response of HCV to alpha interferon/ribavirin (IFN-α/R)-based therapies. An increasing number of host genetic factors, with a well-characterized one being the allelic forms of the IL28B site (310), together with population characteristics of HCV, can affect treatment success. In addition, the viral genotype, a trait that results from long-term HCV evolution, also determines response to therapy. HCV genotype 1 is the most difficult to treat, with approximately 50% patients responding IFN-α/R combined therapy, compared to 80 to 90% for genotype 2 or 3 (505). Similar differences have been found in patients coinfected with HIV-1 (317). Extensive research has been done to correlate nucleotide and amino acid substitutions with the genotype-specific IFN-α response, for example, domains and specific mutations in the E2-, core-, and NS5A-coding regions (116, 249, 644). Analysis of NS5A has been a main focus, especially a 40-amino-acid domain situated in the central part of the NS5A, termed the IFN sensitivity-determining region (ISDR). This region has been described as a focal point of quasispecies variation that can predict treatment outcome. In an initial report, Enomoto and coworkers found a correlation between particular amino acid variations in HCV subtype 1b with sensitivity to IFN-α (249). Japanese patients infected with

HCV genotype 1b who had at least 4 mutations in the ISDR consensus sequence were more likely to achieve a sustained virological response to IFN-α-based therapy than those who had fewer than 4 mutations. While some studies confirmed these results (117, 455, 577), other studies with genotype 1b-infected patients from Europe and the United States did not (644, 718, 731, 868). One of the most relevant interactions of NS5A is with proteins of the IFN signaling pathway, through direct inhibition of the IFN-inducible protein kinase R (PKR), which is normally activated by double-stranded RNA (dsRNA) to trigger a cellular antiviral state. The portion of NS5A interacting with the cellular PKR is known as the PKR-binding domain (PKRBD), which includes the ISDR. Thus, PKR inactivation through a direct interaction of the ISDR may be one mechanism by which HCV avoids the antiviral effects of IFN (303). A larger number of mutations in V3 and the interferon/ribavirin resistance-determining region (IRRDR) of virus from pretreatment serum in early virological responders than in that from non-early responders was quantitated in a cohort of patients infected with HCV of genotype 1b (247). A single amino acid substitution in NS5A might be associated with a sustained viral response and ALT normalization (595). However, a possible relationship between specific mutations in the NS5A and IFN-α therapy outcome remains an open question. In addition to dominant mutations that may relate to treatment outcome, several studies have addressed the role of quasispecies complexity in response to treatment, without uniform conclusions. Puig-Basagoiti and colleagues observed that the complexity and diversity of HCV NS5A quasispecies before treatment were lower in isolates from responders than in those from nonresponder patients, particularly in the domain V3. These differences became more apparent during IFN-α/ribavirin therapy, where complexity and diversity remained stable or tended to increase in nonresponders and to decrease in responder patients (677). Figlerowicz and colleagues found that in children with chronic HCV infection, nonresponders included a restricted number of closely related variants while sustained responders included more distant variant classes present in similar proportions (280). The E2 envelope gene has a hypervariable region (HVR1) that expands over the first 417 nucleotides of the E2-coding region and evolves more rapidly than the rest of the genome (771). Many studies have shown that this region has an important role in immune escape (reviewed in reference 264). Patients chronically infected with HCV usually harbor multiple variants with distinct HVR1 sequences that are present at different frequencies, whereas newly infected patients tend to display virus with lower quasispecies complexity than the virus in the donor (462, 680). Acute resolving HCV maybe a result of the transmission of a minor variant that prevails as the dominant species, which generates a mutant distribution that can be cleared by the immune response (486). In a study using ultradeep pyrosequencing (UDPS), samples from four recently diagnosed HCV-positive individuals (with elevated ALT levels consistent with acute HCV infection) were analyzed (847). Low diversity in HVR1 was seen during the first 5 weeks after diagnosis, probably as a result of transmission of only a few virions. Over time, certain variants became dominant whereas others tended to decline in number (847), a pattern of mutant replacement (543) typical of quasispecies dynamics. Relatively little variation is seen in individuals with deficient humoral immune response (62, 613). It has been suggested that given its tolerance of variations, HVR1 can mediate both the enhancement of cell entry and protection of virions from neutralizing antibodies, rendering this region essential for persistence of HCV (51). This constitutes an example of the biological implications of the overlap between antigenic sites and cell receptor recognition sites (see "Cell Tropism and Host Range Mutants: Biological Alterations and Viral Emergence" above).

Low heterogeneity of the HVR1 is generally associated with normal ALT levels (36, 83), and several groups have described that a higher genetic complexity of the HVR1 quasispecies in pretreatment samples is related to low or no response to IFN-α-based treatment (280, 329, 642, 671, 722, 732, 757). No such correlation was observed in telaprevir (TPV)-based treatments (460). A reduction in genetic diversity in HVR1 is likely to be the result of a more successful and balanced cellular and humoral immune response (266, 642). In nonresponders, Farci and colleagues demonstrated that the initial dominant strain persists for at least the first 12 weeks without changes in viral replication levels. This argues in favor of the hypothesis that inherently IFN-α-resistant HCV variants are present in these patients before initiation of therapy. The reappearance of the original dominant strain seen in patients who had a breakthrough suggests either a temporary viral suppression by IFN-α-modulated immune-mediated mechanisms or a partial degree of sensitivity to IFN-α. However, in patients who had a relapse, new viral strains emerged, suggesting that most of the baseline variants were sensitive to IFN-α. These new variants could represent new mutants or preexisting minor variants present at undetectable levels that may have been less accessible to IFN-α (266). Despite the disappearance of viremia, HCV appears to be able to persist for weeks to months at very low levels, providing a reservoir for virus reactivation after IFN-α treatment (266).

Core is the most conserved protein of the HCV genome, with 96% amino acid sequence homology among subtypes (767). However, amino acid substitutions in the core-coding regions of different genotypes appear to be related to insulin, oxidative stress, or steatosis resistance, variable responses in IFN-α/ribavirin combination therapy, and hepatocellular carcinoma (HCC) (427). Core amino acid 70 and 91 polymorphisms have recently been used to predict IFN-α treatment response; for example, Q/H70 and M91 in patients with genotype 1b predict a slow or no response to IFN-α, whereas patients infected with HCV harboring R70 and L91 are more likely to obtain a rapid sustained response (246, 453, 722).

HCV therapy has entered into a new era with direct-acting antivirals (DAAs) (with boceprevir and TPV licensed in 2011, to be followed by others) (449). As explained for HIV-1 and HBV, the initial results suggest that emergence of viral resistance to DAAs in chronically infected patients will be a significant problem. Again, a central issue is whether drug-resistant mutants preexist in HCV populations. Sanger sequencing, as well ultradeep sequencing, has identified mutations that confer resistance to protease inhibitors in patients not exposed to the drugs (156, 733, 828). Verbinnen et al. assessed the dynamics of variants emerging *in vitro* under low, high, or stepwise-increasing concentrations of a potent macrocyclic NS3/4A protease inhibitor (TMC380765). Distinct concentration-dependent mutation patterns were identified. Low-resistance level patterns, initially observed in the presence of low concentrations of TMC380675, were replaced by high-resistance mutational patterns when the concentration of the compound was increased (828). Resistance level-dependent mutations were first described for picornaviruses (see "Selective

Forces and Escape Mutants" above), and this is probably a widespread occurrence. This confers relevance to the drug concentrations that reach the sites of viral replication, a parameter which is rarely quantified. Hiraga and colleagues investigated emergence of TPV-resistant mutants by ultradeep sequencing using an HCV infectious model in human hepatocyte chimeric mice (367). Mice were injected with HCV genotype 1b (with or without the TPV resistance mutation A156S), and subsequently mice were treated with TPV. Mice infected with the A156S HCV variant developed lower-level viremia than those infected with the wild-type strain but showed strong resistance to TPV. Although mice injected with wild-type HCV showed a rapid decline in viremia at the beginning of therapy, a high frequency (11%) of TPV-resistant V36A variants emerged 2 weeks after the start of treatment, suggesting that rapid emergence of *de novo* TPV resistance was induced by viral mutation and selection (367). Again, no attempt is made here to list the steadily increasing number of mutations related to drug resistance because periodic compilations are published (344, 345, 429, 733).

As extensively documented with many viruses, HCV breakthrough is associated with selection of minor subpopulations present in HCV quasispecies (264, 880). Due to functional connections among HCV proteins, mutational linkage has been observed (880), and it has been suggested that amino acid covariance networks can have a predictive value regarding treatment response (37). Thus, there are at least three major predictors of response to HCV treatment: (i) the alleles at different polymorphic sites in the human genome, (ii) viral population size and mutant spectrum complexity, and (iii) amino acid covariance in the consensus sequences of HCV in patients at the time of treatment.

**Coping with the immune response.** Escape from the host immune response is generally considered a major mechanism of maintenance of HCV persistence (264, 643). The establishment of persistence is probably influenced by several virus and host factors, and the participation of mutant generation in this step has been questioned (643). Similarly to HIV-1, HCV displays a dynamics of continuous escape from neutralizing antibodies (217, 839). Likewise, escape from cytotoxic T cells plays a role in persistence (251, 854; reviewed in reference 264). In a chimpanzee model, HCV control and clearance were determined by the level of HCV-specific IFN-γ-producing T cells in the liver (803), which is suggestive of an arms race between virus and cellular immunity as well described for HIV-1, but for HCV it may lead to clearance or persistence.

Some themes are recurrent in the quasispecies biology of HIV-1, HBV, and HCV, as in probably most RNA viruses as they interact with their hosts. One is the arms race in which viral quasispecies and a correspondingly dynamic immune system engage. An active immune response may have two opposite effects. It may complete viral clearance or it may enhance viral heterogeneity and adaptability, thereby jeopardizing treatment efficacy, with good evidence in the case of HCV (264, 564). Both in the immune response to viruses and in external interventions such as antiviral therapy, there seem to be a number of variables that can tip the balance for or against the virus. Thus, from the point of view of achieving virus clearance, the challenge is to understand what are those critical variables so as to act appropriately once the nature of the viral populations we aim to control begins to be understood.

## CONCLUSIONS, CONNECTIONS, AND PROSPECTS

The multiple viral traits that are influenced by the genetic and phenotypic heterogeneity of RNA viruses demonstrate that quasispecies dynamics stands as a major biological feature that affects virus behavior in decisive manners. Although it is probable that not all the implications of quasispecies dynamics have yet been appreciated, some general consensus can be stated. (i) The presence of complex mutant spectra in viral populations is a reality, and it is not the result of artifactual mutations introduced during *in vitro* nucleic acid amplification reactions or of inadequate processing of nucleotide sequencing data. Each of the procedures requires appropriate controls, and the results may need correction of heterogeneity values, but sequencing errors are not the source of the observed heterogeneities. (ii) Most of the biologically relevant variation observed *in vivo* is the result of genetic variation and competitive selection, together with random events acting on multiple replicative units. (iii) The composition, complexity, and amplitude of the mutant spectrum can exert an influence on the biological behavior of a virus, including medically relevant traits such as disease potential or response to antiviral treatments. (iv) When adaptation to a new environment requires only one or few mutations that do not inflict a high fitness cost, adaptation will be achieved with a virus displaying the usual range of mutation rates, rounds of replication, and population size. Compensatory mutations will often be incorporated in an evolving population. In contrast, when a large number of precisely defined mutations are needed for a phenotypic change, the presence of a mutant swarm will generally be irrelevant, and adaptation to the new environment will not take place. A limited range of adaptability imposed by the portions of sequence space readily available to a virus is what keeps virus identity in our biosphere and justifies taxonomical groupings. (v) Quasispecies dynamics has explained the failure of monotherapy and synthetic antiviral vaccines but has opened new possibilities for antiviral interventions.

Processes of adaptation and deadaptation are brought about by events that affect the composition and complexity of mutant spectra, at times with an invariant consensus sequence. Such events have two complementary aspects: (i) the variation of adaptive capacity depending on the population size and complexity of the mutant spectrum and (ii) fitness modulation through internal interactions established among genomes and expression products within a mutant spectrum. From the mechanisms and specific examples described in previous sections of the present article, it is clear that adaptability is linked to four closely related parameters: replication rate, viral load, genetic heterogeneity, and viral fitness. Under conditions of unperturbed virus multiplication, enhancement of one of these parameters generally goes together with enhancement of the others (Fig. 16). Indeed, as already predicted by the very early formulations of quasispecies theory, when a small amount of virus displaying a mutation rate typical of RNA viruses is allowed unrestricted replication (no limitation of space or resources) in a constant environment, competition among continuously arising variants will result in increasing viral load and viral fitness. If population size has to remain constant but replication can proceed (because of variable host cells and a compensatory turnover of virus generation and degradation), the virus will be enriched in progressively higher-fitness components. This course of events can be modified by three major influences: environmen-

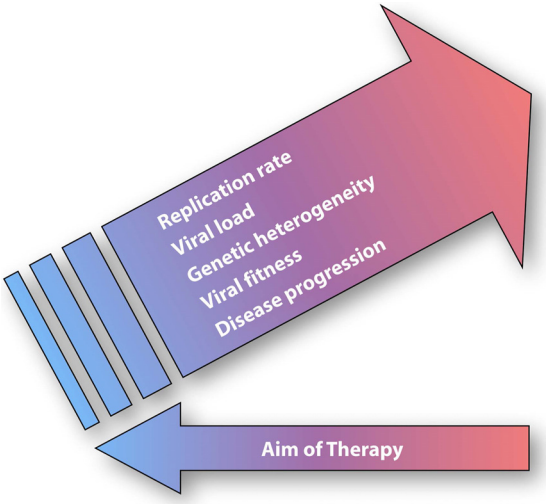


**FIG 16** Interconnected parameters that contribute to disease progression. Replication rate is directly related to the viral load. Viral load, together with population genetic heterogeneity, permits exploration of sequence space for fitness increase. One of the elements of viral pathogenesis is cellular perturbation by virus that displays effective interactions with cells, tissues, and organs, which is increasingly likely when the virus is enriched in potentially functional mutants. Therefore, a major aim of therapy is to oppose increases of viral load or viral fitness. See the text for experimental evidence and exceptions.

tal changes, population bottlenecks, and modification of the mutation rate.

An environmental change can produce deadaptation of a virus that had acquired high fitness in the previous environment. When confronted with an environmental change (immune and other physiological responses), the virus population size confers on the virus the capacity to explore sequence space, an essential ingredient of adaptability (Fig. 8). In connection with viral disease viewed as an adaptation event from the virus side, increased exploration of sequence space often results in disease progression. A general association between viral load and disease severity has been established in many cases, and it can be interpreted as an increased capacity to find a viral subpopulation capable of altering host functions (Fig. 16). Also, the absence of a rapid virological response (decrease in viral load) following treatment initiation is a predictor of virus relapse upon treatment discontinuation (554); host factors may modulate the correlation between viral load decrease and probability of relapse (811). An early start of an antiviral treatment, before the virus increases its intrahost fitness, is the best option for a successful therapy (18, 368). There are exceptions to this picture. Specific viral strains can be particularly pathogenic, independently of any prior or further exploration of sequence space. In the case of PV there are virulent and attenuated strains with genetic signatures associated with these traits, as well as sequence space-dependent virulence determinants, as documented with polymerase fidelity mutants. It may not be easy to distinguish whether a genomic sequence confers high virulence *per se* or because it embodies a capacity to reach neighbor populations of sequence space which constitute disease-associated swarms. We anticipate that application of massive nucleotide sequencing to viral populations as they produce disease in animal models may contribute to clarifying this point.

Exploration of sequence space may result in selection of virus variants that achieve a controlled replication in the face of the host immune response, without disease symptoms. This is the case of chronic, inapparent infections with limited or absent cell damage. What tips the balance between inapparent and overt, disease-causing infections is largely unknown and is probably host dependent and different from virus to virus. In many cases of viruses that tend to produce overt disease, viral load, viral fitness, and exploration of sequence space are interconnected parameters associated with disease progression. Some models of viral ecology suggest that long-term virus survival may select for virus attenuation and persistence strategies. Yet, when acute disease is the outcome of an infection, antiviral therapy should aim at decreasing the values of any of the interconnected parameters implicated in disease progression (Fig. 16).

Bottlenecks can influence the course of events depicted schematically in Fig. 6 and 7 by introducing a stochastic component in which subsets of genomes have to initiate a new cycle of evolutionary events. A third relevant parameter is the mutation rate. Too low a value may limit population heterogeneity and exploration of sequence space, which are in turn connected with fitness gain and viral load. If an infection was initiated by a high-fidelity virus mutant, the prediction is that in successive generations lower-fidelity mutants would be selected, provided that fidelity modifications did not imply impairment of other viral functions. The dominance of lower-fidelity mutants should be more rapid when the virus is confronted with a large environmental heterogeneity during its replication. Tools are now available to test these predictions.

In contrast to low mutation rates, higher-than-basal mutation rates lead to generation of defector genomes which can be detrimental for fitness of the ensemble. Current data suggest a transition between regions of sequence space in which positive, complementing interactions dominate and regions where defection becomes prominent, previous to overt lethality with even higher mutation rates (Fig. 10, 12, and 13). The transition from dominance of complementation toward dominance of defection has been easily observed with modest increases of mutation rate because, from several lines of evidence, RNA viruses replicate close to the error threshold for maintenance of replicative competence. The error threshold is not only an abstract corollary of quasispecies theory; it sets a limit to virus viability when damage to viral genomes and their expression products is no longer compatible with completion of the virus replication cycle. This meaning of error threshold approaches the original formulation by Leslie Orgel, who defined the error threshold in connection with the minimum accuracy of protein synthesis in relation to aging (620, 621).

Because of the proximity of virus replication to an error threshold for virus viability, lethal mutagenesis is a realistic antiviral strategy that can have antiviral effects associated with modest increases of the mutation rate. One of the challenges is to understand the molecular basis of the positive and negative interactions experienced by mutant spectra in order to learn about molecular mechanisms of fitness gain and fitness loss to advance antiviral protocols. Interference was studied extensively with defective interfering (DI) particles, mainly with negative-strand RNA viruses, and those pioneer studies half a century ago can now be applied to understand the consequences of negative interactions within quasispecies exerted by many classes of defective and defector mutants. It is suspected that most interfering interactions at the protein level occur because most viral proteins function as homo- or

heteropolymeric complexes that display decreased activities when some of the monomeric components have amino acid substitutions at sites critical for protein-protein interactions (Fig. 13). While interference exerted either by specific viral mutants or by unfractionated mutagenized viral RNAs has been demonstrated experimentally, the proposed mechanism of malfunctioning polymeric complexes has yet to be proven. Obviously, not only viral proteins but also viral RNA can participate in interference by virtue of its involvement in many RNA-RNA and RNA-protein interactions.

When the implications of quasispecies dynamics were understood, it became clear that synthetic vaccines or antiviral agents used as monotherapy were doomed to failure. It is paradoxical that important advances in biotechnology during the second half of the 20th century have pushed antiviral interventions in the direction contrary to what is required to control diseases associated with viruses that form quasispecies swarms. Indeed, synthetic vaccines consisting of antigenic peptides or proteins expressed from biological vectors became amenable to biotechnological production with the advent of peptide synthesis technology and recombinant DNA methods. Conceptual oversimplifications, together with the ease of production of synthetic antigens, led to hundreds of projects without prospects of being able to effectively prevent diseases associated with quasispecies swarms. Complexity cannot be fought with simplicity. Unless a new paradigm in vaccinology is developed, whole-virus attenuated or inactivated vaccines remain the only ones with some chance to confer protection against infection by highly variable viruses.

Lethal mutagenesis was stimulated by the need to counteract the quasispecies adaptive abilities. A further development has been theoretical and experimental evidence that the efficacy of antiviral treatments can be improved by a judicious use of antiviral inhibitors and mutagenic agents, administered sequentially or in combination, ideally in a personalized manner. Treatment protocols are expected to take into account choice of drugs and drug doses depending on the patient profile, pharmacokinetics, drug compatibilities, viral genotype, and mutant spectrum composition of the virus to be controlled. In using drug combinations, an understanding of possible interactions among drugs is essential, since the effect of two drugs often cannot be accounted for by the effects of the individual drugs (282, 553, 809, 882). This is particularly true in the case of interaction between inhibitors and mutagens, since the former can impede the activity of defectors and the latter can contribute to generate inhibitor-resistant mutants (393, 649, 650) (Fig. 15). Drug interactions that can affect viral deadaptation constitute yet another manifestation of quasispecies acting as a unit of selection.

New developments in the interphase between theory and experiment can be expected in coming years. One important aspect studied theoretically by Manrubia and her associates is the effect of spatial interactions or proximity relationships among replicating quasispecies, in relation to host cell availability, and defense responses. Together with the permissivity or limitation in the spread of genomes, they can affect the outcome of competition among genomes and their survival (9, 98, 158). Although some connections between theoretical models of virus spread and the effects of MOI in experimental infections have been made (508), additional studies in cell culture and *in vivo* are needed. Spatial effects are likely to influence viral survival, and, in principle, this should be approachable through genome comparisons at different sites in an infected host. Subpopulation frequency should be interpreted in relation to local concentrations of components of the immune response or antiviral agents.

Quasispecies theory had its major impact in interpreting the behavior of RNA viruses, but the theory can be applied to other biological systems due to two related facets of the theory: (i) the impact of high mutation rates and (ii) the collective behavior of biological ensembles. The extension to DNA viruses is obvious, since DNA viruses whose replication is catalyzed by a low-fidelity DNA-dependent DNA polymerase will display quasispecies dynamics with the consequences described in previous sections. A less clear situation is encountered with the complex DNA viruses whose replication is catalyzed by high-fidelity polymerases. Despite all viruses displaying adaptability to different environments, it is not obvious whether complex DNA viruses form mutant swarms. Among other issues, to what extent cellular postreplicative repair pathways can cope with the repair needs in viral DNA accumulated in replication factories needs to be investigated. A point to be stressed is that the larger the complexity of a genome, the lower its relative capacity of exploration of sequence space. Thus, the majority of consequences of quasispecies dynamics for virus adaptation and deadaptation will have a higher temporal impact for short RNA genomes than for large DNA genomes, even if they display comparable mutation rates (additional discussion of quasispecies for DNA viruses can be found in references 192, 197, and 204).

A similar argument applies to cells, which have genomes orders of magnitude larger than those of viruses. However, the advantage of high mutation rates for biological systems has been documented with selection of mutator subpopulations of bacteria, often associated with increased pathogenesis (reviewed in references 192 and 619). Bacterial collectivities display cooperative interactions that can modulate their virulence. Genetic heterogeneity and population complexity of cancer cells are important determinants of their adaptability, including the capacity to invade distant organs in metastasis (121, 348). Even if the term quasispecies is used mainly in the field of virology, cells in which a heterogeneous ensemble is the target of selection follow the same conceptual principles of quasispecies theory (for reviews of extension of the quasispecies concept to cell populations, see references 530 and 619 and references therein).

The connection between evolution of viral quasispecies and of early life forms in an RNA world may have yet another remarkable confluence. In the discussions on the tree of life and a putative cellular RNA world (285), a mode of precellular evolution reminiscent of complementary intramutant spectrum interactions has been proposed (870). In this view, early cellular evolution involved frequent exchanges of genetic material at a stage prior to the establishment of those barriers that defined the genetic lineages that evolved toward the ones we see today. This model proposes a rapid Darwinian evolution, with high rates of lineage extinction and generation, close to mechanisms described in this article.

An intriguing extension of quasispecies to a non-nucleic acid system has been to conformation heterogeneity in prions as a determinant of host range (482, 483, 759, 857). Strains of prions are defined by differences in conformation of the same host prion protein ($PrP^C$). Recent data on Darwinian behavior of prions suggest that a prion consists of a "quasispecies" of conformers and that the conformer that "replicates" (that is, converts into the

aggregated pathological form [$PrP^{SC}$] of $PrP^{C}$) is more efficiently selected. In this extension of quasispecies, "mutation" is not an inheritable change in a nucleic acid but a change of conformation of the prion protein. According to this model, a prion consists of a major conformation and a spectrum of conformation variants (147, 482, 857). The quasispecies behavior of prions encompasses selection of inhibitor-resistant mutants and preexistence of inhibitor-resistant mutants in prion populations never exposed to the inhibitor, a remarkable parallelism with viral quasispecies behavior. Rather than being separated by mutation, substrains of prions are separated by the activation energy barrier needed to convert one conformation into another. Adaptation can be viewed as a movement in "conformation space." As in the case of viruses, recognition of quasispecies in prions may open new therapeutic approaches to prion disease, although no effective therapy is yet foreseen (857).

Conversion among conformations in a protein originates in alterations of actions among amino acids, dependent on environmental conditions. Quantum mechanical fluctuations in chemical bonds that determine mutations in genetic systems may also determine conformations in proteins.

The extensions of concepts conveyed by quasispecies to disparate systems such as viruses, cell collectivities, and prions speak of the power of the theory. Despite this review having fundamentally addressed experimental quasispecies evolution, there are ongoing developments in quasispecies theory, with a remarkable cross talk between theory and experiment.

Eigen stated that "Theory cannot remove complexity, but it shows what kind of 'regular' behavior can be expected and what experiments have to be done to get a grasp on the irregularities. This is more true in biology than in any other field of the physical sciences" (233). In this area of research, theory has inspired experiments and vice versa. There is much to be gained from the study of biological and macromolecular entities as spectra or related variants, since in such spectra lie their adaptedness.

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