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INTERACTION OF VIRUS POPULATIONS WITH THEIR HOSTS

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ABBREVIATIONS

A	adenine
ABV	avian bornavirus
ACE 2	angiotensin-converting enzyme 2
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 infection
mRNA	messenger RNA
N	population size
Ne	effective population size
NGS	next generation sequencing
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
TCID₅₀	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, and other times they become part of the innate immune response can be interpreted as a result of a long-term coevolution between viruses and cells, and the basic mechanisms by which biological evolution is working in our biosphere. The concept of evolutionary tinkering proposed by F. Jacob in the last century (Jacob, 1977) is most 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)-prey (host) relationship, with a very distinctive attribute: the disproportionate difference in population size between viruses and their hosts. The difference is 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) 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). More recently, 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 also 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 (TCID₅₀/ml) were determined in nasopharyngeal fluids 1 day after infection (Murphy and Webster, 1985). In children with type B IV, titers of 10^4 TCID₅₀/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, but 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 the hosts that viruses infect. Considering mammals, each of many primate species include 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} - 10^{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} to 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).

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 having generally evolved to produce vast numbers of progeny. Using terminology of ecology, viruses as *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 Q β 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.

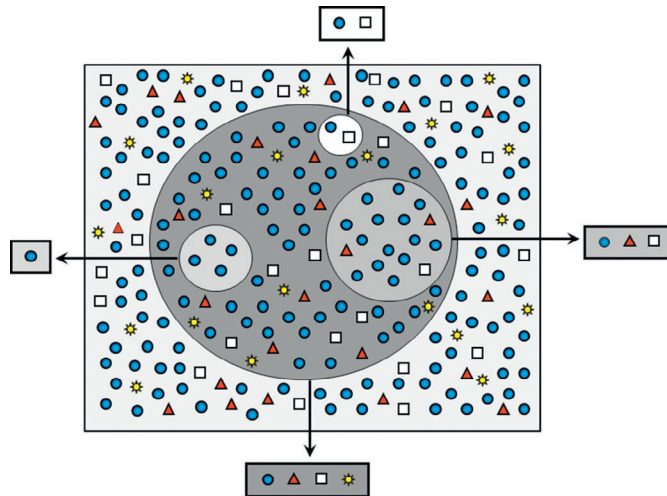
In the course of infections by HIV-1, it has been estimated that 10^{10} to 10^{11} new virions are produced each day (Coffin, 1995; Ho et al., 1995). The average life span of cells productively infected with HIV-1 has been estimated in 1-2.2 days, with a half-life ($t_{1/2}$) of about 1.5 days. The 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). A rapid turnover of virions occurs also during HCV and HBV infections. 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 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 establish also 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 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 is not dependent on reinfections by virus particles produced from 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 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 a 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 Borna virus 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 substitutions rates for ABV than the mammal-infecting BDV (He et al., 2014).

**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 a 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 et al. (2012), with permission from the American society for Microbiology, Washington DC, USA.

The maintenance of a large population size of viruses in cytolitic or acute infections *in vivo* is conditioned to the absence of population bottlenecks (strong reductions in population size that can alter the course of selective events, as discussed in Chapter 3) (Figure 4.1). Bottlenecks have been characterized during viral transmission from an infected into a susceptible host, and also within infected hosts (reviewed in Domingo et al., 2012; Gutierrez et al., 2012; Forrester et al., 2014). During host-to-host transmission, bottlenecks are favored by aerosol spread of viruses in the case of respiratory viruses, but also when infection is initiated through contact with small volumes of secretions or excretions. The microdroplets in aerosols should contain a very small proportion of the total number of viruses present in an infected individual (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). 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 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 a contaminated fluid, but a massive amount of initial virus facilitates adaptation of the quasispecies to the recipient host.

Severe population bottlenecks occur during plant leave inoculation, and seed or aphid transmission of plant viruses. Studies have included the transmission of cucumber mosaic virus, tobacco mosaic virus, pea seed-borne mosaic virus, and potato virus Y variants (Li and Roossinck, 2004; Ali et al., 2006; Moury et al., 2007; Betancourt et al., 2008; Sacristan et al., 2011; Fabre et al., 2014;

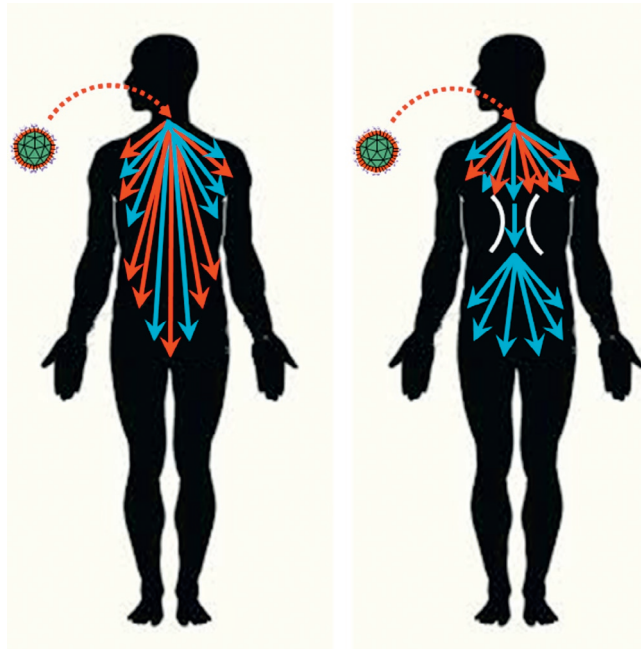


FIGURE 4.2

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.

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 patients studied a single founder genome initiated the infection while in the others a minimum of two to five viruses were involved ([Keele et al., 2008](#)). Bottlenecks have been identified also during transmission and early stages of HCV infections ([Quer et al., 2005](#); [Bull et al., 2011](#)).

Bottlenecks happen within infected host once the infection is already ongoing ([Foy et al., 2004](#); [Scholle et al., 2004](#); [Quer et al., 2005](#); [Ali et al., 2006](#); [Kuss et al., 2008](#); [Smith et al., 2008](#); [Haaland et al., 2009](#); [Forrester et al., 2014](#)). 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](#)) ([Figure 4.2](#)). 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](#)). Additional studies are needed to elucidate how variations in population size can affect evolutionary outcomes in viruses. Application of next generation sequencing (NGS) should provide new insights in the consequence of bottlenecks for the genetic heterogeneity of viral populations (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 Figure 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 only 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 in some particular cases or for other biological systems.

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 a process of selection, will reduce the diversity of the postbottleneck population. A few rounds of replication are needed to restore the mutant spectrum amplitude of the initial populations (Figure 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 a subset of alleles 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).

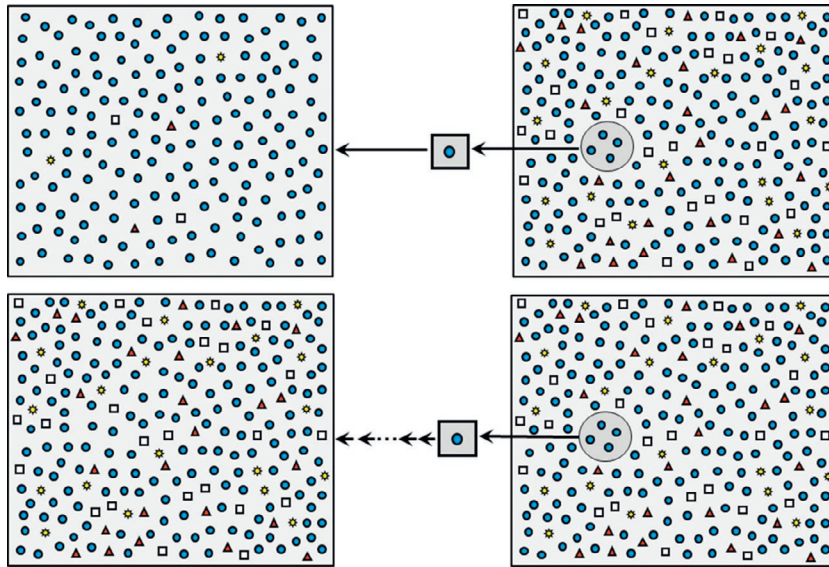


FIGURE 4.3

A population bottleneck results in 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.

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 long-term survival of both. The term constraint is used to refer to multiple mechanisms that prevent generalized and devastating infections by viruses, notably 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 (specific examples and some introductory references can be found in [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. Basal constraints prevent the deterioration of the “core information” that determines viral identity.

- Selective constraints 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.
- 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 radicals, or other perturbations, phenotypic heterogeneity among cells of the tissues where virus replicates, among others.
- 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.

4.2.1 LONG-TERM HISTORY DICTATES BASAL CONSTRAINTS

According to our current understanding of cellular and viral genomics, a present day virus is the result of a long evolutionary history. Using 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. As in the developmental program of an organism, a present day virus can be viewed as a result of a historical process (Maynard Smith et al., 1985). A viral genome was shaped by natural selection that involved transfers of functional modules, duplication of genomic regions, constellations of mutations acting in concert, and other molecular events. It is not easy to find pathways 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.

High-order structures include the internal ribosome entry site (IRES) for the initiation of protein synthesis of some viruses, and pseudoknot structures involved in protein recognition and ribosomal frameshifting. These structures generally tolerate limited numbers of mutations, and very often compensatory mutations occur to maintain their folding (Olsthorn 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 be imposed also 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 DNA and RNA 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 or from a donor organism into a recipient organism. Constraints to variation of

surface residues in the viral particle is one of the mechanisms proposed to explain antigenic stability of some viruses despite their displaying high mutation rates and frequencies (e.g., measles or rabies virus), or the presence of widely different numbers of serotypes among picornavirus genera (Chapter 7). Constraints introduce conflicts regarding 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 function of interacting with integrin receptors and with neutralizing antibodies (Acharya et al., 1989; Verdaguer et al., 1995). 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 viral 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 successfully confronted a selective constraint, as illustrated with specific examples in Chapters 5 and 8, but a universal optimization is impossible. 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). Data for virus evolution suggest that due to the extremely compact information packed 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 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 as 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 reveal 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 (compare with the theories of virus origins that favor long-term coexistence of viral elements with precellular and primitive cell organizations discussed in Chapter 1).

4.2.3 CONSTRAINTS IN HOST ORGANISMS: CONTRAST WITH MAN-MADE ANTIVIRAL INTERVENTIONS

Concerning constraints internal to organisms, the latter have evolved 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 under the new environment, must not compromise viral survival. 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 intended to limit their replication, typically during antiviral treatments, can be divided in 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 has been the realization that viruses are capable of selecting mutants resistant virtually to any drug used in therapy. 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 (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 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 Borja 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). Given their ancient nature and 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 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; Aragonés 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. The specific infectivity is the ratio between infectious and physical particles, an important parameter that is further discussed in Chapter 9. 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), among others]. 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.

In contrast to PV, hepatitis A virus (HAV) uses rare synonymous codons (those that correspond to tRNAs that are present at low concentrations) 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 and capsid stability (Sánchez et al., 2003; Aragonés 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 this 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 were 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 has also 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 to be taken into consideration for the choice of expression systems. When elevated expression levels are not obtained because of codon usage-related effects on translation efficiency, the problem may be circumvented by engineering genetic forms of the virus with a modified codon composition that matches the requirements for expression in the desired host (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). In fact, 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) (Figure 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, and the latter codon 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). Furthermore, in several viral populations that have been examined by deep sequencing, a remarkably high frequency of termination codons is observed that presumably denotes the presence of defective genomes that represent either dead-end evolutionary pathways or are maintained by complementation during quasispecies replication (Rodríguez-Frías

		Middle nucleotide					
		U	C	A	G		
First (5') nucleotide	U	UUU Phe UUC UUA Leu UUG	UCU UCC Ser UCA UCG	UAU Tyr UAC UAA STOP UAG	UGU Cys UGC UGA STOP UGG Trp	U C A G	
	C	CUU CUC Leu CUA CUG	CCU CCC Pro CCA CCG	CAU His CAC CAA Gln CAG	CGU CGC Arg CGA CGG	U C A G	
	A	AUU AUC Ile AUA AUG Met	ACU ACC Thr ACA ACG	AAU Asn AAC AAA Lys AAG	AGU Ser AGC AGA Arg AGG	U C A G	
	G	GUU GUC Val GUA GUG	GCU GCC Ala GCA GCG	GAU Asp GAC GAA Glu GAG	GGU GGC Gly GGA GGG	U C A G	

FIGURE 4.4

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

et al., 2012). The frequency of termination codons may increase as a result of treatment with antiviral agents and may decrease 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 sequences space can actually affect the phenotypic space (Schuster, 2011). Position in sequence space is 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 a 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 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 productive infection is highly restricted to the epithelial cells of the respiratory tract (Bergelson, 2010).

Early and more recent measurements indicate that for several viral receptors about 10^3 to 10^4 molecules are present per cell (Lonberg-Holm et al., 1976; Consigli et al., 1986; Thulke et al., 2006). Although the MOI *in vivo* 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 virus that reaches a target tissue or organ than to 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).

Here we describe cell tropism changes of viruses that document the relevance of viral population size in the process. 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 not 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. Also, members of the same virus family, or viruses associated with related disease manifestations, may use different receptors. Among other examples, consider the different viruses that 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.

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 Figure 4.5)	Hueffer et al. (2003)
Change in receptor recognition by foot-and-mouth disease virus <i>in vivo</i> and in cell culture (see also text and Figure 4.6)	Baranowski et al. (2000, 2001) , Ruiz-Jarabo et al. (2004)

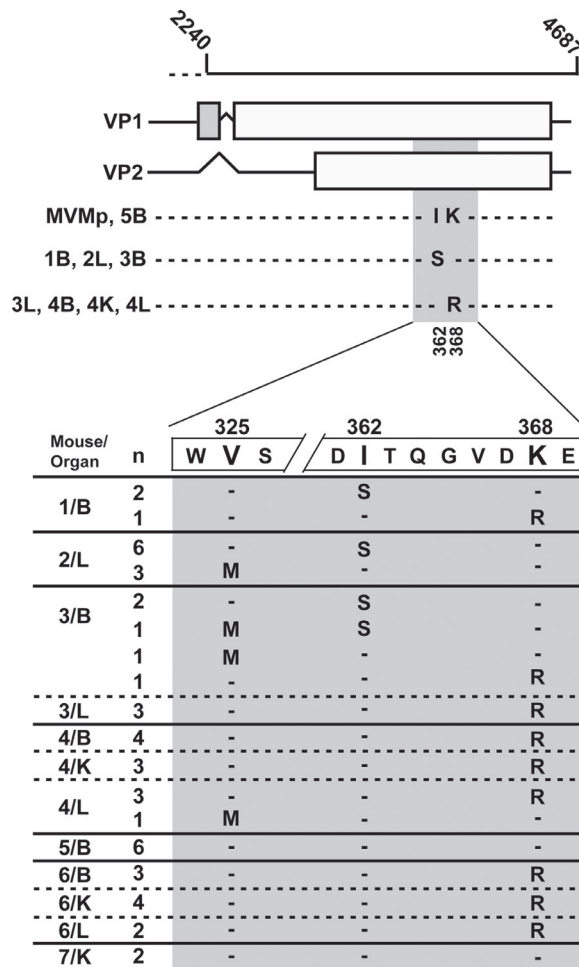
Next we comment on a few specific cases with the aim of deriving general concepts. Some residues of the IV hemagglutinin (HA) dictate the specificity for sialic acid linked to galactose by either an α -2, 3 or an α -2, 6 linkage, and linkage preference is a determinant of host specificity (a preference 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 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) (Figure 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 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 a receptor type 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) (discussed also in Chapter 9 in connection with antiviral agents directed to cellular targets). The bicyclam AMD3100, a selective antagonist of CXCR4, led to 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 sequence) and expansion of cell tropism (Ruiz-Jarabo et al., 2004) (Figure 4.6). Thus, paradoxically, repeated replication in a cell line may lead to relaxation of virus specialization for that cell line. Despite this example of tropism expansion, one of the tenets of quasispecies dynamics, the generation of a mutant spectrum as a source of minority variants that can become dominant in response to environmental


FIGURE 4.5

Amino acid substitutions found in the capsid protein of the prototypic strain of the parvovirus minute virus of mice (MVMp) upon 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 et al. (2006), with permission from the American Society for Microbiology, Washington DC, USA.

demands, probably underlies several cases of tropism changes in RNA and DNA viruses. Application of NGS to probe into the minority levels of viral quasispecies *in vivo* should help establishing whether low-frequency mutants with altered receptor recognition sites are frequently present in evolving quasispecies or they remain below the level of detection. Their basal frequency will depend on their fitness relative to other components of the mutant spectrum. Here again the relevance of virus population

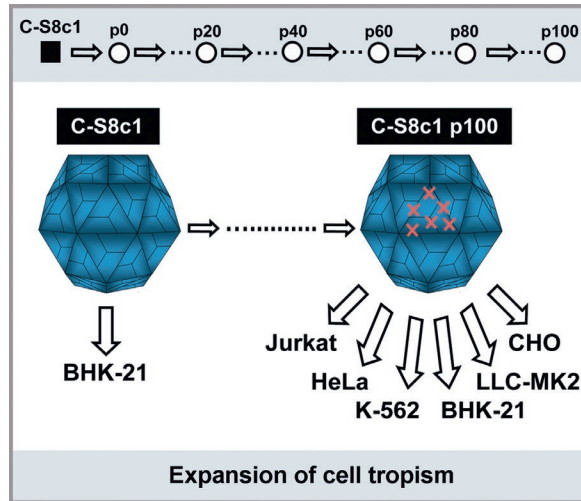


FIGURE 4.6

Expansion of host cell tropism. FMDV biological clone C-S8c1 (filled square at the top) was passaged in BHK-21 cell at a MOI of 1 to 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 et al. (2004).

numbers is evident: the larger the population size, the higher the probability that mutants with altered cell recognition are present. The fitness cost associated with amino acid substitutions in viral proteins is discussed in Chapter 5.

4.4.1 NONSTRUCTURAL VIRAL PROTEINS AND RNA IN CELL TROPISM AND HOST RANGE OF VIRUSES

The event of being able to penetrate a new cell type has consequences for the host range of viruses, and the potential emergence of new viral pathogens (Chapter 7). In the present section, we document that 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) (Figure 4.7). Despite the conservation of the LTR of a macrophage-tropic strain of the lentivirus equine infectious anemia virus (EIAV) *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 can 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














FMDV	Lesion score	Amino acid		
		VP1 (147)	2C (248)	3A (44)
C-S8c1		L	I	Q
V 2.1		L	I/T(10%)	Q
V 2.2	 +	L	T	Q/R (40%)
V 2.3	 +	L	T	R
V 2.4a	 +	L/P(40%)	T	R
V 2.4b	 ++	L/P(20%)	T	R
V 2.5a		P	T	R
V 2.5b	 +	L/P(50%)	T	R
V 2.6a	 ++	P	T	R
V 2.6a		P	T	R
V 2.6b		P	T	R
V 2.7b	 ++	P	T	R
V 2.7b	 ++	P	T	R
V 2.10b	 ++	P	T	R

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 et al. (2001), with permission from the American Society for Microbiology, Washington DC, USA.

IV may influence host range, and, probably, gene constellations affects the relative replication capacity of viruses in different hosts (Parrish and Kawaoka, 2005).

Dominance of viral subpopulations *in vivo* can be due to cells offering higher permissivity to variant forms of a virus, or to 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 a persistent infection of FMDV in cell culture.) Not only nonstructural proteins, 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).

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 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 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 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). 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). A MIC value of zero means independent evolution of the two amino acids tested while increasingly positive values denote enhanced covariation (Korber et al., 1993).

Structural studies with several viruses have documented 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 the virus to cell culture may result in antigenic variation and in the use of heparan sulfate as a molecule that facilitates 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 selection of variants with altered receptor binding (Laeq et al., 1997). The hemagglutinating activity of type C IV can be modulated by amino

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	Anti-idiotypic antibodies bind to cellular receptors	Thaker et al. (1994), Varthakavi and Minocha (1996)
Human cytomegalovirus	Anti-idiotypic antibodies bind to cellular receptors	Keay et al. (1989), Keay and Baldwin (1991)
Herpes simplex virus	Anti-idiotypic antibodies bind to cellular receptors	Huang and Campadelli-Fiume (1996)
Hepatitis B virus	Overlap between a receptor binding domain in gD and an antigenic site	Whitbeck et al. (1999)
	Anti-idiotypic antibodies that mimic cellular structures bind to small HBV surface antigen. Synthetic peptide analog is recognized by anti-HBV antibodies and cell receptors	Neurath et al. (1986)
Duck hepatitis B virus	Anti-idiotypic antibodies bind to cellular receptors	Petit et al. (1992), Hertogs et al. (1994), Budkowska et al. (1995)
	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)

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 reactivity of monoclonal antibodies with the HA (Yamada et al., 1984). 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 that are extremely frequent because of the continuous confrontation of viruses with antibodies, may contribute to 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 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

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 Critical role of VP1 BC loop in receptor interaction	Murray et al. (1988), Harber et al. (1995) Yeates et al. (1991)
Human rhinovirus	Neutralizing antibody to HRV14 penetrates the receptor-binding canyon Exposed VP1 BC- and HI-loops covered by footprint of very low-density lipoprotein receptor	Smith et al. (1996) Hewat et al. (2000)
Theiler's encephalomyelitis virus	Neutralizing antibodies map close to putative receptor-binding site Substitutions of adaptation to some cells map in antigenic sites	Sato et al. (1996) 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	Anti-idiotypic antibodies bind to cellular receptors Residues critical for neurotropism are involved in antibody binding	Hanham et al. (1993) Coulon et al. (1998)
Bovine viral diarrhea virus	Anti-idiotypic 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	Anti-idiotypic 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	Anti-idiotypic antibodies bind to cellular receptors	Co et al. (1985), Gaulton et al. (1985), Williams et al. (1988, 1989, 1991)
Bluetongue virus	Anti-idiotypic antibodies bind to cellular receptors	Xu et al. (1997)

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 D₂ of FMDV. The conformation of this site in FMDV O₁BFS and C₁ is conserved despite different primary sequences. The substitutions in antibody-escape mutants of D₂ 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). 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. By modulation we mean the expression of viral gene products that by any mechanism can alter 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. By escape we mean mutations in the viral genome that render the virus resistant to inactivation by components of the immune response, typically 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).

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 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 resistance to IFN- α (Perales et al., 2013). The resistant HCV displayed 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 to respond 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 fact that fitness decrease due to amino acid substitutions in several proteins will be accentuated by the multifunctionality of most proteins. The observations with HCV in cell culture agree with multiple possible IFN- α -resistance mutations identified in clinical practice (reviewed in Perales et al., 2014).

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. 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). They have been attributed to two possible mechanisms: (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 selection of an unrelated trait or through random drift) 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 above). Antigenic variation may follow from the hitchhiking of mutations that encode amino acid substitutions at antigenic sites, following selection sweeps

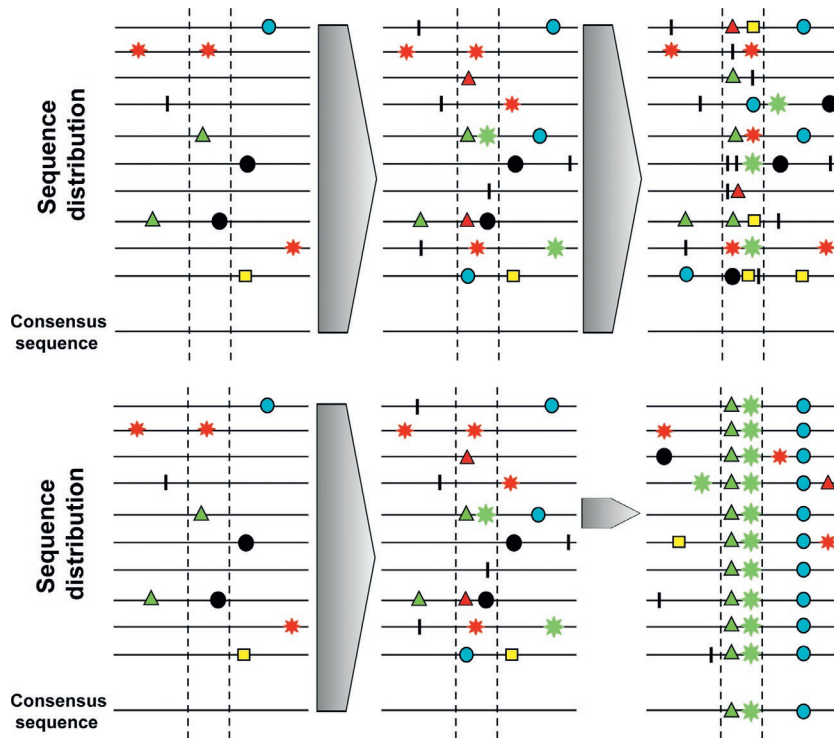


FIGURE 4.8

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 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 et al. (1993).

(Chapter 3) (Figure 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 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 (Figure 4.9).

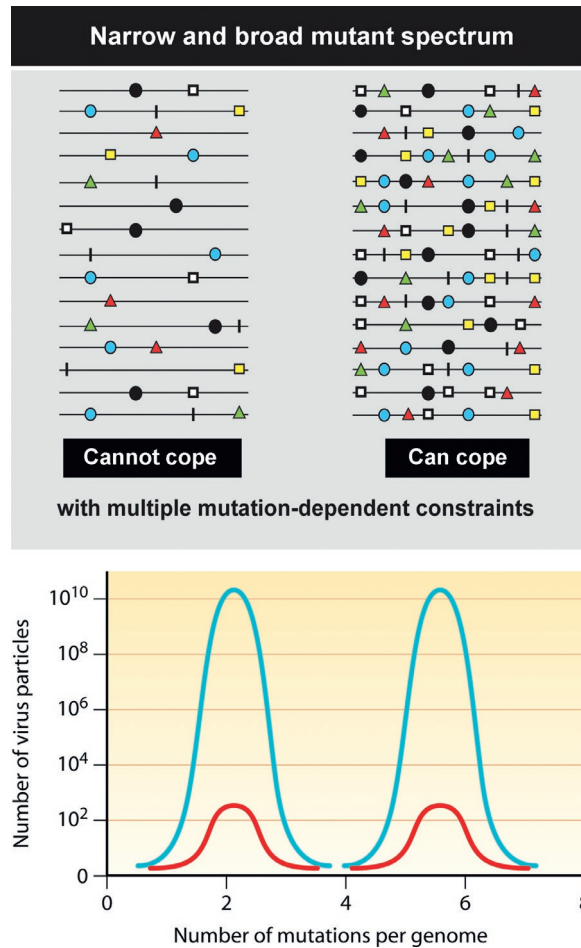


FIGURE 4.9

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 et al. (2012)* with permission from the American Society for Microbiology, Washington DC, USA.

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 in mice (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 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) but not a trait directly selected for adaptation, ignore the necessity of viruses to periodically respond to complex (multiple) selective constraints for which high mutation rate should be positively selected, and we know that mutation rate is an evolvable trait. In particular, following a population bottleneck, an event which from all evidence is very frequent in nature (Section 4.1.2), a single infectious virus must repopulate a mutant spectrum to provide a range of phenotypes that can mediate adaptation. The need to rapidly provide a mutant repertoire following population bottlenecks may by itself justify that high mutation rates in RNA viruses have been positively selected (Vignuzzi and Andino, 2010). In addition, mutant viruses displaying higher or lower than standard template-copying fidelity generally display lower fitness than their standard parental genomes. Again, evidence suggests that the observed mutation rate values are a consequence of evolutionary adaptation of viruses.

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 repertoires of a single protein have been documented. A few of many examples 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 reduction of cytopathology and to an increase of virus budding and release from the cells (reviewed in Roy, 2008).

Picornavirus nonstructural protein 2C constitutes another dramatic example of multifunctionality. 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 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 initiation of picornaviral RNA

replication (Arias et al., 2010). 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 acts also to regulate nucleotide incorporation during RNA synthesis (Ferrer-Orta et al., 2015). The nucleoprotein (NP) of arnaviruses 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. This region folds in a way similar to DEDDh family of 3'-5' exonuclease, 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). NP mutants lacking this activity display remarkable decreases in viral fitness (Martinez-Sobrido et al., 2009; reviewed in Grande-Perez et al., 2016). There are many additional examples of multifunctionality of proteins encoded by DNA and RNA viruses, again a manifestation of the need to exploit any coding stretch for completion of the replication cycles.

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 needed to overcome a specific selective pressure may also affect unrelated functions performed by the same 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. Multifunctionality may limit the number of possible amino acid substitutions that can be used for such an increase, because the same substitutions that may compensate 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), or upon replication of the same clone in mice (Sanz-Ramos et al., 2008). Substitutions that serve a virus to increase its fitness under different environmental circumstances are termed “joker” substitutions. Joker substitutions (in the sense of the extra playing card in some games) map at amino acid positions apt to affect positively one or more activities of one protein, leading to a general increase in replication efficiency under different environmental demands. Joker substitutions may compensate for fitness costs endured by the virus, even those due to malfunction of an unrelated viral protein.

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

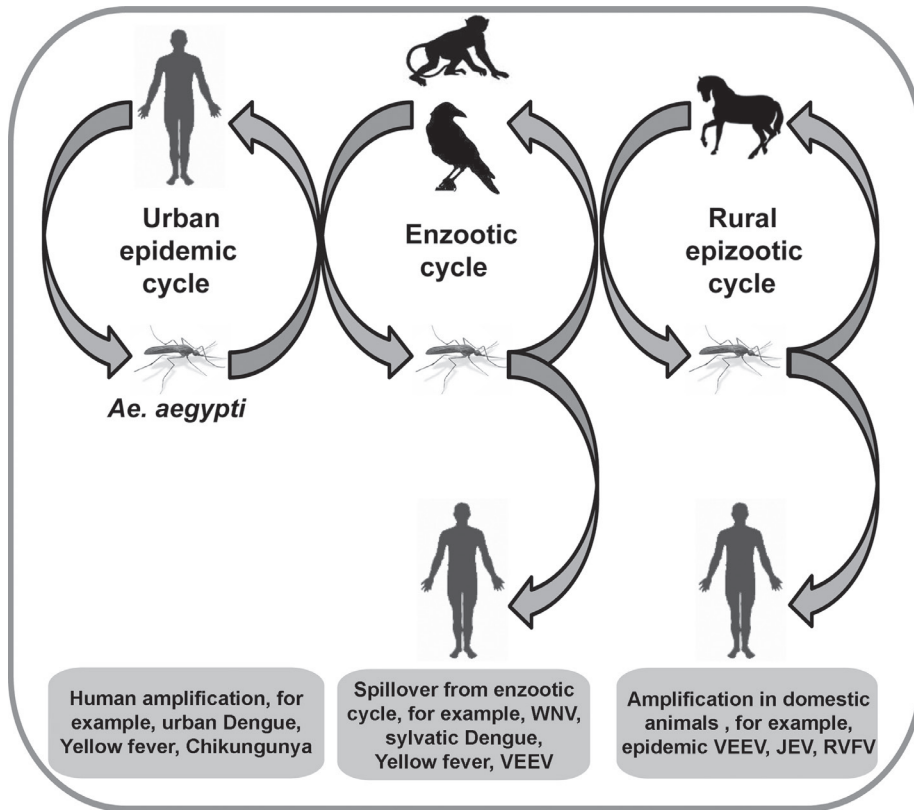
that may not differ significantly from the constraints operating on the products expressed from overlapping genes (Domingo et al., 2012).

4.10 ALTERNATING SELECTIVE PRESSURES: THE CASE OF ARBOVIRUSES

Selective pressures that viruses encounter when infecting their hosts in nature 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. Expressed in a simple way, 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 successfully 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. During the circulative-propagative style (ii), 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, accentuate the stochasticity in evolutionary outcomes (Hanley and Weaver, 2008; Gutierrez et al., 2012; Forrester et al., 2014).

Zoonotic vector-borne flaviviruses 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 for 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 (Figure 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).

**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 and Weaver (2008) with permission from the authors.

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 a 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. Reductions of population size accentuate the effect of chance in virus evolution. This is because out of a large mutant repertoire that is produced during an infection, 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 due to limitation in the number of transmitted viruses. Bottlenecks result also 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. It has been suggested that one of the reasons why high mutation rates are maintained in viruses is the need to restore 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.

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 has opened a new prospects for the design of live-attenuated antiviral vaccines.

The modulation and evasion strategies of viruses to overcome immune response have been summarized, and emphasis put on fitness costs and the multifunctionality of viral proteins that act as immune modulators. The studies on evasion strategy, based on selection of antibody- and CTL-resistant mutants have been instrumental for the understanding of quasispecies dynamics. Viruses can use one 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 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 (see [Summary Box](#)).

SUMMARY BOX

- 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.

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