Interaction of virus populations with their hosts

Abbreviations

A Adenine
ABV Avian Bornavirus
ACE two Angiotensin-Converting Enzyme two
AIDS Acquired Immune Deficiency Syndrome
Arg (R) Arginine
BDV Borna Disease Virus
C Cytosine
CAR Coxsackievirus and Adenovirus Receptor
CTL Cytotoxic T Lymphocyte
CVB Group B Coxsackieviruses
DAF Decay-Accelerating Factor
EIAV Equine Infectious Anemia Virus
FMDV Foot-and-Mouth Disease Virus
G Guanine
HA Hemagglutinin
HAV Hepatitis A Virus
HBV Hepatitis B Virus
HCV Hepatitis C Virus
HIV-1 Human Immunodeficiency Virus Type 1
IFN Interferon
IV Influenza Virus
LCMV Lymphocytic Choriomeningitis Virus
LTR Long Terminal Repeat
MHC Major Histocompatibility Complex
MIC Mutual Information Criterion
MOI Multiplicity of Iinfection
mRNA Messenger RNA
N Population Size
Ne Effective Population Size
NK cells Natural Killer Cells
NTPase Nucleoside-Triphosphatase
PV Poliovirus
PVR Poliovirus Receptor
RGD Arginine-Glycine-Aspartic Acid

RNAi RNA Interference
SARS CoV Severe Acute Respiratory Syndrome Coronavirus
SIV Simian Immunodeficiency Virus
T Thymine
TCID$_{50}$ Tissue Culture Infectious Dose 50 (the amount of virus needed to infect 50% of a cell population)
tRNA Transfer RNA
Trp (W) Tryptophan
U Uracil
VPg Viral Protein Genome Linked
VSV Vesicular Stomatitis Virus

4.1 Contrasting viral and host population numbers

Although we often refer to viruses as "autonomous" genetic elements, their replication is dependent on host functions. This dependence occurs at two levels: at the stage of replication within individual cells and at the stage of dissemination among populations of susceptible cells, animals, or plants. Host cells and organisms have mediated survival of the viruses that we can isolate and study, but they have conditioned their persistence to the capacity to overcome selective constraints imposed by the cellular world. The paradox that represents that host functions are sometimes recruited by the virus

to ensure its replication, while other times they become part of the innate immune response, can be interpreted as a result of long-term coevolution between viruses and cells, and the basic mechanisms that mediate biological evolution in our biosphere. The concept of evolutionary tinkering proposed by F. Jacob in the last century (Jacob, 1977) is adequate to interpret the paradoxical interplay of viral and cellular functions (Domingo, 2011). In this view, viruses and cells, in their coevolutionary race for survival, must have taken advantage, as needed, of what existed at any given stage of the evolutionary process. Here, again, viral population numbers and molecular instructions to ensure genome diversity have played essential roles.

The infection of a host by a virus can be viewed as a specific example of a predator (virus)-pray (host) relationship, with a very distinctive attribute: the disproportionate difference in population size between viruses and their hosts, dramatically in favor of viruses. The large population size of many viral populations (both, as replicative ensembles inside individual cells and as particles available for new rounds of infection and transmission in the field) favors their adaptability, as a consequence of the ease of exploration of sequence space (Section 3.7 in Chapter 3). It is well established in ecology that, except in the case of being a pathogenic agent, the predator cannot be more numerous than the prey (Remmert, 1980). A virus in an infected organism can reach 10^9 to 10^{12} potentially infectious particles at a given point in time. Early calculations were made in cattle infected with foot-and-mouth disease virus (FMDV) (Sellers, 1971), and during togavirus infections (Halstead, 1980). Similar estimates have been obtained for human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), and hepatitis C virus (HCV) (Wei et al., 1995; Nowak et al., 1996; Neumann et al., 1998) or for a tobacco leaf infected with tobacco mosaic virus (review in Gutierrez et al., 2012). Large amounts of virus can be present in excretions and secretions from infected individuals, where the total amount of virus and its concentration is a factor of virus spread. In adult volunteers infected with influenza virus (IV) type A, maximum titers of 10^3 to 10^7 tissue culture infectious dose 50 per ml (TCID$_{50}$/ml) were determined in nasopharyngeal fluids 1 day after infection (Murphy and Webster, 1985). In children with type B IV, titers of 10^4 TCID$_{50}$/ml were present in nasal washings also 1 day after infection (Hall et al., 1979). In the laboratory, for viruses that infect cells in culture, a visible viral plaque on a cell monolayer (or a focus formed in a cluster of infected cells) contains a variable number of infectious units that generally exceeds 10^3. A range of 10^3 to 10^9 infectious units per plaque is quite frequent for cytopathic viruses, such as FMDV and VSV used in studies of experimental evolution (Chapter 6). The lytic plaque and infected cell focus size, and the number of viral particles in them, depends on the replication rate of the viral clone, the way of transmission from cell to cell (either exit into the intercellular medium prior to penetration into a neighbor cell, or direct cell-to-cell transmission), virus stability in the extracellular environment (agar overlay or other medium), and the host cell viability during the time of plaque or focus development. Culture medium added to confluent bacteriophage plaques on a bacterial lawn is an effective method to achieve high bacteriophage titers for viral purification and physical studies. For animal viruses that infect cells in culture, infections of cell monolayers or cells in suspension can be scaled up to produce viral populations of 10^{10} to 10^{11} particles in the case of the most fecund viruses.

The viral population numbers have been given to underline the sharp contrast with the population numbers of their hosts. Considering mammals, each of many primate species includes a total of hundreds to a few thousand individuals. Among the most abundant, the gibbon *Hylobates muelleri* is represented by 3×10^5 to 4×10^5 individuals, and humans by 7×10^9 individuals, with a projection of 9×10^9

individuals for 2040, values which are orders of magnitude lower than the total number of HCV particles in a liver acutely infected with HCV. Mammals are, however, modest in representation as compared with other types of organisms. The number of insect species is uncertain, with estimates broadly ranging from 1×10^6 to 20×10^6, with about 10^{18}-1^{19} individual insects alive in our planet at a given time. Despite these impressive numbers (imagine how many viruses might be hosted by insects that have never been analyzed!), the estimated number of individual insects is still 10^{13}-fold lower than the total number of viral particles on Earth (compare with figures given in Chapter 1). Insects are only exceeded by zooplankton (about 10^{21} individuals) and nematodes (about 10^{22} individuals). To give some additional comparative figures that will become pertinent when dealing with zoonotic transmissions and viral disease emergence (Chapter 7), the total number of livestock is 2.4×10^{10}, and the total number of birds, mammals, reptiles, amphibians, or fish is 10^{10}-10^{13}. Estimates of the number of biological species and of individuals within species are regularly published, and the reader will find numbers that are all extremely modest compared with the VIROME (Viral Informatics Resource for Metagenome Exploration) regarding virus diversity and anticipated number of individual viral particles per group (Wommack et al., 2012; Virgin, 2014; Zhang et al., 2018).

Long-term virus survival has been based not only in multiple strategies to cope with the host immune response but also in their life cycles generally having evolved to produce vast numbers of progeny. From the terminology of ecology, viruses are *r* strategists in the sense that they base their success in rapid reproduction to confront multiple habitats (intrahost compartmentalization and multiple selective constraints, as discussed in Section 4.2). In contrast, large animals are *K* strategists that produce limited progeny, have a long life span, and inhabit relatively stable environments (Remmert, 1980).

4.1.1 Productive power of some viral infections

The exploration of sequence space is commensurate with the number of newly synthesized viral genomes per unit time in infected organisms. Only for a few virus-host systems, the velocity of genome replication (number of nucleotides incorporated into a growing viral RNA or DNA genome per unit time) has been calculated. Early studies indicated that the average time needed to synthesize an entire plus strand of bacteriophage Qβ RNA (4220 nucleotides) in vivo was about 90 s (Robertson, 1975). For poliovirus (PV) it has been estimated that it takes about 1 min to synthesize a full-length genomic RNA (7440 nucleotides) and that when PV RNA synthesis reaches its maximum, 2000−3000 RNA molecules are produced per cell and minute (Richards and Ehrenfeld, 1990; Paul, 2002). HCV polymerase incorporates 5 to 20 nucleotides per second (reviewed in Fung et al., 2014) These values imply that with the mutation rates and frequencies typical of RNA viruses (Chapter 2) mutant distributions of 10^5 to 10^7 genomes can be produced in infected cell cultures or host organisms in minutes.

In the course of infections by HIV-1, it has been estimated that 10^{10} to 10^{11} new virions are produced each day (Coffin, 1995; Ho et al., 1995). The average life span of cells productively infected with HIV-1 has been estimated in 1−2.2 days, with a half-life ($t^1/_2$) of about 1.5 days. The average life span of HIV-1 virions in plasma is about 6 h, with a $t^1/_2$ of 2−4 h (Ho, 1995; Wei et al., 1995; Perelson et al., 1996; Markowitz et al., 2003). The half-life of HCV particles circulating in infected individuals is about 2.7 h, and about 10^{12} particles are produced and cleared every day (Neumann et al., 1998; Ramratnam et al., 1999). A typical active HBV infection can produce 10^{13} viral particles per day; with an average mutation rate of 10^{-4} mutations per nucleotide, 10^9 new mutations can be

tested every day in the 3200 bp HBV genome (Whalley et al., 2001) (review in Quer et al., 2008). Thus, replication of some important viral pathogens is extremely rapid; viruses undergo continuous genetic change and are constantly replaced by new variants (rapid turnover). Genetic and phenotypic diversification is fast and observable. This has been directly noticed in the case of HIV-1 upon the reconstruction of the genomic nucleotide sequence of the transmitted (or founder) virus in a number of patients (Keele et al., 2008; Salazar-Gonzalez et al., 2009). These studies revealed a rapid diversification of the founder, biologically active HIV-1 into multiple replication-competent and defective progeny. There is little question that quasispecies dynamics, as defined in Chapter 3, is operating in vivo, and it implies an effective exploration of the permissive area of sequence space. Rapid, error-prone replication is the basis of virus behavior as *r* strategists, an adaptation to their long-term survival in heterogeneous environments.

## 4.1.2 Population size limitations and the effect of bottlenecks: the effective population size

High viral yields are not universal during viral infections. Viral production can be very high in acute infections in vivo and in cytopathic infections in cell culture. However, viruses can also establish latent infections with intermittent periods of virus production and intervals without detection of infectious virus. In latent infections by DNA viruses or retroviruses, the virus can be undetectable or present in minimal quantities until recurrence of the infection by activation of the latent reservoir takes place. Latency can occur with or without the integration of viral DNA into the host DNA. Chronic infections involve continuous but variable production of infectious virus, with or without disease manifestations that may become apparent only after prolonged chronicity.

An acute infection can be followed by a persistent stage, sometimes producing highly mutated forms of the acute virus that give rise to new pathologies. This is the case of subacute sclerosing panencephalitis, a rare brain disease associated with hypermutated variants of measles virus (Chapter 2).

Persistent infections in cell culture have been divided into steady-state and carrier cell infections. The maintenance of a steady-state system depends on the continued division of cells carrying the replicating virus, and not on reinfection of a susceptible subset of uninfected cells. In contrast, in carrier cell cultures, there is a continuous supply of a small number of uninfected cells that engage in a sustained, low-level viral production. Persistent infections in cell culture have been instrumental to learn about the consequences of virus-host cell interactions, and they are studied in Chapter 6.

In the steady-state persistent infections, cells often produce and release a limited amount of virus, while cells divide with little metabolic affectation. Persistent infections by Borna disease virus (BDV) are particularly illustrative because as little as 0.01−0.05 infectious units are present per infected cell (Pauli and Ludwig, 1985). This behavior may relate to the frequent occurrence of asymptomatic infection of several animal species by this unique viral pathogen (de la Torre, 2002). Limited Bornavirus replication may also explain its relative evolutionary stasis in some hosts, while its rate of evolution and extent of diversification appear to be larger among newly described avian bornaviruses (ABVs) (Philadelpho et al., 2014), with 17-fold higher substitution rates for ABV than the mammal-infecting BDV (He et al., 2014). A requirement of rapid replication is not universal for the different lifestyles exhibited by viruses.

The maintenance of a large population size of viruses in cytolytic or acute infections in vivo is conditioned to the absence of population bottlenecks (strong reductions in population size that
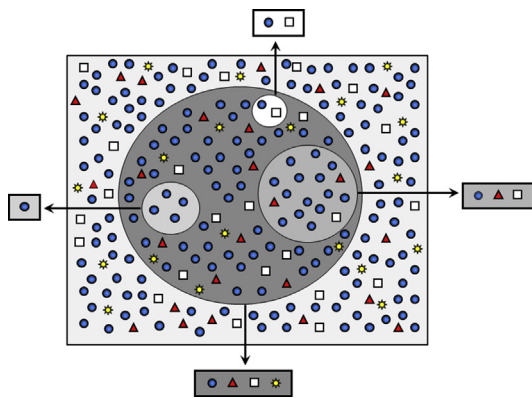
FIGURE 4.1 A depiction of population bottlenecks of different intensity. The big rectangle represents a viral population with multiple variants distinguished by various symbols. Internal circles denote reductions in population size that result in the isolation of one, two, three, or four variant types (different external rectangles) to initiate the next round of infection. The evolutionary outcome will depend on the number of founder variants that participate in the infection. *The figure is modified from Domingo, E., Sheldon, J., Perales, C., 2012. Viral quasispecies evolution. Microbiol. Mol. Biol. Rev. 76, 159—216, with permission from the American Society for Microbiology, Washington DC, USA.*

can alter the course of selective events, as discussed in Chapter 3) (Fig. 4.1). Bottlenecks have been characterized during viral transmission from an infected into a susceptible host, and also within infected hosts (Foy et al., 2004; Scholle et al., 2004; Quer et al., 2005; Ali et al., 2006; Pfeiffer and Kirkegaard, 2006; Kuss et al., 2008; Smith et al., 2008; Haaland et al., 2009; Bull et al., 2011; Domingo et al., 2012; Gutierrez et al., 2012; Forrester et al., 2014). As emphasized by S. Gutierrez, S. Blanc, and colleagues, a variety of population bottleneck sizes is probably encountered during virus-host interactions (Gutierrez et al., 2012). Aerosols produced by an infected individual can spread a very small proportion of the total number of infectious particles, thus representing strong bottleneck restrictions (estimates were reported in Artenstein and Miller, 1966; Gerone et al., 1966; see also Clarke et al., 1994 and

Chapter 6 for the effect of serial bottlenecks, as assessed in laboratory experiments). Bottlenecks are also intense when an infection is initiated through contact with small volumes of secretions or excretions. Different evolutionary outcomes can be expected when a virus is transmitted through a small or large amount of infected fluids, for example, sharing a syringe versus a transfusion with contaminated blood in the case of HIV-1 or HCV when blood screening for these viruses had not been implemented (Shepard et al., 2005; Sharma and Sherker, 2009; De Cock et al., 2012). Not only the probability of infection is higher when a susceptible individual is exposed to a large amount of contaminated fluid, but a massive amount of initial virus facilitates the adaptation of the quasispecies to the recipient host, by providing multiple initial genomes from which to initiate adaptive walks.

Severe population bottlenecks occur during plant leave inoculation, and seed or aphid transmission of plant viruses, such as cucumber mosaic virus, tobacco mosaic virus, pea seedborne mosaic virus, and potato virus Y variants, among others (Li and Roossinck, 2004; Ali et al., 2006; Moury et al., 2007; Betancourt et al., 2008; Sacristan et al., 2011; Fabre et al., 2014; reviewed in Roossinck, 2008; Gutierrez et al., 2012). Aphids transmit an average of 0.5-5 virus particles into the recipient plant host, a range of values that is very similar to that estimated for HIV-1, with evidence that in about 75% of HIV-1-infected patients a single founder genome initiated the infection while in the others a minimum of two to five viruses was involved (Keele et al., 2008). In other cases, no severe transmission bottlenecks or major barriers for dissemination seem to operate during intrahost virus expansion (Murcia et al., 2010; Erickson and Pfeiffer, 2013) (Fig. 4.2). Additional studies are needed to elucidate how variations in population size can affect evolutionary outcomes in viruses. Deep sequencing should provide new insights toward the consequence of bottlenecks for the genetic heterogeneity of viral populations
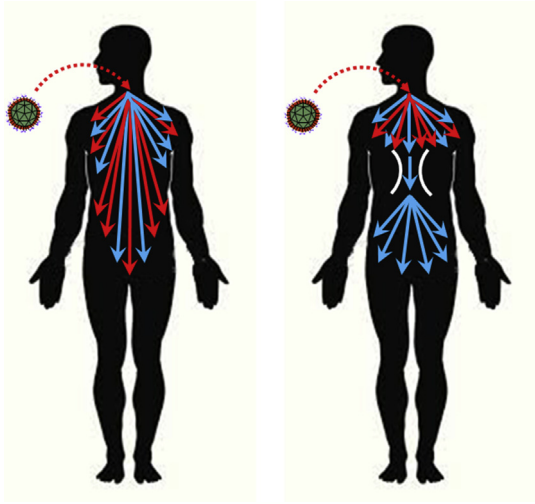
FIGURE 4.2   A simplified scheme of the consequences of a bottleneck event in vivo. On the left, a virus spreads in an infected individual without intervening bottlenecks. Any variant produced (here only two are represented by red and blue arrows) can reach any target organ. On the right, the occurrence of a bottleneck restricts the types of variants (here represented by a blue arrow) that can reach some target organs.

(see Section 4.10 on bottlenecks during the arbovirus life cycle).

Not all viral particles present at a given time in an infected organism are productively replicating. The term effective population size (N_e) has been used to indicate the fraction of the total viral population (N) that contributes to progeny. N_e in virology was adopted from a concept initially introduced by S. Wright in population genetics to mean the number of breeding individuals, and therefore, the number of the total N that contributes to the next generation. It is considered important to evaluate the variability in a population and the relative participation of selection and random drift in evolution (Charlesworth, 2009). N_e is also relevant for conservation biology since it can predict the rate of inbreeding and the level of genetic diversity in wildlife (Palstra and Ruzzante, 2008).

The application of N_e to viral populations is not easy and has not been without controversy.

Measurements of N_e for HIV-1 have yielded values that range from 10^3 to 10^6 (Rouzine et al., 2014). There are at least two reasons to account for the fact that not all viral particles present in an infected individual are replicating to produce infectious progeny at any given time: (i) despite all infectious virus being potentially replication-competent, for stochastic reasons not all of them make their way into a susceptible cell. (ii) A proportion of viral particles that are counted in virus censuses based on quantification of genetic material are intrinsically noninfectious (they could not infect despite having available permissive host cells). The reasons for defectiveness are multiple, encompassing genetic lesions, and virion assembly defects. It is very difficult to estimate the proportion of defective viruses in the different host compartments in which replication takes place. Regarding transmission, it means that when one to four particles are transmitted (external rectangles in Fig. 4.1) some particles may be defective or unfit, thus contributing to possible unproductive infections. Viral N and N_e values may vary locally in different tissues and organs, rendering the interest of the actual N_e value highly dependent on the purpose of the determination (i.e., during viremia to interpret the neutralizing activity of circulating antibodies, or in a specific host compartment to evaluate pathogenic consequences of the infection or the probability of generation of mutants resistant to an inhibitor). The distinction between N and N_e has been attempted with a few virus-host systems (Gutierrez et al., 2012; Rouzine et al., 2014, and references therein). In the cases in which estimates of N_e have been obtained, the values are such that both selection and random drift can influence the evolutionary outcomes. For all these reasons, in the present book, we refer only to virus population size, without attempting a distinction between N and N_e. The interpretation of several observations related to differences in population size that will be discussed in Chapters

5 and 6 is not critically dependent on such a distinction, further justifying our simplification, despite acknowledging the relevance of N_e for nonviral systems, and in some particular cases for virus evolution (for example, the population size version that contributes to fitness gain during viral replication; Chapter 5).

Differences in population size and the severity and frequency of bottleneck events determine the contributions of random drift versus selection as evolutionary influences (Chapter 3). In addition, a bottleneck event, irrespective of its perturbing effect in the process of selection, will reduce the diversity of the post-bottleneck population. A few rounds of replication will generally be needed to restore the mutant spectrum amplitude of the initial populations (Fig. 4.3). It has been proposed that one of the driving forces for RNA viruses to maintain as a universal trait high mutation rates is to favor a rapid repertoire of mutants

to ensure adaptability following bottleneck events that they have to undergo as part of their life cycles (Vignuzzi and Andino, 2010).

Despite having population sizes far smaller than viruses, differentiated organisms can also undergo bottleneck events, for example, through geographical isolation of a subset of individuals of an animal species. Such founder events, by which progeny with different alleles from those dominant in a large parental population has a chance to proliferate, can contribute to geographical differentiation of host species, and it is one of the models proposed for the generation of new species (speciation).

4.2 Types of constraints and evolutionary *trade-offs* in virus-host interactions

Infection of host organisms by a vast amount of viruses must be necessarily limited to ensure
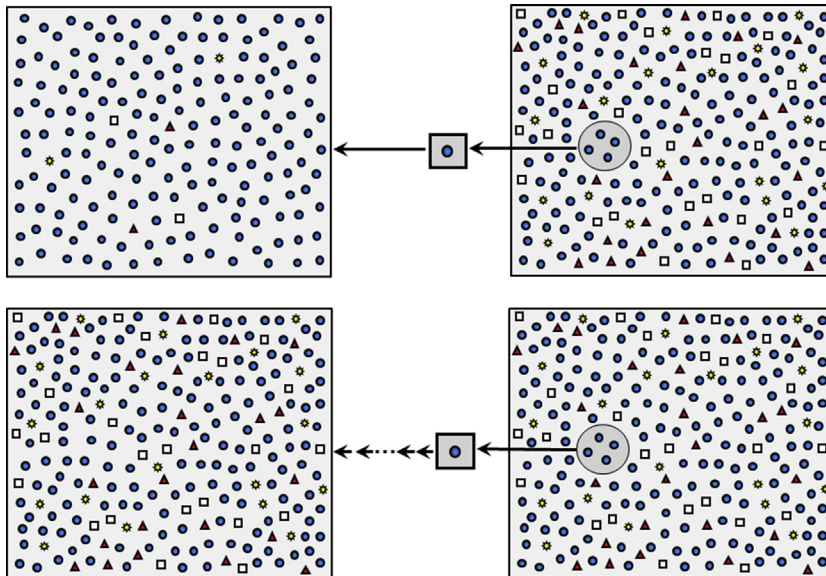
FIGURE 4.3   A population bottleneck results in the reduction of population complexity, and complexity restoration may require multiple rounds of infection. A severe bottleneck is represented by a single type of virus (blue sphere in the small square). In the upper drawing, a single infection leads to limited relevant phenotypic diversification (although virtually any progeny virus can be a mutant). In the bottom drawing, multiple infective cycles have restored a phenotypic complexity similar to that of the parental population.

the long-term survival of both, viruses and hosts. The term, constraint, is used to refer to multiple mechanisms that prevent generalized and devastating infections by viruses, notably, barriers for virus penetration into organisms, and the host immune response. In Chapter 3, positive and negative selection acting on viruses were discussed in conceptual terms, using some specific examples, but without detailing types of constraints that viruses must face in their hosts. At the cellular level, superinfection exclusion provides a set of mechanisms for an infected cell to prevent the infection by other related viruses. However, virus mutants can be selected to partly overcome the exclusion mechanism (Webster et al., 2013; Campbell et al., 2014).

In addition to immune responses and exclusion mechanisms, viruses are continuously and unavoidably subjected to functional and structural constraints, even when replication occurs in relatively constant environments, such as those provided by established cell lines in culture. Several classes of selective constraints can be distinguished (Morse, 1994; Gibbs et al., 1995; Domingo et al., 2001; Simmonds et al., 2004):

- Basal constraints inherent to the virus, and to the requirements for viral genome replication that operate independently of the environment in which a virus is immersed. Such constraints prevent the deterioration of the "core information" that determines viral identity.
- Selective constraints are intrinsic to individual host cells. They include the availability of membrane structures, nucleotide pool levels, tRNA abundances, ionic composition, accessibility of proteins that can act as receptors for viruses, host factors that have to be incorporated into replication complexes or used for viral protein synthesis, presence of RNA or protein chaperones, etc. A balance of many host cell-dependent influences is

necessary for a virus to complete its replication cycle inside individual cells.
- Selective constraints internal to the host as an organism understood as an integrated aggregate of cells, tissues, and organs. These constraints include immune responses, metabolic alterations that may result in fever or abnormal concentrations of metabolites, increased levels of reactive oxygen species, phenotypic heterogeneity among cells of the tissues where the virus replicates, among other organism-dependent perturbations.
- Selective constraints due to external influences exerted on the host. They relate to human intervention and include administration of antiviral drugs or therapeutic antibodies, vaccination, immunosuppressant treatments, and others.

Next, the origin and features of the different classes of constraints are examined.

4.2.1 Long-term history dictates basal constraints

As expressed for the developmental program of an organism (Maynard Smith et al., 1985), present-day viruses can be viewed as the result of a long historical process. From the molecular mechanisms and the building blocks available at the different evolutionary periods, viruses have attained a viable solution as autonomous, cell-dependent, genetic elements. A viral genome was shaped by natural selection acting on phenotypes that were the result of transfers of functional modules, duplication of genomic regions, constellations of mutations acting in concert, and other molecular events that changed the genetic materials. It is not easy to partially dismantle the coordinated set of elements without destroying the replicative capacity of the construct. The requirement of a finely tuned assemblage of elements introduces what we refer to as basal constraints. Their existence at multiple

structural and functional levels has become more and more evident as knowledge of the molecular biology of viruses has progressed. This is one of the reasons why synonymous mutations are not necessarily neutral, as justified in Chapter 2, and why manipulation of viral genomes in the laboratory (changes of gene order, construction of chimeric viruses, codon deoptimizations, etc.) generally results in fitness decrease or even lethality.

High-order RNA structures (such as the internal ribosome entry site for the initiation of protein synthesis of some viruses, or pseudoknot structures involved in protein recognition and ribosomal frameshifting) tolerate limited numbers of mutations, and when they occur, compensatory mutations are selected to maintain their folding (Olsthoorn et al., 1994; Pleij, 1994; Escarmís et al., 1995; Arora et al., 1996; Belsham and Sonenberg, 1996; Martinez-Salas et al., 1996). Open-reading frames may include regulatory signals that are independent of the protein-coding function of the same RNA region. Restrictions to the fixation of silent substitutions within open-reading frames may also be imposed by codon usage and translation efficiency (Britten, 1993; Eyre-Walker, 1996) (Section 4.3). The long evolutionary history of viruses (Chapter 1) has derived in the array of basal constraints that we are beginning to characterize at the molecular level.

4.2.2 Cell-dependent constraints: No free lunch

Other constraints for viruses are a consequence of the complete dependence of virus replication on the cell, and the need for the virus to be competent for transmission from a donor cell into a recipient cell. Constraints to variation of surface residues in the viral particle are one of the mechanisms proposed to explain antigenic stability of some viruses despite their displaying high mutation rates and frequencies (e.g., measles virus or rabies virus), or the presence of widely different numbers of serotypes among picornavirus genera (Chapter 7). Constraints introduce conflicts regarding the evolutionary change. A viral capsid must confer stability to virions outside the cell, but be labile enough (or capable of a transition toward a labile form) to permit uncoating and release of the viral genome as a result of some intracellular environmental change.

The integrin-recognition site of FMDV illustrates constraints acting on this rapidly evolving virus. Contrary to other picornaviruses, the capsid of FMDV lacks a canyon or pit where residues involved in cell receptor recognition lie. FMDV has a smooth surface with a protruding, mobile loop that has the dual activity of interacting with integrin receptors as a means to enter cells and binding to neutralizing antibodies that impair infectivity (Acharya et al., 1989; Verdaguer et al., 1995; Mateu, 2017). Thus, antibody escape through amino acid substitutions in this loop must be compatible with receptor recognition. This explains the limited repertoire of amino acid substitutions at this multifunctional loop among field isolates and laboratory populations of FMDV (Martínez et al., 1992; Borrego et al., 1993; Mateu, 1995) (Section 4.4).

Other types of conflicting requirements are becoming evident as we learn about the multiple molecular interactions between viruses and cells. They force viruses to evolutionary *trade-offs* by which some nucleotide or amino acid substitutions are introduced to confront a constraint, despite rendering the genome suboptimal for another trait. Suboptimality, however, has a limit, and any *trade-off* must fulfill a balance regarding how a genetic change is positive for a trait and negative for other traits. Most virus functions (replication rate, viral protein synthesis, protein processing, particle assembly, etc.) are unlikely to have been optimized for maximum rate; rather, they are the result of *trade-offs* to fulfill multiple requirements to complete the virus life cycle, and each individual function has a performance level, which is conditioned by the requirements of other viral functions.

In the context of *trade-offs*, the theorem of "no free lunch," amply used in economy and algorithm solution searching, applies. Stated in simple terms, a person or a society cannot obtain "something for nothing." There is always a cost, even if that cost is hidden. A virus will endure a fitness cost as a result of having confronted a selective constraint successfully (as illustrated with specific examples in Chapters 5 and 8), and a universal optimization is highly unlikely. Application of the "no free lunch" theorem to complexity theory is currently under investigation regarding its applicability to biological processes, such as genetic optimization algorithms (Whitley and Watson, 2005; Manning et al., 2013; Buenno et al., 2015; Popovici, 2018). Data of viral RNA and protein functions suggest that due to the extremely compact biological information imprinted into a physically small genome, each nucleotide is exploited for multiple functions, even those nucleotides that do not belong to overlapping genes (alternative open-reading frames with two different proteins encoded in part by the same nucleotide sequence). As a consequence, possible neutral sites ("neutral" meaning that their modification does not entail any functional difference) are probably very rare, as explained in Chapters 2 and 3. Some sites may be referred to as neutral only in the sense that a modification in them still allows the virus to survive. Few nucleotides of an RNA virus genome will conform to the definition of neutral alleles in the sense that "one could be substituted for the other …. without affecting the altered individual's prospect of survival and reproduction under any environmental circumstance" (Reeve et al., 1990). The scarcity of neutral mutations is one of the major reasons for fitness landscapes being extremely rugged for viruses (Chapter 5). Finding *trade-offs* is a way of life for viruses. No free lunch for individuals, societies, or viruses.

The constraints often cited as inherent to the host cells (supply of cellular membranes, components of the translation apparatus exploited by viruses for their own replication machinery, etc.) constitute only a minority of the cellular functions that are, one way or another, involved in virus replication. Application of microarray hybridization analysis to quantitate the alteration of host gene expression during viral infections typically shows upregulation or downregulation of hundreds of host genes. Testing the influence of cellular functions by RNAi (interference) screens often reveals unsuspected effects of host gene products in virus replication, even when appropriate controls to exclude off-target effects of the interfering RNAs are used. Part of the host modifications in gene expression observed in infected cells may be just the indirect consequence of other cellular perturbations, and thus, they may not be essential to the progression of the infection. However, the massive response of collectivities of genes illustrates the multiple connections that a replicating virus establishes with cells and suggests that disarrangements of some cellular functions lead to other (perhaps compensatory) changes in the cells. Viruses are structurally and functionally deeply integrated into the cells in which they replicate, in line with theories of virus origins that favor long-term coexistence of viral elements with precellular and primitive cell organizations (Chapter 1).

4.2.3 Constraints in host organisms: contrast with man-made antiviral interventions

Constraints internal to organisms have evolved to display at least three lines of defense against pathogens: intrinsic (preexisting factors that restrict virus replication), innate (activated when a virus enters the organism; i.e., several interferons, apoptosis, NK cells, etc.), and adaptive (immune interferon, T cells, B cells, that expand and evoke a specific cellular and antibody

response against the invading virus, among other activities). Long-term coevolution of host organisms and their viruses must have contributed to the survival of both (Woolhouse et al., 2002; Switzer et al., 2005; Villarreal, 2005). Viruses may respond to short-term perturbations by the dominance of subsets of variants that, in addition to increasing fitness in the new environment, must not compromise viral survival in the presence or absence of the perturbation. Many examples can be cited: an amino acid substitution permitting antibody escape should be compatible with virion stability and receptor recognition; a substitution that decreases virus affinity for a soluble cellular receptor should still allow recognition and binding to the cell-anchored receptor for cell entry, or allow an alternative entry pathway, etc. They are changes that must conform to the *trade-off* concept described in Section 4.2.2.

The external influences exerted on host organisms with the aim of limiting viral replication can be divided into two major groups: those that consist of inducing or mimicking an immune response (vaccination or passive immune therapy, respectively), and those that consist of challenging the virus with antiviral inhibitors which are not among the metabolites of their hosts. Viruses do not have an evolutionary history of confrontation with man-made antiviral agents, and nevertheless, viruses can overcome the effect of this class of inhibitors. This reflects that viruses have evolved survival mechanisms of such a general nature (basically the different genetic variation strategies described in Chapter 2) that they constitute a flexible "tool-box" ready for contingencies. One of the major problems in antiviral therapy is the selection of viral mutants resistant to drugs used in therapy. There is virtually no antiviral agent for which no resistance mutations have been described. In fact, the capacity of a drug to select resistant mutants has been traditionally considered a proof of the selectivity of the drug (Herrmann and Herrmann, 1977). Although many studies on inhibitor-resistant mutants have involved HIV-1, HBV, and HCV

infections, all viruses respond to specific antiviral agents by selecting escape mutants, as described in Chapter 8. Remarkably, different mutations in the same viral genes may determine resistance to, or dependence on, an antiviral agent (de la Torre et al., 1990; Baldwin et al., 2004; Baldwin and Berkhout, 2007, and references therein). This adaptive flexibility is obviously of great practical relevance, and it has encouraged the exploration of new antiviral strategies that aim at avoiding selection of virus mutants resistant to antiviral agents (Chapter 9).

There are antiviral inhibitors that establish a connection between external and internal constraints. They target a cellular metabolic pathway whose inhibition results in a stimulation of innate immune response genes. Inhibitors of pyrimidine biosynthesis (for example, A3, DD264, and brequinar) may affect specific replicative steps of some viruses, and they trigger induction of components of the innate immune response. They confront the virus with a broad (multifactorial) antiviral response (Lucas-Hourani et al., 2013; Ortiz-Riano et al., 2014, and references therein). For this reason, this class of inhibitors are broad-spectrum antiviral agents, and the hope is that they may be less prone than standard inhibitors to select resistant mutants. If this expectation is confirmed, this class of inhibitors could be incorporated into new antiviral strategies to control viral quasispecies (Chapter 9).

4.3 Codon usage as a selective constraint: virus attenuation through codon and codon-pair deoptimization

Codon use bias in relation to cellular tRNA abundances was listed as one of the mechanisms by which synonymous mutations can affect virus behavior (Section 2.3 in Chapter 2), and the possibility of codon choice is part of the relevant information harbored by the genetic code (Maraia and Iben, 2014). Here we expand the discussion of

codon usage as a constraint for viral infections and its implications for long-term virus-host interactions. Several computations have established that the choice among synonymous codons is not random in many biological systems, not only in viruses. Evolutionary events must have resulted in the preferential use of some codons over others, and several possibilities have been proposed. One is that misincorporation tendencies of nucleic acid polymerases may have led to the selection of codons rich in the bases preferentially introduced during genome replication. Long-term priority for some mutation types (because of the catalytic and fidelity properties of cellular or viral polymerases) may decant synonymous codons in favor of those containing the nucleotides that arise more frequently as a consequence of the mutational bias. An alternative, but not mutually exclusive possibility, is that codon bias might have been an evolutionary outcome for optimal RNA secondary structure or RNA-RNA interactions. RNA is rarely a linear unstructured polynucleotide (as usually drawn for simplicity), but rather a complex molecule with a number of high-order structures (stem-loops, pseudoknots, kissing loops, etc.) that play functional roles, and contribute to RNA stability. The appearance of an RNA molecule is certainly closer to that of a protein than to double-stranded DNA (as examples, see Cantara et al., 2014; de Borba et al., 2015 and references therein). Regulatory signals in viral RNA may be located either in untranslated regions or within open-reading frames. In the latter case, preservation of the higher-order structure may restrict the possibility of some triplets to mutate to synonymous ones. This occurrence would constitute an example of negative selection acting on synonymous mutations amply documented during RNA virus evolution (Chapter 2).

tRNAs are among the oldest biological molecules dating back to the time in which the genetic code was developed (Eigen, 1992). In accordance with their ancient nature and with the critical role they play in the transmission of information,

tRNAs are extremely conserved among cellular organisms, and their sequences have served, to date, the origin of the genetic code (discussed in Chapter 1). tRNAs must have been extremely restricted with regard to nucleotide sequence changes, due to folding requirements and their role as "adaptor" molecules. Regulation of translation could not be achieved (at least in an effective manner) through tRNA sequence modifications. Instead, regulation could be attained through differences in the abundances of tRNAs that recognize different synonymous codons.

The accommodation of synonymous codon usage to the cellular tRNA pool is known as translational selection. Other mechanisms related to selection of base and dinucleotide frequencies, composition of enhancers of splicing, or translation kinetics have also been proposed as underlying the variation of codon usage (dos Reis et al., 2004; Chamary and Hurst, 2005; Lavner and Kotlar, 2005; Shackelton et al., 2006; Yang and Nielsen, 2008; Aragones et al., 2010). Picornaviruses have been studied regarding synonymous codon usage and the response of a virus when codon frequencies are artificially altered. Introduction of unpreferred synonymous codons in the capsid-coding region of PV resulted in fitness decrease, attributed to alteration of an early step in the virus replication cycle (Burns et al., 2006). The relative fitness of the modified virus, measured in HeLa cells, decreased in proportion to the number of replaced codons. Codon deoptimization resulted in reduced viral RNA yields and decreased specific infectivities of purified virus (Mueller et al., 2006). The specific infectivity is the ratio between infectious and physical particles, an important parameter that is further discussed in Chapter 9 because its decrease is associated with the extinction of viruses by lethal mutagenesis. Not only codon usage, but also codon-pair frequencies can affect PV fitness. It has been suggested that viruses deoptimized for codon pairs may open the way to a new generation of antiviral vaccines

(Coleman et al., 2008), and this approach is currently investigated with other viral systems [(Martrus et al., 2013; Le Nouen et al., 2014; Nogales et al., 2014; Cheng et al., 2015; Li et al., 2018), among other investigations]. These observations reflect once again the multiple ways in which a viral RNA per se can be part of the viral phenotype, independently of its protein-coding function.

There is no clear picture of the molecular mechanisms involved in fitness recovery of codon-deoptimized viruses. Evidence for HIV-1 suggests that phenotypic reversion can come about through true reversion of some of the initial modifications, together with additional mutations (Martrus et al., 2013). Information on the mutational pathways for fitness recovery of these highly debilitated viruses is essential to quantify the stability of the attenuated phenotype of candidate vaccines based on codon modifications.

In contrast to PV, hepatitis A virus (HAV) uses rare synonymous codons (those that correspond to tRNAs that are present at low concentrations in the cells it infects) to control the rate of translation. The adequate combination of common and rare codons allows HAV to regulate ribosome traffic and to slow down the synthesis of capsid proteins to facilitate their proper folding, thus contributing to capsid stability (Sánchez et al., 2003; Aragones et al., 2010; Costafreda et al., 2014). In an elegant study, HAV was replicated in cells treated with actinomycin D (a specific inhibitor of DNA-dependent RNA polymerases), which provided an altered cellular environment in which the tRNA pool available for translation of viral RNA was increased. HAV adaptation to the modified environment resulted in a new deoptimization of codon usage in the capsid-coding region, again supporting translation kinetics selection as the basis for biased codon usage by HAV. Proper protein folding may be essential for nonenveloped viruses that are transmitted via the fecal-oral route to survive for prolonged time periods in the external

environment (Aragones et al., 2010). For viruses whose infectivity is maintained in the external environment, the time-dependent difference between intra and interhost evolutionary rate might be accentuated (discussed in Chapter 7). The differences between PV and HAV regarding fitness effects of codon usage modification were reviewed by Bosch et al. (2010).

In the course of the studies with HAV, evidence of quasispecies memory (explained in Chapter 5) and of selection for fine-tuning translation kinetics acting on the mutant spectrum as a whole was obtained (Aragones et al., 2010). Also, the continuous re-deoptimization of HAV to the new environment to maintain fitness constitutes further support of the "Red Queen" hypothesis (Van Valen, 1973; Krakauer and Jansen, 2002), one of the concepts of population genetics shown to operate with RNA viruses. (Concepts first proposed in general population genetics and then shown to operate in viruses are treated in Chapter 6).

Codon usage also has biotechnological implications. When the codon frequencies in an expression system do not match the codon usage of the viral genomes to be expressed, viral yields may be diminished. This is a relevant factor, which is taken into consideration for the choice of expression systems, including the use of synthetic genes with optimized codon composition (Lanza et al., 2014 and references therein).

4.3.1 The synonymous codon space can affect an evolutionary outcome

It is worth expanding on the concept outlined in Chapter 2 that synonymous codons may lie at a different distance from a nonsynonymous codon in sequence space, and this may modify the extent of genetic change needed for adaptation to a new environment (e.g., to reach an amino acid substitution to confer resistance to an antiviral inhibitor). Some mutations have been termed *quasisynonymous* because, despite

not leading to an amino acid replacement, they can affect the evolutionary course (Salemi and Vandamme, 2004). As an example, there are six triplets that encode the amino acid R (Arg), and they all have a G in the middle position (AGG, AGA, CGG, CGA, CGC, CGU); out of these, only two (AGG and CGG) are within a one nucleotide distance from W (Trp) (UGG); transversion A → U is required to change the triplet AGG (R) to UGG (W), and transition C → U is required to change CGG (R) to UGG (W). The only triplet encoding W (UGG) is within a single nucleotide distance from two termination codons (UGA and UAG) (Fig. 4.4). Thus, a difference in the R codons that a virus uses is not a neutral trait since two of them are within a one nucleotide distance of the codon for W, which in turn is at one nucleotide distance of two termination (stop) codons. The latter may be reached as a result of increased mutational pressure, either due to a decrease of polymerase fidelity or to antiviral lethal mutagenesis treatments (Chapter 9). A strategy to attenuate virus consists in engineering triplets at one mutation distance from a stop codon, or expressed more generally, in transferring genomes to unfavorable regions of sequence space (Moratorio et al., 2017; Moratorio and Vignuzzi, 2018).

In several viral populations that have been examined by deep sequencing, a remarkably high frequency of termination codons is observed, denoting the presence of defective genomes that represent either dead-end evolutionary pathways or genomes that are maintained by complementation during quasispecies replication (Rodriguez-Frías et al., 2012). The frequency of termination codons may increase as a result of treatment with antiviral agents and may decrease the fitness of the quasispecies ensemble.

The studies and concepts summarized in this section bear on the increasing realization of the potential biological impact of synonymous codon modifications (Hunt et al., 2014), and how movements in a neutral sequence space can actually affect the phenotypic space (Schuster, 2011). Position in sequence space is

FIGURE 4.4 The genetic code with an indication of triplets and encoded amino acids. The triplets in red are those discussed in the text in connection with the phenotypic nonequivalence of synonymous codons, regarding the proximity to STOP (protein synthesis terminating) codons.

pertinent to the viral dynamics in the infected hosts.

4.4 Modifications of host cell tropism and host range

Any step in the replication cycle can determine the permissivity of a cell type to sustain the replication of a virus. However, recognition by viruses of cellular receptors is a key determinant of cell tropism. Macromolecules that act as viral receptors are diverse, and they include cell adhesion and cell-to-cell contact proteins, extracellular matrix components, sugar and lipid derivatives, chemokine and G-protein-coupled receptors, growth factor receptors, complement control protein superfamily, low- and high-density lipoprotein receptor, tumor necrosis factor-related proteins, and transporter proteins, among others (as review, see Baranowski et al., 2003; Bhella, 2015). The expression of a virus receptor is a necessary but not sufficient condition for the virus to infect a cell. The PV receptor (PVR or CD155) belongs to the immunoglobulin-like superfamily of cell adhesion molecules that constitute an important group of the cellular receptor for viruses (Mendelsohn et al., 1989; Bhella, 2015). Many tissues that express PVR are not infected by PV in vivo. Likewise, the sialic acids that act as IV receptors are common in cell surfaces, and the virus attaches to them, but the productive infection is highly restricted to the epithelial cells of the respiratory tract (Bergelson, 2010).

About 10^3 to 10^4 receptor molecules can be present per cell (Lonberg-Holm et al., 1976; Consigli et al., 1986; Thulke et al., 2006). Although the MOI during in vivo infections is difficult to estimate, it is unlikely that the number of receptor molecules represents a limitation to an infection. Bottlenecks are more probably due to restrictions of the number of infectious viruses that reaches a target tissue or organ than to an insufficient number of receptor molecules on the cell surface. The interaction of a virus with one or several receptors (or a receptor and a coreceptor) will generally allow virus entry, which is a multistep process that involves changes in virion structure, a succession of low- and high-affinity binding to one or more cellular proteins, and membrane fusion in the case of enveloped viruses (Verdaguer et al., 2014; Strauss et al., 2015).

Features of virus-receptor interactions relevant to viral evolution are summarized in Box 4.1, based on concepts reviewed in Baranowski et al. (2003). Of note is that differentiated organisms do no express the same set of cell surface macromolecules in different tissues and organs. This implies not only compartmentalization of viral infections but also the possibility of selection of viral subpopulations in specific host compartments, a translation in vivo of one of the tenets of quasispecies dynamics. A significant example is the cyclical adaptation of measles virus quasispecies to epithelial and lymphocytic cells (Donohue et al., 2019).

Members of the same virus family, or viruses associated with related disease manifestations, may use different receptors. Among other examples, consider the diversity among viruses that can cause brain or liver disease. Since tropism changes are one of the biologically most relevant consequences of viruses existing as mutant spectra, several studies have been listed in Table 4.1 to emphasize that tropism changes are not exceptional; the list is by no means exhaustive. Some residues of the IV hemagglutinin (HA) dictate the specificity for sialic acid linked to galactose by either an α-2,3 or an α-2,6 linkage, and linkage preference is a determinant of host specificity (a preference for α-2,6 linkage by human IVs, and for α-2,3 linkage by avian IVs) (Skehel and Wiley, 2000; Parrish and Kawaoka, 2005; Yang et al., 2015). In lymphocytic choriomeningitis virus (LCMV), high-affinity binding to its receptor α-dystroglycan is associated with immunosuppression and viral persistence in mice, whereas low-affinity binding results in

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BOX 4.1

Facts related to receptor usage by viruses

- Different compartments within an organism do not express the same surface macromolecules that can act as viral receptors.
- A virus may use different receptors and coreceptors.
- A receptor type can be shared by different viruses and other microbial pathogens.
- A phylogenetic position or biological features of a virus do not predict the use of some

receptor types. Members of the same virus family or viruses that are associated with similar disease manifestations may use different receptors.

- One or a few amino acid substitutions in capsid or surface proteins may modify receptor recognition, with consequences for viral pathogenesis.

TABLE 4.1   Examples of one or few amino acid substitutions that can modify virus cell tropism.

Observation	References
Host restriction of avian polyomavirus budgerigar fledgling disease virus	Stoll et al. (1994)
Colonic tropism and persistence of murine norovirus	Nice et al. (2013)
N versus B tropism of murine leukemia virus	Jung and Kozak (2000)
Tissue tropism of adeno-associated viruses	Wu et al. (2006)
Cellular tropism of feline immunodeficiency virus	Verschoor et al. (1995), Vahlenkamp et al. (1997), Lerner and Elder (2000)
Loss of enteric tropism of transmissible gastroenteritis coronavirus by two amino acid substitutions in the spike protein	Ballesteros et al. (1997)
HIV-1 tropism by a single amino acid substitution in gp120	Takeuchi et al. (1991), Boyd et al. (1993)
SARS coronavirus recognition of ACE2 receptor (see also text)	Li et al. (2005)
Receptor preferences of herpes simplex virus	Spear et al. (2000)
Hemagglutinin residues in influenza virus tropism (see also text)	Rogers et al. (1983), Connor et al. (1994)
Substitutions in poliovirus capsid expand receptor recognition	Colston and Racaniello (1995)
Conversion of encephalomyocarditis D into a diabetogenic variant through altered cell tropism	Bae and Yoon (1993)
High-affinity binding of measles virus to CD46	Hsu et al. (1998)
Decreased neurovirulence of Sindbis virus through impaired receptor recognition in neural cells	Tucker and Griffin (1991), Lee et al. (2002)
Parvovirus host range (see also text and Fig. 4.5)	Hueffer et al. (2003)
Change in receptor recognition by foot-and-mouth disease virus in vivo and in cell culture (see also text and Fig. 4.6)	Baranowski et al. (2000, 2001), Ruiz-Jarabo et al. (2004)

clearance of infection (Sevilla et al., 2000, 2002; Smelt et al., 2001).

One or few amino acid substitutions have been associated with changes in cell tropism and host range of parvoviruses (Hueffer and Parrish, 2003; Parrish and Kawaoka, 2005; Shackelton et al., 2005; Lopez-Bueno et al., 2006) (Fig. 4.5), adenoviruses (Huang et al., 1999), herpes simplex viruses (Spear et al., 2000), and lentiviruses. HIV-1 coreceptor usage varies in the course of infection in humans. Most primary HIV-1 isolates belong to the R5 receptor specificity (they use coreceptor CCR5), and as the infection progresses, dualtropic (R5X4) and X4 variants (that use coreceptor CXCR4) arise and often become dominant. The expansion by HIV-1 of coreceptor usage to include CXCR4 is associated with loss of $CD4^+T$ cells, and to progression to AIDS (Connor et al., 1997). The R5 to X4 transition in coreceptor usage constitutes an example of tropism change with a direct impact in viral pathogenesis, a change that is observed in about half of HIV-1-infected patients. Multiple, additional coreceptors can be used by HIV and simian immunodeficiency virus (SIV) variants, and transitions in receptor usage vary with virus types and subtypes. Two amino acids in the spike (S) protein of the SARS coronavirus (SARS CoV) modulate the binding to either human or palm civet angiotensin-converting enzyme 2 (ACE 2), a functional receptor for the virus (Li et al., 2005).

It must be clarified that the examples in Table 4.1 and those commented in the text are centered on the effects of a few amino acid substitutions on cell tropism. Some of these substitutions and additional ones may affect virus pathogenesis, which is often (but not always) related to tropism alterations. Moreover, other genetic lesions, such as insertions or deletions (not only point mutations) may also affect receptor recognition and pathogenic potential.

The selective forces that trigger a modification of cell tropism are not easy to identify although several observations suggest that the availability of the type of receptor may select a variant subpopulation out of a mutant distribution. HIV-1 variants enter $CD8^+$ cells at late disease stages (Saha et al., 2001). In a mouse model, a modified form of RANTES, a natural ligand for CCR5, selected HIV-1 mutants that used CXCR4 as a coreceptor (Mosier et al., 1999) (also discussed in Chapter 9 in connection with antiviral agents directed to cellular targets). The bicyclam AMD3100, a selective antagonist of CXCR4, led to the suppression of X4 variants in cell culture and prevented the switch from R5 HIV-1 to X4 HIV-1 (Este et al., 1999). Group B coxsackieviruses (CVB) use the coxsackievirus and adenovirus receptor to infect cells (Bergelson, 2010). When a CVB was passaged in a cell line expressing a limited amount of CAR, the virus expanded its cell receptor specificity to bind multiple molecules including CAR and decay-accelerating factor (DAF). The modification involved a limited number of amino acid substitutions in the viral capsid (Carson et al., 2011).

Multiplication of a virus in a given cell line (or primary culture) may select virus subpopulations present as a minority in a biological sample from a naturally infected host. Passage of biological clone of FMDV in BHK-21 cells resulted in the dispensability of the RGD (integrin recognition Arg-Gly-Asp sequence) and expansion of cell tropism (Ruiz-Jarabo et al., 2004) (Fig. 4.6). Thus, paradoxically, repeated replication in a cell line may lead to the relaxation of virus specialization for that cell line. Application of deep sequencing to describe mutant spectra in vivo should help in establishing whether low-frequency mutants with altered receptor recognition sites might be present in evolving quasispecies. Their basal frequency will depend on their fitness relative to other components of the mutant spectrum. Here again, the relevance of virus population numbers is evident; the larger the population size, the higher the probability that variants with altered cell recognition are present as minority components of the mutant cloud.

2240   4687

VP1

VP2

MVMp, 5B - I K - - - - - - -

1B, 2L, 3B - S - - - - - - - -

3L, 4B, 4K, 4L - - - - - - - - - - - - - - - - - - - R - - - - - - -

362
368

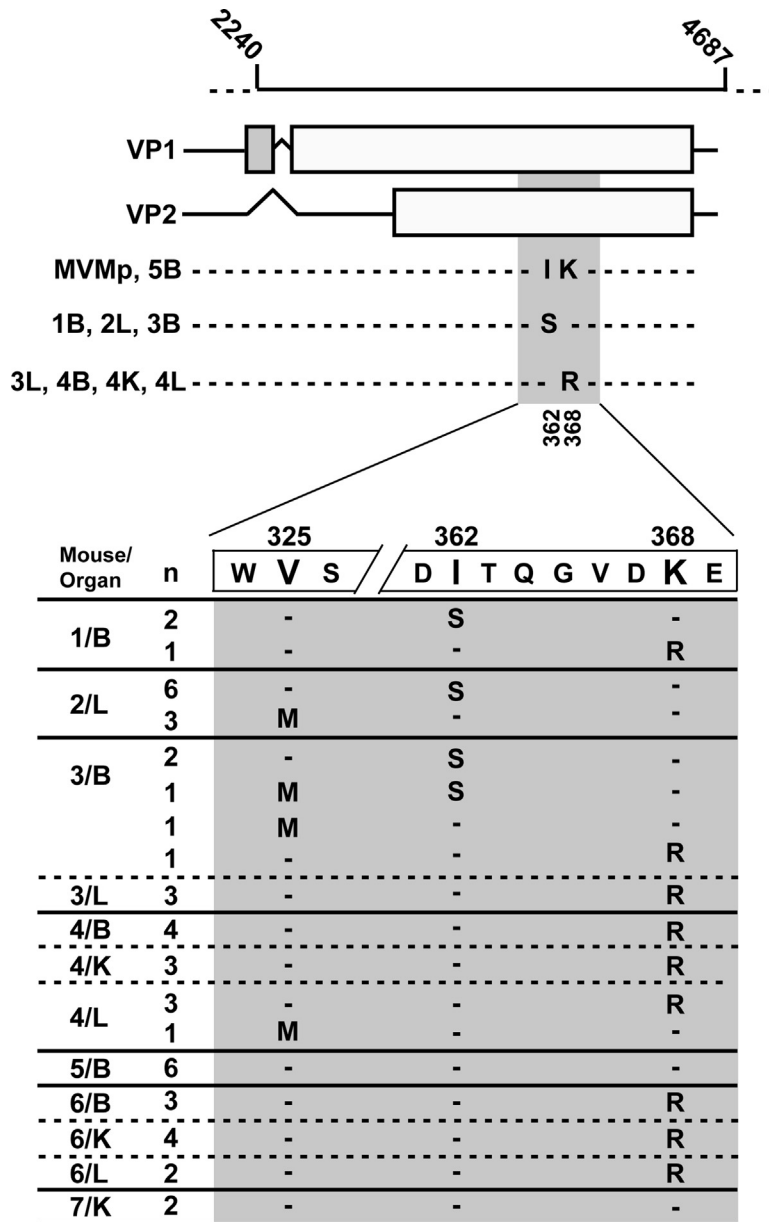| Mouse/Organ	n	325 (W **V** S)	362 (D **I** T Q G V D)	368 (**K** E)
1/B	2	-	S	-
1/B	1	-	-	R
2/L	6	-	S	-
2/L	3	M	-	-
3/B	2	-	S	-
3/B	1	M	S	-
3/B	1	M	-	-
3/B	1	-	-	R
3/L	3	-	-	R
4/B	4	-	-	R
4/K	3	-	-	R
4/L	3	-	-	R
4/L	1	M	-	-
5/B	6	-	-	-
6/B	3	-	-	R
6/K	4	-	-	R
6/L	2	-	-	R
7/K	2	-	-	-

FIGURE 4.5   Amino acid substitutions found in the capsid protein of the prototypic strain of the parvovirus minute virus of mice (MVMp) upon the passage in immunodeficient mice. At the top, genomic residue numbers and amino acid substitutions (single letter code) found in virus from several organs are listed. At the bottom, a more detailed list of substitutions found in many individual clones is given. The numbers (*n*) indicate the number of clones analyzed, and the letters the organ from which the clones were isolated (B, brain; K, kidney; L, liver). *The figure is modified from Lopez-Bueno, A., Rubio, M.P., Bryant, N., McKenna, R., Agbandje-McKenna, M., et al., 2006. Host-selected amino acid changes at the sialic acid binding pocket of the parvovirus capsid modulate cell binding affinity and determine virulence. J. Virol. 80, 1563–1573, with permission from the American Society for Microbiology, Washington DC, USA.*
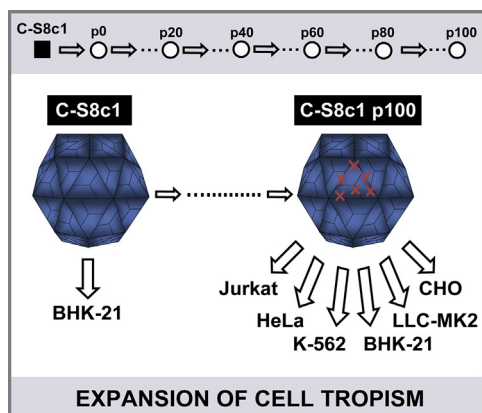
FIGURE 4.6 Expansion of host cell tropism. FMDV biological clone C—S8c1 (filled square at the top) was passed in BHK-21 cell at an MOI of 1—5 PFU per cell (open circles at the top). The resulting population included amino acid substitutions in the viral capsid and acquired the capacity to infect additional cell lines (large arrows below the capsid structures). *Scheme based on the results reported in Ruiz-Jarabo, C.M., Pariente, N., Baranowski, E., Dávila, M., Gómez-Mariano, G., et al., 2004. Expansion of host-cell tropism of foot-and-mouth disease virus despite replication in a constant environment. J. Gen. Virol. 85, 2289—2297.*

4.4.1 Nonstructural viral proteins and RNA in cell tropism and host range of viruses

The capacity of a virus to penetrate a new cell type has consequences for its host range, and the potential emergence of new viral pathogens (Chapter 7). Nonstructural proteins (those that are not present in the viral particles) and regulatory regions in viral genomes may also mediate cellular tropism and host range changes in viruses. Deletions and point mutations in the nonstructural protein 3A of FMDV have been associated with attenuation for cattle (reviewed in Baranowski et al., 2003). A single amino acid substitution in 3A was critical for the adaptation of a swine FMDV to guinea pig (Núñez et al., 2001) (Fig. 4.7). Despite the conservation of the LTR of a macrophage-tropic strain of the lentivirus equine infectious anemia virus (EIAV)

FIGURE 4.7 Amino acid substitutions in proteins VP1, 2C, and 3A in the course of adaptation of swine FMDV C—S8c1 to the guinea pig. Sequential virus isolates from different animals are represented in the first column. The lesion score indicates the presence of a vesicle at the inoculation site (+) or the presence of vesicles at the inoculation site and other sites (secondary lesions) (++). The amino acid substitutions (single letter code) in VP1, 2C, and 3A (amino acid position given for each protein) are indicated in the three columns on the right. Results are based on consensus sequences; the presence of amino acid mixtures (percentage in parenthesis) is based on the peaks of the sequencing experiments. *The figure has been adapted from Núñez, J.I., Baranowski, E., Molina, N., Ruiz-Jarabo, C.M., Sánchez, C., et al., 2001. A single amino acid substitution in nonstructural protein 3A can mediate adaptation of foot-and-mouth disease virus to the Guinea pig. J. Virol. 75, 3977—3983, with permission from the American Society for Microbiology, Washington DC, USA.*

in vivo, the stepwise generation of a new transcription factor-binding motif within the enhancer element was associated with the expansion of tropism to endothelial cells and fibroblasts (Maury et al., 2005). A comparison of the minimal promoter-enhancer element of HIV-1 of clades B, C, and E, engineered into the same SIV genetic background, indicated that this element could modulate viral replication in different cell subsets in vivo (Centlivre et al., 2005; Sala et al., 2006). Polymorphism in the binding sites of transcription factors is probably involved in differential expression of HIV-1

clades in different tissues. The polymerase genes of IV may influence host range, and probably, gene constellations affect the relative replication capacity of viruses in different hosts (Parrish and Kawaoka, 2005).

The dominance of viral subpopulations in vivo can be due to cells offering higher permissivity to variant forms of a virus, or depletion of cells due to cytolytic infection, and survival of other cell types permissive to other viral variants (Centlivre et al., 2005; Sala et al., 2006). Cell dynamics can exert an important influence on short-term virus evolution and pathogenesis (See Chapter 6 for a description of the dependence on host cell variation for the initiation of persistent infection of FMDV in cell culture). Not only nonstructural proteins but modifications in viral RNA may also contribute to host-specific viral fitness. RNA structural elements and mutations within the elements may influence the adaptation of arboviruses to alternative hosts (Ventoso, 2012; Villordo et al., 2015; Filomatori et al., 2017).

From an evolutionary perspective, the capacity of viruses to use alternative receptors and to modify receptor specificity or intracellular preferences by modest genetic change—involving short distances in the genotypic sequence space (Chapter 3)—may manifest a necessity of viruses to parasitize increasingly differentiated organisms. Any virus whose capacity to expand cell tropism was limited by genetic constraints (e.g., the need to move to long distances in sequence space, incompatible with viral population numbers) would have a lower probability of long-term survival in an increasingly differentiated cellular world. A large virus population size is the key to provide a sufficient number of variants with new potential cell recognition specificities. That is, the application of the Darwinian principles to the long-term evolution of viruses is expected to have produced a flexible virus-host cell interaction in the sense that it may be modified by a limited genetic change in the virus.

4.5 Trait coevolution: mutual influences between antigenic variation and tropism change

Coevolution between organisms and their pathogenic agents means that reciprocal and adaptive genetic modifications have occurred in them because of their interaction as biological systems that have shared space and resources over prolonged time periods. Coevolution is a general concept that applies not only to host-parasite relationships but also to other interacting biological species that have shaped biological systems up to the present epoch (Futuyma and Slatkin, 1983; Woolhouse et al., 2002; Gomez et al., 2015). Long-term coevolution has probably led to increasingly subtle molecular mechanisms to deal with the host immune response, based on virus-coded proteins that interact with host proteins. In addition, mutual evolutionary influences may be exerted among different sites within a virus. Such influences are favored by overlaps between domains involved in distinct functions in viral nucleic acids or proteins that may also introduce additional evolutionary constraints. Coevolution of amino acids at or around important functional domains may contribute to functional stability (Gloor et al., 2005). Homologous structure analyses can be applied to identify functional residues in proteins (Du et al., 2016). Intramolecular coevolution can be measured for amino acid pairs by the probability of their occurrence at some defined positions, also termed mutual information criterion (MIC) (Korber et al., 1993).

The three-dimensional structure of several viruses has evidenced that frequently, there is an overlap between antibody and receptor recognition sites (reviewed in Baranowski et al., 2003) (Tables 4.2 and 4.3). As mentioned in Section 4.2.2, the FMDV capsid includes an Arg-Gly-Asp (RGD) triplet at an exposed mobile loop in protein VP1 (Acharya et al., 1989; Fry and

Stuart, 2010) which is involved in the binding of neutralizing antibodies and in recognition of integrin receptors (Verdaguer et al., 1995). Functional alterations in FMDV can be interpreted as a consequence of the overlap between a major antigenic site and the integrin recognition domain: variants selected with neutralizing monoclonal antibodies displayed altered integrin recognition (Martinez et al., 1997; Baranowski et al., 2000; Ruiz-Jarabo et al., 2004). Adaptation of FMDV to cell culture may result in antigenic variation and in the use of heparan sulfate as a molecule that facilities virus entry; some of the residues involved in heparin-binding map at antigenic sites (Curry et al., 1996; Sa-Carvalho et al., 1997; Fry et al., 1999; Baranowski et al., 2000). Cattle that were partially immunized with synthetic peptides, representing the VP1 loop sequence, selected FMDV mutants with substitutions within the RGD or at neighboring sites. The mutants showed altered host cell tropism (Taboga et al., 1997; Tami et al., 2003) (see also Chapter 8).

Studies with IVs have shown several consequences of the close connection between antigenic and receptor interaction sites. HA antigenic variants were selected upon egg adaptation of the virus (Robertson et al., 1987). Treatment with antibodies resulted in the selection of variants with altered receptor binding (Laeeq et al., 1997). The hemagglutinating activity of type C IV can be modulated by amino acid residues involved in antibody binding (Matsuzaki et al., 1992). Passage of this virus in MHV-II cells resulted in antigenic variants that displayed an advantage in receptor binding (Umetsu et al., 1992). The receptor-binding specificity of IV can modify the antigenic profile of the virus as analyzed by the reactivity of monoclonal antibodies with the HA (Yamada et al., 1984).

TABLE 4.2 Examples of overlap between antigenic sites and receptor recognition sites in DNA viruses.

Virus	Observation	References
Adenovirus-3	Fiber knob includes receptor-binding and antigenic sites	Liebermann et al. (1998)
Adeno-associated virus (serotypes 1 and 5)	Virus-antibody complex structures show epitopes at receptor recognition sites	Tseng et al. (2015)
Bovine herpesvirus-1	Antiidiotypic antibodies bind to cellular receptors	Thaker et al. (1994), Varthakavi and Minocha (1996)
Human cytomegalovirus	Antiidiotypic antibodies bind to cellular receptors	Keay et al. (1989), Keay and Baldwin (1991)
Herpes simplex virus	Antiidiotypic antibodies bind to cellular receptors	Huang and Campadelli-Fiume (1996)
	Overlap between a receptor-binding domain in gD and an antigenic site	Whitbeck et al. (1999)
Hepatitis B virus	Antiidiotypic antibodies that mimic cellular structures bind to small HBV surface antigen. Synthetic peptide analog is recognized by antiHBV antibodies and cell receptors	Neurath et al. (1986)
	Antiidiotypic antibodies bind to cellular receptors	Petit et al. (1992), Hertogs et al. (1994), Budkowska et al. (1995)
Duck hepatitis B virus	Residues involved in the interaction with cells are also critical for virus neutralization	Tong et al. (1995), Li et al. (1996), Sunyach et al. (1999)

TABLE 4.3   Examples of overlap between antigenic sites and receptor recognition sites in RNA viruses.

Virus	Observation	References
Poliovirus	Receptor recognition influenced by residues of antigenic sites	Murray et al. (1988), Harber et al. (1995)
	Critical role of VP1 BC loop in receptor interaction	Yeates et al. (1991)
Human rhinovirus	Neutralizing antibody to HRV14 penetrates the receptor-binding canyon	Smith et al. (1996)
	Exposed VP1 BC- and HI-loops covered by footprint of very low-density lipoprotein receptor	Hewat et al. (2000)
Theiler's encephalomyelitis virus	Neutralizing antibodies map close to putative receptor-binding site	Sato et al. (1996)
	Substitutions of adaptation to some cells map in antigenic sites	Jnaoui and Michiels (1998)
Foot-and-mouth disease virus	Overlap of integrin- and antibody-binding sites (Additional studies presented in the text)	Verdaguer et al. (1995)
Human influenza virus	Amino acid residues of the sialic acid-binding pocket are accessible to neutralizing antibodies (Additional studies presented in the text)	Stewart and Nemerow (1997)
Newcastle disease virus	Monoclonal antibodies to HN glycoprotein prevent virus attachment	Iorio et al. (1989)
Rabies virus	Antiidiotypic antibodies bind to cellular receptors	Hanham et al. (1993)
	Residues critical for neurotropism are involved in antibody binding	Coulon et al. (1998)
Bovine viral diarrhea virus	Antiidiotypic antibodies bind to cellular receptors	Xue and Minocha (1993), Minocha et al. (1997)
Dengue virus	Residues critical for mouse neurovirulence are involved in antibody binding	Hiramatsu et al. (1996)
Yellow fever virus	Residues critical for neurotropism are involved in antibody binding	Jennings et al. (1994)
Murine coronavirus	Overlap between epitopes and receptor-binding sites	Kubo et al. (1993, 1994)
Middle East respiratory syndrome (MERS) coronavirus	Antibodies bind to receptor recognition site	Ying et al. (2014)
Sindbis virus	Antiidiotypic antibodies bind to cellular receptors	Ubol and Griffin (1991), Wang et al. (1991), Strauss et al. (1994)
Ross River virus	Binding of antibodies to cell-receptor recognition regions	Smith et al. (1995)
Reovirus	Antiidiotypic antibodies bind to cellular receptors	Co et al. (1985), Gaulton et al. (1985), Williams et al. (1988, 1989, 1991)
Bluetongue virus	Antiidiotypic antibodies bind to cellular receptors	Xu et al. (1997)

The mutual influence between antigenic variation and receptor site modifications has been extended to recent IV isolates (Koel et al., 2013, 2014). These summarized accounts for FMDV and IV, in addition to the studies listed in Tables 4.2 and 4.3 provide incontestable evidence that antigenic changes, which are extremely frequent because of the continuous confrontation of viruses with antibodies, may contribute to the modification of cell-binding preferences.

Two facts facilitate antigenicity-cell tropism coevolution: (i) the limited number of amino acid substitutions, and hence of mutations, that are needed for an antigenic change (one of the parameters that render the quasispecies nature of viruses biologically relevant; see Box 3.3 and text in Chapter 3). (ii) surface residues tend to be less constrained structurally than internal residues in viral capsids or envelopes. Their tolerance to accept amino acid substitutions is one of the mechanisms of antigenic variation in the absence of immune selection (Section 4.7). Surface residues appear as the most variable when all the historically recorded substitutions are depicted on the three-dimensional structures of viral particles. In the case of FMDV high variability of surface amino acids has been corroborated with several virion structures determined by D. Stuart, E. Fry, D. Rowlands and their colleagues (one of the comparisons between two isolates of a subtype and an antigenic variant is described in Lea et al., 1995). Conformational antigenic sites, similarly to internal capsid residues, are involved in interactions, which may be necessary for virion stability, and therefore, their tolerance of amino acid substitutions is more restricted than in linear, continuous epitopes. This is the case with discontinuous epitopes that have been characterized within antigenic site D_2 of FMDV. The conformation of this site in FMDV O_1BFS and C_1 is conserved despite different primary sequences. The substitutions in antibody-escape mutants of D_2 map in amino acids which are not involved in interactions with surrounding residues. Significantly, the only substitution found in a residue involved in hydrogen bonding led to an amino acid that maintained the hydrogen bond with the same neighbor amino acid, according to the modeling of the change based on crystallographic data (Lea et al., 1994). Epitope plasticity may allow antibody escape while maintaining the interaction with cellular ligands (Lindesmith et al., 2019). It is expected that disordered protein regions free of structural constraints can tolerate amino acid substitutions. In some cases, surprisingly, even a viral polymerase may contain domains that can be extensively changed and remain functional (Gitlin et al., 2014).

4.6 Escape from antibody and cytotoxic T cell responses in viral persistence: fitness cost

Viruses use two major strategies to cope with the host response: modulation and escape. Modulation consists of the expression of viral gene products that by any mechanism can alter the components of the immune response. The consequence is to facilitate virus survival to increase the probability of transmission, or virus persistence in the infected organism. The escape strategy is based on the exploitation of mutations in the viral genome that confer the virus increased resistance to inactivation by components of the immune response. Amino acid substitutions may prevent neutralization of viral particles by antibodies or elimination of infected cells by specific cytotoxic T cells. Both modulation and escape strategies are exploited by DNA and RNA viruses, although complex DNA viruses encode several proteins whose primary function is to interfere with the host defense mechanisms. They include homologues of cytokines, chemokines, viral proteins that act as a decoy for antiviral antibodies, proteins that block complement activation, that suppress MHC class I and II molecules, that interfere with ubiquitin-dependent proteolysis, that induce or inhibit apoptosis, among other proteins and activities (Alcami,

2003; Seet et al., 2003; Rustagi and Gale, 2014). Viral proteins block interferon induction (i.e., influenza virus NS1, Ebola virus P35, and others) (Basler and Garcia-Sastre, 2002; Katze et al., 2002; Weber et al., 2004). Some of the proteins involved provide remarkable examples of protein multifunctionality. The leader L proteinase of FMDV catalyzes its own cleavage from the polyprotein, cleaves the host cell translation factor eIF4G—leading to the shutoff of host cell translation dependent on capped mRNAs—and inhibits IFN induction in the infected cells (de Los Santos et al., 2006). HIV-1 Nef contributes to HIV-1 evasion of immune surveillance through interaction with membrane traffic regulators (Pawlak and Dikeakos, 2015). Several additional multifunctional viral proteins one of whose roles has an impact on the host antiviral response have been described.

Despite expressing proteins that interfere with the immune response, RNA viruses exploit evasion through genetic variation as a major means to cope with host defenses. This is probably an evolutionary coadaptation of high mutability and genome compactness. Genomic compression is evidenced by overlapping reading frames, ambisense RNA genomes, RNA editing, partial read-through of termination codons, overlap between regulatory and protein-coding regions, leaky ribosome scanning with initiation of protein synthesis at two in-frame AUGs, ribosome frameshifting, hopping, shunting and bypassing, synthesis of polyproteins whose partial or complete processing leads to several functional proteins, etc (Domingo et al., 2001; Alberts et al., 2002). Evolution has offered high mutation rates, small genome size, and escape pathways as an alternative to the modulation strategy.

Mutations that mediate escape from neutralizing antibodies and from CTLs can be readily observed in vivo (Weiner et al., 1995; Borrow et al., 1997; McMichael and Phillips, 1997; Ciurea et al., 2000; Sevilla et al., 2002). Rather than being a secondary phenomenon in the course of viral infections, the generation of antibody- and CTL escape mutants may indeed contribute to viral persistence (Gebauer et al., 1988; Ciurea et al., 2000; Richman et al., 2003; Domingo, 2006). Evasion of an immune response, as the outcome of a selection event in viruses, may entail a fitness cost. Such a cost may bring about reversion to the initial sequences when selective forces (antibodies or CTLs) are no longer present (Borrow et al., 1997). Viruses often display multiple antibody-escape routes, and the preferred pathway may be imposed by the number and concentration of the antibodies (Borrego et al., 1993; Keck et al., 2014).

There is an important difference between the selective constraint imposed by antiviral drugs and by the host immune response. While drugs inhibit a specific step of the virus replication cycle (or two or more steps if two or multiple drugs are administered simultaneously), the immune response gives rise to multiple constraints that act upon the virus. As an example, 100 HCV passages in cell culture in the presence of IFN-α were necessary to select HCV mutants displaying significant resistance to IFN-α (Perales et al., 2013). The resistant HCV exhibited higher fitness than the populations passaged in the absence of IFN-α when fitness was measured in the presence of IFN-α, but not in its absence. Sequence analysis documented that amino acid substitutions that contributed to resistance were present in most viral proteins, and many substitutions differed among parallel viral lineages (Perales et al., 2013). It is expected that viruses find higher genetic and phenotypic barriers for responding to multicomponent antiviral responses than to a single inhibitor with a defined target (Perales et al., 2014). A high genetic barrier is due to the requirement of multiple mutations, while a high phenotypic barrier reflects the fitness cost inflicted by the mutations once they have been selected. The fact that IFN-α resistance requires several amino acid substitutions implies a high barrier to resistance since each of the substitutions may affect a multifunctional protein. The observations with HCV in cell culture agree

with multiple possible IFN-α-resistance mutations identified in clinical practice, thus providing an interpretation of the absence of a catalog of IFN-α-resistance mutations that are available for other HCV inhibitors [reviewed in (Perales et al., 2014; Perales, 2018)].

It is an open question whether the concept of high fitness cost to overcome an interferon response can be extended to other systems. I. S. Novella, J.J. Holland, and colleagues showed that passage of VSV in IFN-treated cells selected only variants of limited IFN resistance (Novella et al., 1996). However, field isolates of VSV appear to contain clones with different capacity to resist or to induce IFN, to the point that interferon induction was used as a quasispecies marker for the virus (Marcus et al., 1998).

Quantification of fitness cost is described in Chapters 5 and 8, and the use of broad-spectrum antiviral agents that promote a multicomponent antiviral state is discussed in Chapter 9.

4.7 Antigenic variation in the absence of immune selection

Several cases of antigenic variation of DNA and RNA viruses in the absence of immune selection have been described (Domingo et al., 1993, 2001, and references therein). Two possible underlying mechanisms have been proposed: (i) tolerance of antigenic sites to accept amino acid replacements by virtue of being relatively free of structural constraints (Section 4.5); fluctuations of mutant distributions (through the selection of an unrelated trait or through random drift) that may raise antigenic variants to dominance. (ii) Not mutually exclusive with the previous mechanism, selective forces other than an immune response may result in amino acid replacements at antigenic sites (as in coevolution of receptor recognition specificity and antigenicity, discussed in Section 4.5). Antigenic variation may follow from the hitchhiking of mutations

that encode amino acid substitutions at antigenic sites, following selection sweeps (Chapter 3) (Fig. 4.8). Therefore, a consequence of quasispecies dynamics is that amino acid substitutions observed at the antigenic sites of viruses are not necessarily the result of immune selection.

4.8 Constraints as a demand on mutation rate levels

The nature of the selective constraint influences the requirements—in terms of mutation rate and complexity of the mutant spectrum—to be fulfilled by a virus to overcome that specific selective constraint. For a virus to lose sensitivity to an antiviral inhibitor directed to a viral protein, when such a loss depends on a single transition mutation (low genetic barrier), the standard mutation rate, or even a mutation rate lower than the standard, may suffice to generate a resistant mutant. In contrast, when a virus has to adapt to a complex environment, multiple mutations located at different genomic sites might be required for effective replication in the new environment.

The occurrence of multiple mutations in the same viral genome will be favored by low-fidelity polymerases because they generate mutant spectra characterized by a larger average number of mutations per genome (higher mutation frequency and nucleotide diversity) than an enzyme with standard copying fidelity. We refer to "broad" or "narrow" mutant spectra to indicate whether they include many types of variants or a restricted repertoire of variants, respectively (Fig. 4.9). A selective disadvantage due to a narrow mutant spectrum was elegantly documented with a high-fidelity PV mutant and its inability to cause neurological disease using a mouse model (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006) (see also Chapter 3). Examples of complex environments for a virus—those that often require a broad mutant spectrum for a virus to confront them successfully—are the invasion of
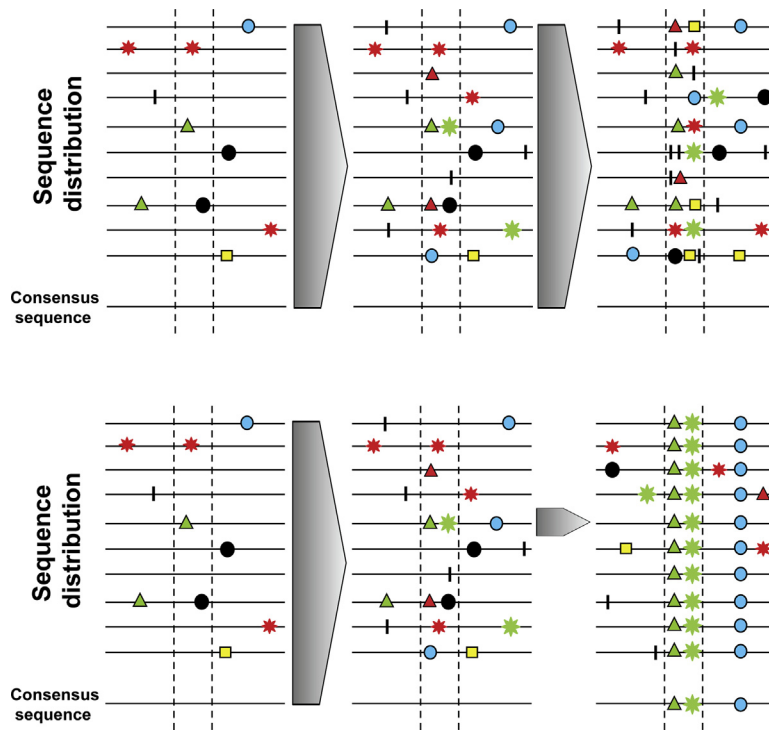
FIGURE 4.8 Antigenic variation in the absence of immune selection. The genomic region encoding an antigenic site is delimited by two discontinuous vertical lines. At the top, extensive replication of the virus leads to the accumulation of amino acid substitutions at the antigenic site because of its structural flexibility. At the bottom, a genetic bottleneck or a selection event results in the fixation (hitchhiking) of two amino acid substitutions (green triangle and asterisk) at the antigenic site. *The figure has been modified from Domingo, E., Díez, J., Martínez, M.A., Hernández, J., Holguín, A., et al., 1993. New observations on antigenic diversification of RNA viruses. Antigenic variation is not dependent on immune selection. J. Gen. Virol. 74, 2039—2045.*

tissues or organs to ensure extended replication (that may or may not lead to disease) or survival in face of the immune response of the host, with its multiple (humoral and cellular) branches (Section 4.6).

Arguments occasionally put forward by theoretical biologists that high mutation rates are a consequence of some other parameter [i.e., rate of RNA genome replication (Fitzsimmonds et al., 2018)] but not a trait directly selected for adaptation, has the problem that it is very difficult to demonstrate experimentally in a sound manner with several viral systems; substantiation of this proposal would require showing a fitness advantage of high fidelity mutants using a fitness measurement that does

not involve short-term replication rate. Even assuming that high replication rate was a general need for viruses, it would be a case of trait coadaptation. The necessity of viruses to periodically respond to complex (multiple) selective constraints suggests that high mutation rates have been positively selected (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). To my knowledge, there is no evidence that persistent viruses—that in most cases would benefit from a limited replication rate—have selected high fidelity mutants in nature or the laboratory. Error-prone replication is maintained after prolonged viral persistence despite mutation rate being an evolvable trait and that copying fidelity can be modulated by proteins other than the
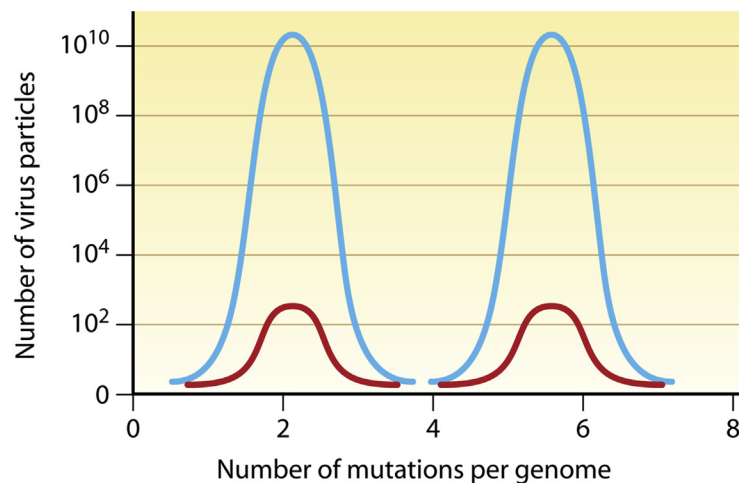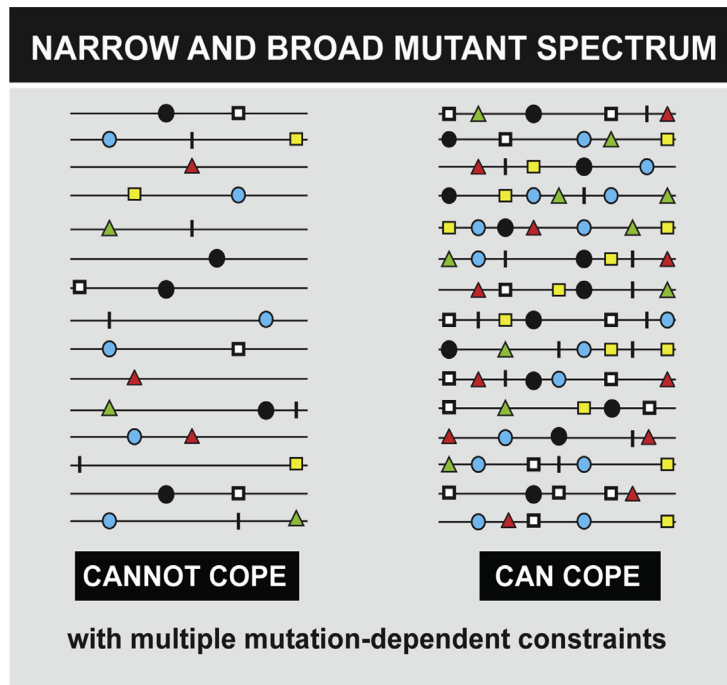
FIGURE 4.9 Two representations of viral populations with different amplitude and size of the mutant spectrum. At the top, a narrow mutant spectrum that cannot cope with a complex selective constraint is represented (left), in contrast with a more complex mutant distribution that can cope with a constraint (right). Genomes are depicted as horizontal lines and mutations as symbols on the lines. At the bottom, mutant distributions of different size (large in the blue and small in the red curves) and complexity (average number of mutations per genome given in the abscissa) are represented. *The bottom part is reproduced from Domingo, E., Sheldon, J., Perales, C., 2012. Viral quasispecies evolution. Microbiol. Mol. Biol. Rev. 76, 159–216 with permission from the American Society for Microbiology, Washington DC, USA.*

polymerase (Section 4.9). Following a population bottleneck, an event which from all evidence is very frequent (Section 4.1.2) a single infectious virus must repopulate a mutant spectrum to provide a range of phenotypes that can mediate adaptation. A selective advantage over mutants that cannot provide a comparable phenotypic repertoire can be anticipated (Vignuzzi and Andino, 2010). Mutant viruses displaying altered template-copying fidelity generally exhibit lower fitness than their standard parental genomes. Therefore, current evidence favors the view that the observed mutation rate values are of benefit to the survival strategies employed by viruses, and probably a consequence of their evolutionary adaptation to changing environments.

4.9 Multifunctional viral proteins in interaction with host factors: joker substitutions

Many viral proteins are multifunctional, a feature that may be regarded as an adaptation of compact viral genomes to exploit, as much as possible, the phenotypic possibilities of each coding nucleotide (a point mentioned in Section 4.6 in connection with functions exerted by proteins that modulate the host immune response). Multifunctionality of proteins is not unique to viruses since it is increasingly documented for cellular proteins. However, it is in the case of viruses in which the broadest functional repertories of a single protein have been documented. Of many examples, a few are briefly mentioned here.

Proteins NS1 and NS2 of orbiviruses play roles in replication, assembly, and morphogenesis. NS1 is synthesized abundantly in bluetongue virus-infected cells, the protein assembles as tubular structures, and a single-chain antibody against NS1 expressed intracellularly can lead to the reduction of cytopathology and an increase of virus budding and release from the cells (reviewed in Roy, 2008).

Picornavirus nonstructural protein 2C constitutes another dramatic example of multifunctionality that extends even to influence the fidelity properties of the viral polymerase. 2C includes nucleoside-triphosphatase (NTPase) and RNA-binding activities, acts as a chaperone during picornaviral replication, and participates in viral RNA encapsidation, the uncoating of viral particles, and host cell membrane rearrangements required for picornavirus replication (reviewed in Ehrenfeld et al., 2010). Amino acid substitutions in 2C may affect the killing of cells in culture (Herrera et al., 2007) or virulence in vivo (Sanz-Ramos et al., 2008). A substitution in 2C acted as a compensatory mutation of a defect in cytopathology displayed by deletion mutants of FMDV that lack two copies of 3B, the gene encoding VPg, which is the protein involved in the initiation of picornaviral RNA replication (Arias et al., 2010). Mutations in FMDV 2C can modulate the incorporation of nucleotides in the absence of mutations in the viral polymerase (Agudo et al., 2016). 2C is the target of guanidine hydrochloride, a protein-denaturing agent that at low concentrations is an inhibitor of picornavirus replication. This inhibitor has been instrumental to estimate mutation rates of picornaviruses and to understand the molecular basis of virus extinction by enhanced mutagenesis (Chapter 9).

A specific protein domain can participate in two or more functions. The FMDV polymerase 3D includes in its N-terminal region a nuclear localization signal that also acts to regulate nucleotide incorporation during RNA synthesis (Ferrer-Orta et al., 2015; de la Higuera et al., 2018). The nucleoprotein (NP) of arenaviruses is the main structural element of the viral ribonucleoprotein that directs viral RNA synthesis. In addition, NP counteracts the activity of type I IFN, and this function was mapped in the C-terminal region of the protein. The latter region folds in a way similar to the DEDDh family of 3–5′ exoribonucleases (named after a conserved core domain with amino acids DEDD), the type of activity that in replicative DNA polymerases

and coronaviruses RNA polymerase is responsible for proofreading repair of mismatched nucleotides at the growing 3'-end of the genome (Chapter 2). Arenavirus NP mutants lacking this activity display remarkable decreases in viral fitness (Martinez-Sobrido et al., 2009; reviewed in Grande-Perez et al., 2016).

Multifunctionality of viral proteins has several implications for the modulation of host cell functions during infections, virus evolution, viral genome stability, and the response of viruses to selective constraints. An amino acid substitution in a viral protein needed to overcome a specific selective pressure directed to that protein may also affect unrelated functions performed by the protein. This may result in a fitness cost, frequently observed with viral mutants selected for their capacity to overcome an inhibitory activity. When the selected mutants are allowed to continue their replication, additional mutations may occur to increase fitness. Involvement in two or more functions may limit the number of possible amino acid substitutions that a protein can accept for fitness increase, because the same substitutions that may compensate for a limitation of one of the functions may adversely affect other functions exerted by the same protein. Again, *trade-offs* (the virus *modus vivendi*) enter the picture (Section 4.2). Restrictions imposed by multifunctionality may underlie the observation that a single amino acid replacement I248T in protein 2C was selected repeatedly and independently when FMDV had to respond to different environmental demands: in the course of adaptation of a biological clone of the virus to guinea pig (Núñez et al., 2001), upon replication of the same clone in mice (Sanz-Ramos et al., 2008), or to modulate the relative rate of incorporation of different nucleotides by the FMDV polymerase (Agudo et al., 2016). Substitutions that serve a virus to increase its fitness under different environmental circumstances are termed "joker" substitutions. They lead to a general increase in replication efficiency under different environmental demands.

The expectation that the genomic nucleotide sequences that belong to overlapping genes (i.e., polynucleotide stretches that contribute to the coding of more than one protein) would be much more conserved than standard open-reading frames for a single protein has not been confirmed, at least to the extent of being able to derive a general conclusion. A reason for the limited differences in conservation of these two categories of coding regions may lie in protein multifunctionality, in combination with the phenotypic involvement of the viral RNA itself, independent of its coding function (Section 4.3 and Chapter 2). Indeed, if the same protein performs different functions and the RNA is also involved in regulatory activities, each nucleotide may be subjected to constraints that may not differ significantly from the constraints operating on the products expressed from overlapping genes (Domingo et al., 2012).

4.10 Alternating selective pressures: the case of arboviruses

The selective pressures that viruses encounter when infecting their hosts in nature are difficult to identify, but those that have been recognized are rarely constant and uniform. The experimental designs in which a virus is subjected to a specific selective pressure in an established cell line in culture are a gross simplification of reality that has nevertheless allowed quantification of the effects of well-defined variables (Chapter 6). However, the first point to note in dealing with the interaction of viral populations with their hosts is the multitude of selective pressures, often conflicting, that viruses must confront. Selective pressures vary in kind and intensity in space and time.

An interesting, biologically relevant case of alternating selective pressures is provided by the animal and plant arboviruses which alternate replication in vertebrate animals or in plants and insect hosts, and have successfully persisted in

nature as disease agents (reviewed in different chapters of Morse, 1994; Gibbs et al., 1995; Domingo, 2006). Three strategies have been distinguished regarding the part of the virus life cycle that elapses in the vector: (i) the virus attaches to the vector, usually at its external organs, but does not reach the internal milieu, and does not undergo replication. This style has been termed noncirculative. (ii) The virus enters the vector through specific receptors and multiplies in it. This style is often termed circulative-propagative. (iii) The virus cycles inside the vector, but does not propagate in it. This is the case of the plant nanovirus in its aphid vectors. Interactive styles (i) and (iii) must be distinguished from purely mechanical transmission because inside the vector the virus may meet conditions that alter particle stability relative to the outside environment, or it may induce changes in the insect expression profile. During the circulative-propagative style, the virus undergoes several bottleneck events. In mosquito vectors, the virus must transit from one compartment into another, and most of the compartments are separated by basal lamina that limits the penetration of viruses (Forrester et al., 2014). Bottlenecks may affect fitness, limit the number of particles that can be transmitted and, in consequence, contribute to the unpredictability of evolutionary outcomes (Hanley and Weaver, 2008; Gutierrez et al., 2012; Forrester et al., 2014). The consequences of stochasticity are accentuated by the cloud nature of viral populations since it is virtually impossible that two bottleneck events acting on the same virus population will isolate two genomes with an identical sequence. Quasispecies ensures diverse initial genomes to pursue evolutionary trajectories following bottleneck episodes.

Zoonotic vector-borne flaviviruses and alphaviruses have been extensively studied because of their pathogenic potential for humans. They include yellow fever virus, Dengue virus, Chikungunya virus, and Venezuelan equine encephalitis virus, among others.

Several types of insect-mammalian infection cycles have been characterized by these viruses. A sylvatic cycle is defined as the one that involves nonhuman animal host and insects. Phylogenetic evidence suggests that sylvatic Dengue was the precursor of Dengue viruses that infected humans and established enzootic and endemic cycles (Fig. 4.10). Here we return to the importance of virus and host population numbers that were discussed in the first section of this Chapter. In the case of Dengue virus, it has been estimated that efficient human-to-human transmission required a minimum human population size in the range of 10^4 to 10^6 individuals, a size that was attained only with the advent of urban life (Gubler, 1997). Critical population numbers of hosts and vectors, as well as numbers of infectious particles in viremic hosts, are necessary for the emergence and maintenance of viral diseases (Chapter 7). Dengue virus appears to be highly adaptable to new animal and vector hosts, notably the transition from *Aedes albopictus* to *Aedes aegypti* as mosquito vectors (reviewed in Hanley and Weaver, 2008). Replication in different environments is the rule for the great majority of viruses, even those that have a preferred host species. Perhaps because of this, there are no salient features in the patterns of evolution of different viruses (overall rate of evolution, proportion of conserved genomic residues or protein domains, frequencies of antibody- or inhibitor-escape mutants, etc.) that allow a clear, quantitative and general distinction between viral pathogens that are directly transmitted among individual vertebrate hosts and those that require an invertebrate vector.

4.10.1 The sophistication of pathogen-vector-host interactions in plant viruses

Since plants do not have an independent capacity to move, virus transmission from one individual plant to another or from a cultivar to
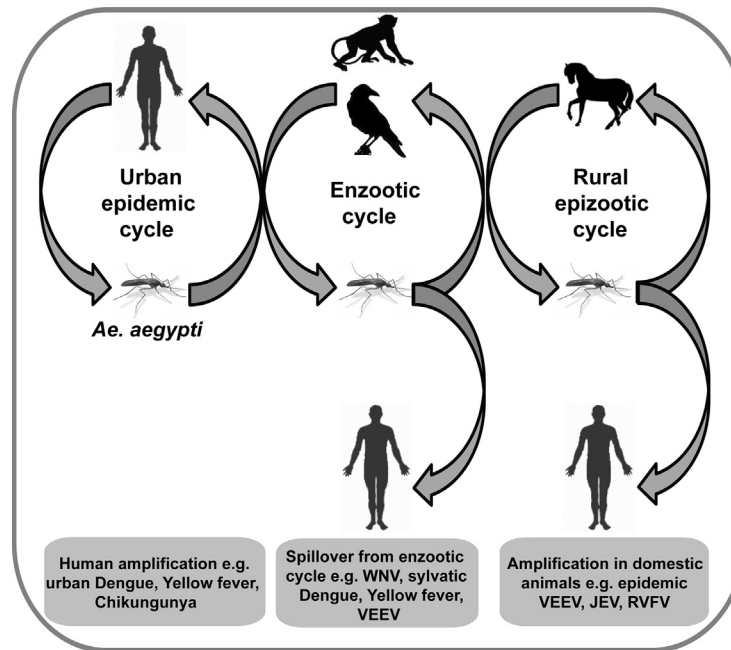
FIGURE 4.10 Simplified representation of three types of infectious cycles (epidemic, enzootic, and rural epizootic) identified for zoonotic arboviruses. Host alternations between vertebrate and mosquito hosts are depicted by curved arrows. Examples of viruses documented to participate in these cycles are shown at the bottom (WNV, West Nile virus; VEEV, Venezuelan equine encephalitis virus; JEV, Japanese encephalitis virus; RVFV, Rift Valley fever virus). *Figure modified from Hanley, K.H., Weaver, S.C., 2008. Arbovirus evolution. In: Domingo, E., Parrish, C.R., Holland, J.J. (Eds.), Origin and Evolution of Viruses second ed. Elsevier, Oxford, pp. 351–392 with permission from the authors.*

another can rarely rely on direct interhost contacts and never on plant movements, as is the case with most animal viruses. Instead, viruses are transmitted among plants either via nematodes in the soil or flying insects [compilation in (Hogenhout et al., 2013)]. Virus particles have to survive during the process of insect-mediated transmission, an adaptation that has probably been shaped over the 300 million years since the first insects evolved. A surprising array of plant-insect-virus interactions has been described, with the exploitation of biochemical pathways that at the organism level rival with the adaptations attained by mutant spectra of the infecting viruses at the molecular level. In some cases, infected vectors are attracted to noninfected plants while uninfected vectors are attracted to infected plants, as a result of

molecular signals whose result is the enhancement virus transmission (Mauck et al., 2010; Carr et al., 2018). Virus factories and the transmission body of cauliflower mosaic virus can reversibly disassemble to enhance virion distribution, and favor aphid-mediated transmission (Bak et al., 2013). A true arms race and coevolutionary events at many levels are established among viruses, their host plants, and terrestrial or airborne insect vectors, to achieve the survival of all participants.

4.11 Overview and concluding remarks

Virus population numbers are orders of magnitude greater than the numbers of individuals in the host species that they infect. Large population

numbers, however, are not constant during the virus life cycles. Viruses undergo periodic bottleneck events, that is, drastic reductions in population size as they spread within individual hosts and during host-to-host transmission. Bottlenecks accentuate the effect of chance (that promotes genetic drift) in virus evolution, made more severe by the mutant spectrum (cloud) nature of viral populations. Out of the large mutant repertoire that is produced during an infection, bottlenecks dictate that only a minority contributes to the next round of progeny production either in the same host (because of a change of compartment) or in a different host individual. It is unlikely that two bottleneck transmissions will involve the same genome repertoire, even when applied to the same population. Bottlenecks also result in transient reductions of population heterogeneity (a decrease in the amplitude of mutant spectra) because only a few founder viral particles will originate the progeny repertoire, and the latter necessitates some replication rounds to restore the population size and complexity that the population had prior to the bottleneck. It has been suggested that one of the reasons why high mutation rates are maintained in viruses is the need to reinstate population heterogeneity following a population bottleneck. This proposal is in line with the adaptive value of complex mutant spectra documented in Chapters 2 and 3. Despite several models on the evolutionary origins of high mutation rates, current evidence favors that high mutation rates of RNA viruses (and some DNA viruses) serve the adaptive needs of viruses; virtually, every chapter of this book includes examples.

Viruses are subjected to several classes of constraints during their multiplication in cellular hosts, an obvious condition for long-term virus-host coexistence given the disparity of their population numbers. Basal constraints are those imposed by the core replicative needs of viruses. Other constraints are due to responses of the individual cells or entire organisms in the event of an infection. Still, other constraints are the result of human intervention such as vaccination or the administration of antiviral drugs. Emphasis has been put on codon usage and codon-pair associations as a basal constraint for virus replication, for two reasons. Because it constitutes an example of the nonneutral character of synonymous mutations in viral RNAs, and because codon and codon-pair deoptimization have opened new prospects for the design of live-attenuated antiviral vaccines.

A major problem in evolutionary biology is the identification of the selective constraints that, together with the random drift of genomes, mold evolutionary outcomes. This is an immense problem often solved by shallow assumption. We often propose a selective advantage for the brain to acquire the capacity of consciousness, for the giraffe to have a long neck, or for a virus to assemble a stable capsid. Yet, successful creatures can lack consciousness and can have a short neck; some viruses that have survived to our times have a labile capsid. I suggest that the difficulty of identifying the selective constraints that viruses confront in their multiplication cycles is one of the major contributory factors to our fragmentary understanding of virus evolution.

Modulation and evasion are two distinct strategies used by viruses to overcome immune responses. Viral proteins that act as immune modulators are often multifunctional. The studies on evasion strategy, based on the selection of antibody- and CTL-resistant mutants, have been instrumental for the understanding of quasispecies dynamics. Viruses can use several receptors that belong to different families of cell surface proteins that mediate virus internalization. Viruses display remarkable flexibility to modify their cell target preference. Alterations of cell tropism and coevolution of antigenicity and receptor recognition specificity have been outlined as a consequence of quasispecies dynamics and the overlap between antigenic sites and receptor-recognition domains in viruses. The adaptability of a viral quasispecies

is also reflected in the capacity of viruses to confront changing and alternating selective pressures. The arboviruses that alternate between insect and vertebrate or plant hosts constitute an example of adaptation to multiple hosts as a standard way of life, although to some extent any virus must confront different environments (see Summary Box).

S u m m a r y   B o x

- Virus population numbers are several orders of magnitude larger than those of their host organisms.
- Viruses are subjected to constraints at multiple levels that limit their replication and contribute to maintaining their biological identity.
- Viruses exploit a variety of molecular mechanisms to overcome external constraints, such as therapeutic interventions.
- Codon usage and codon-pair frequencies represent constraints for viral gene expression that can be used to design new generation vaccines.
- Viruses can modify their cellular tropisms through amino acid substitutions in surface protein residues and other mechanisms. Overlap between antigenic sites and receptor recognition sites facilitates coevolution of antigenicity and cellular tropism.
- Viruses use two major strategies to cope with the host immune response: modulation and escape. Escape mutations generally imply a fitness cost for the virus.
- Viruses have evolved means to cope with multiple constraints, including remarkable environmental alternations. This is the case of pathogenic arboviruses whose natural infectious cycles involve replication in vertebrate and insect hosts.
- An important limitation to interpret evolutionary trajectories of viruses is that many selective constraints confronted by viruses in their life cycles have not been identified.

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