CHAPTER

6 VIRUS POPULATION DYNAMICS EXAMINED WITH EXPERIMENTAL MODEL SYSTEMS

CHAPTER CONTENTS

ABBREVIATIONS

BHK	baby hamster kidney cells
DI	defective interfering
FMDV	foot-and-mouth disease virus
HIV-1	human immunodeficiency virus type 1
IRES	internal ribosome entry site
IV	influenza virus

Virus as Populations. http://dx.doi.org/10.1016/B978-0-12-800837-9.00006-X

miRNA micro-RNA
MAb monoclonal antibody
MOI multiplicity of infection
NCCR noncoding control region
NGS next generation sequencing
PBMCs peripheral blood mononuclear cells
PV poliovirus
VSV vesicular stomatitis virus

6.1 VALUE OF EXPERIMENTAL EVOLUTION

There are good reasons to suspect that we are not aware of many of the influences that guide the evolution of viruses in nature, and that only a few of the influences can be properly quantified. A rapid review of the major concepts expressed in previous chapters (the chance origin of mutations, multiple selective forces and random events that alter population compositions in an almost incessant manner, potential fidelity modifications of viral polymerases, rapid diversification of viruses even within an individual host, fitness variations due to changes in genomic sequences or in the environment, etc.) forces us to realize that the interpretation of how and why viral populations evolve in nature must be based on largely indirect evidence and a considerable number of assumptions. An understanding of the basic principles that preside over the generation of viral diversity and how some viral forms replace others should be based not only on indirect evidence, but also on the design of experiments in which a limited number of variables can be examined. Ideally, we need to investigate the operation of one variable at a time in an evolutionary outcome, and then try to extend the simplified approach to integrate and interpret the effect of multiple variables acting conjointly.

Some of the concepts explained in previous chapters can indeed be translated into one defined variable. If we ignore intrinsic population heterogeneity, we can compare the effect of viral population size by examining the outcome of an infection of a cell culture or an animal with 0, 1, 10, 10^2, or 10^3 infectious units of a virus. We can fix the amount of virus to 10^2 and examine the influence of prior treatment with 0, 1, 10, 10^2, or 10^3 doses of any compound that might modify the virus, the cell, or the virus-cell interaction with the aim of investigating the virus life cycle or finding a new antiviral target. Parallel replicas may help controlling the possible effects of differences in virus population composition. We may also consider physico-chemical variables such as temperature or ionic composition of the cell culture medium. A diagram of some experimental possibilities with viruses that can grow in a cell culture is depicted in Figure 6.1. For some virus-cell systems, persistent infections are readily established because the virus displays limited cytopathology. In other cases, persistence is only established from cells that survive killing by the virus (Section 6.4). Remarkable insights have been obtained during the last four decades by subjecting viral populations to defined constraints in the laboratory, inspired by the types of constraints likely to operate *in vivo*. This is one of the major objectives of experimental evolution in virology. The studies included in this chapter serve the dual purpose of identifying the experimental origin of some of conclusions drawn in other chapters, and of providing additional complementary information on basic concepts of virus evolution.

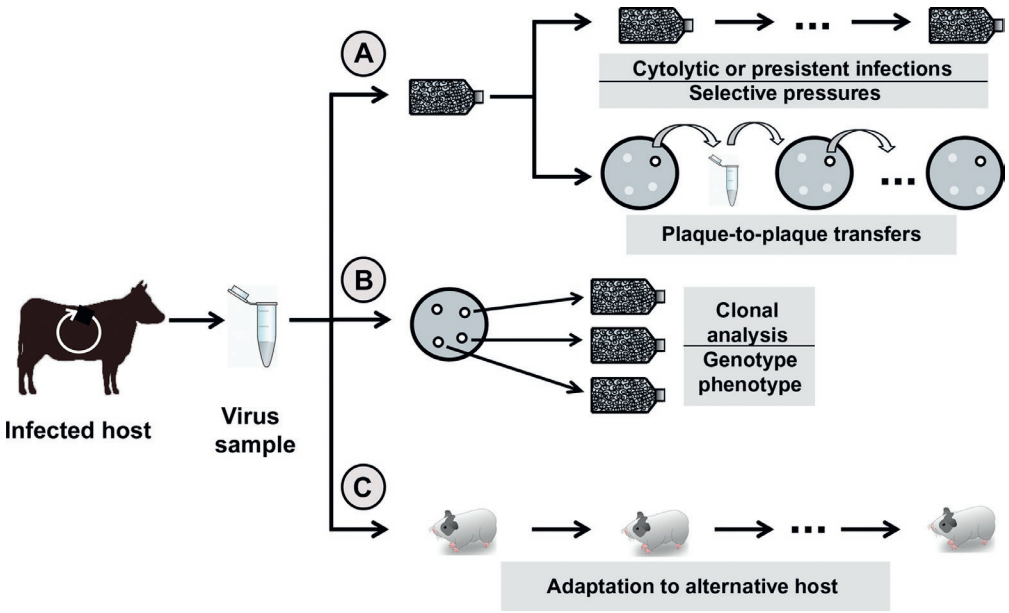

FIGURE 6.1

Scheme of possible laboratory experiments with a virus that can grow in cell culture. A virus sample from an infected host can be used to infect a cell culture (upper branch A) and the progeny can be passaged serially in cytolytic or persistent infections under different selective pressures. The virus can be diluted and plated to characterize biological clones from the population (middle branch B). The virus may also be adapted to an alternative host by serially passaging the progeny virus produced in the new host (branch C, at the bottom). These and additional possible designs form the basis of experimental evolution, and several examples are discussed in the text, with literature references.

6.2 EXPERIMENTAL SYSTEMS IN CELL CULTURE AND *IN VIVO*

For viruses that grow in cell culture, the experimental designs may involve serial passages of cytolytic viruses or of persistently infected cells under different passage conditions (i.e., virus or cell population numbers) or environmental alterations (absence or presence of drugs or antibodies). Passage of a cytolytic virus involves the infection of fresh (uninfected) cells at each passage, implying that no cell evolution can take place. In contrast, passage of persistently infected cells involves successive rounds of cell multiplication with the virus replicating in the cells. The number of duplications that the cells undergo depends on the number of cells seeded on the plate and their number when they reach confluence or are taken for the next passage. In this type of system both the cells and the resident virus may evolve (Section 6.4). When bottlenecks are introduced (meaning a reduction of the number of cells per passage), they will affect both viruses and cells, with consequences for the evolutionary outcome.

During cytolytic infections in which only the virus can evolve, population size in experimental designs has two components: the multiplicity of infection (MOI, or number of infectious particles added per cell) or the total amount of virus used in each infection. In the case of plaque-to-plaque transfers, first performed by L. Chao using bacteriophage φ6 (Chao, 1990), the population size is reduced to one in

each transfer, representing an extreme passage regime that has been highly informative of the profound molecular alterations associated with fitness decrease underwent by the components of mutant spectra (Section 6.5). The viral population size has yet another relevant influence that can be explained with a numerical example. In standard virus passages (not plaque-to-plaque transfers), infection of 10^3 cells with 10^4 infectious particles yields the same MOI than infection of 10^6 cells with 10^7 particles (in both cases the MOI is 10 infectious particles per cell). Yet, the two infections may lead to different outcomes because the number of genomes that initiate infection is 1000-fold larger in the second case. Specifically, any relevant mutant present at a frequency of 10^{-4} in the viral populations will have a high probability of exclusion from participation in the next infection round if the latter involves 10^3 infectious particles. In contrast, the mutant will be included in infections started with 10^7 particles. [The same concept applies to the loss of memory genomes in viral quasispecies due to intervening bottlenecks that exclude subsets of minority and memory genomes for the next infection rounds (Section 5.5 in Chapter 5).]

The presence and maintenance of a specific mutant in a viral population depends on the virus population size and, therefore, it is an "extrinsic" property of a viral population, as opposed to the "intrinsic" properties that are independent of the population size (Domingo and Perales, 2012) ("intrinsic" vs. "extrinsic" properties of mutant spectra were discussed in Chapter 3 in connection with biological complexity as applied to viruses). A natural isolate of a virus may also be adapted to some alternative hosts *in vivo* depending on the barriers to be confronted by the virus in the potential new host (Figure 6.1 and Chapter 4). The basic designs depicted in Figure 6.1 can be extended and modified including complex scenarios such as environmental heterogeneity (alternations or migrations among different cell lines, or between cell lines and host organism, etc.), including the alternacy between mammalian and insect cells in the study of arbovirus evolution (Coffey and Vignuzzi, 2011; Coffey et al., 2008; Novella et al., 1999a, 2007, among other investigations). In a study with vesicular stomatitis virus (VSV) populations passaged in different cell lines, virus adaptation was cell specific in the absence of cell flow, and fitness in all environments decreased with migration rate (Cuevas et al., 2003). The scope of possibilities of designed experiments to learn about virus evolution is truly remarkable. However, we have to be aware that viruses change continuously in the course of experiments.

6.2.1 "TO CULTURE IS TO DISTURB"

The transfer of a viral isolate into an alternative host (either a cell culture, an explant, or an intact organism) implies a perturbation regarding the representation of the parental quasispecies in the new host for two reasons: (i) the involvement of a bottleneck event whose intensity depends on the amount of virus in the biological sample relative to the total amount in the infected parental host, and (ii) the change of biological environment from the donor into the recipient host (Figure 6.2). Concerning (i), a virus sample contains only a subset of the genomes present in the infected host, and the relative fitness of subsets may not represent the fitness of the entire population, if it were feasible to introduce the entire population into the recipient host. Both chance and selection events will modify the genome composition that will enter subsequent rounds of multiplication (Figure 6.2). This important point was first noted by A. Meyerhans, S. Wain-Hobson, and their colleagues in a comparison of the *tat* gene from sequential human immunodeficiency virus type 1 (HIV-1) isolates, and from peripheral blood mononuclear cells (PBMCs) infected with the natural isolates. The study unveiled the difficulties of defining HIV-1 infections in molecular terms, and the authors coined the following sentence that became popular in virus evolution: "to culture is to disturb" (Meyerhans et al., 1989). It gives a very pertinent image of what is hidden behind quasispecies dynamics.

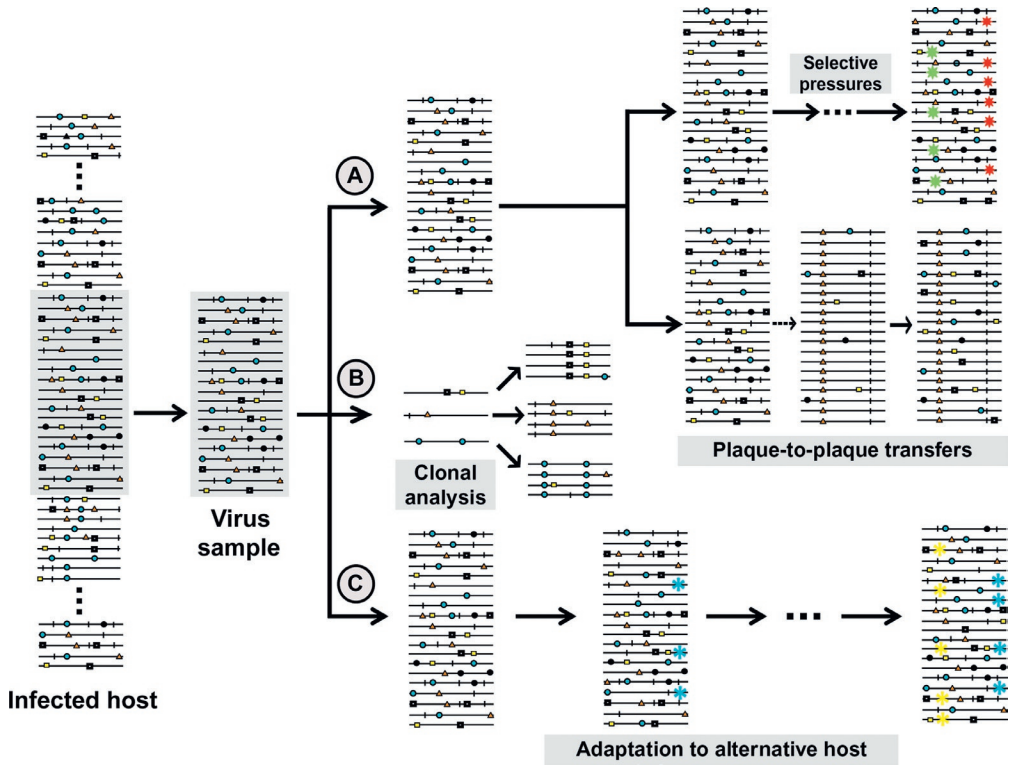

FIGURE 6.2

The implication of mutant spectra and population size in experimental designs. This scheme is parallel to that shown in Figure 6.1, except that infected objects have been replaced by mutant spectra in those same objects. From left to right: the infected host has a huge mutant spectrum whose depiction would occupy millions of columns as the one drawn on the left. The virus sample taken from the infected host includes only a subset of genomes (even if they amount to hundred-thousands), here shadowed in gray. Upper branch A: When this genomic subset is passaged in cells under a selective pressure, the mutant spectrum will be modified, and genomes with specific mutations (green and red asterisks) will increase in dominance. If the same subset is subjected to plaque-to-plaque transfers, mutations accumulate beginning with a founder genome that will hitchhike two initial mutations (red triangle and horizontal line). In the middle branch B the three biological clones will have different initial mutations, and heterogeneity will increase upon expansion of the population. In the bottom branch C, adaptation to a new host will imply increasing dominance of new mutations (yellow and blue asterisks). Many examples of genetic variation due to the types of effects shown here in a diagrammatic form are discussed in the text and in other chapters of the book.

Important genetic and phenotypic differences between natural isolates and their cell culture-adapted counterparts have been observed in several viruses, including DNA viruses. Polyomaviruses are widespread in humans, but they are rarely pathogenic except for immunocompromised individuals. One of the regions of the polyomavirus genome, the noncoding control region (or NCCR), is quite conserved among natural, usually nonpathogenic isolates. When these viruses are adapted to cell culture, the

NCCR undergoes rearrangements involving deletions and duplications. Rearrangements are observed in variants that cause pathology, and they can be readily grown in cell culture. An interesting possibility is that NCCR variants display high replication rates associated with pathogenic potential (Gosert et al., 2010). The connection between replication rate, viral load, and disease progression is discussed in Chapter 8. Genome rearrangements were also identified in regions with repeated sequences in cell culture-adapted cytomegaloviruses (Murphy and Shenk, 2008). These observations with DNA viruses, as well as their relevance for viral persistence and the possible implication of micro-RNA (miRNA) expression have been reviewed (see Imperiale and Jiang, 2015, and references therein). It appears as virtually impossible to maintain a virus population invariant when it enters a different environment.

6.2.2 EXPERIMENTAL EVOLUTION *IN VIVO*

The field of experimental evolution includes designed experiments with viruses in their natural or alternative potential hosts. The objective is to probe concepts of viral evolution and pathogenesis, which are viewed as increasingly interconnected. There is an extensive literature with plant and animal viruses that has contributed to the major concepts discussed in this book. Because the studies are very numerous, here we will discuss some selected examples, again with the objective of underlining some general conclusions.

Studies that rose considerable controversy relate to the elucidation of amino acid substitutions in avian influenza virus (IV) that mediated pathogenicity and human-to-human transmission potential of the virus (reviews in Bouvier and Lowen, 2010; Schrauwen et al., 2014). The ferret is a useful animal model for IV research since it produces respiratory symptoms similar to those in humans. The use of ferret as animal model showed that amino acid substitutions in the receptor-binding domain of the hemagglutinin and the polymerase PB2 were important for transmission of an avian IV among ferrets. In one of the experiments to unveil critical determinants for human-to-human transmission, an avian H9N2 IV was adapted to replication in mammals by serially passaging the avian IV in ferrets. Considerable IV heterogeneity that was diagnostic of active population dynamics in the animals was recorded. The ferret-adapted virus was transmitted efficiently among ferrets, indicating, that, not surprisingly, an avian IV can adapt to be transmissible among humans (Sorrell et al., 2009). Likewise, a number of different mutations, including those that affect receptor preference are needed for effective airborne transmission of an avian H5N1 IV (Herfst et al., 2012). These and other experiments to unveil transmissibility of avian IV in humans open the possibility of next generation sequencing (NGS) screening of avian IVs in search for mutations that may approximate avian viruses to represent a zoonotic threat for humans. The controversy surrounding the need to carry out these types of experiments ("gain of function" experiments) has, in my view, two major components: the uncertainties derived from specifying and publicizing mutations that may render avian IV a biological weapon, and the danger of laboratory escape of a modified IV that can be highly pathogenic and transmissible among humans, causing a devastating disease, in the shadow of the 1918 influenza pandemic. With regard to the first concern, unfortunately, there are many biological weapons available without the need of new ones, and it is very likely that pathogen-enhancing mutations in general (and for IV in particular) do not have a universal value and may change even for closely related isolates of a virus (even, we may speculate, in the course of preparing new stocks for ill-intentioned purposes). Concerning the second concern, indeed, high containment facilities and strict protocols must be used for experiments on directed changes of virus virulence and transmissibility.

A virus that naturally displays a broad host range, and that has contributed examples of biological modifications *in vivo* is foot-and-mouth disease virus (FMDV). In Chapter 4, a molecular analysis of adaptation of a swine FMDV to the guinea pig was used as an example of the influence of substitutions in nonstructural viral proteins in host range (Section 4.4.1). An earlier analysis of FMDV evolution *in vivo* involved a collaboration between Madrid, Rio de Janeiro, and Buenos Aires teams. It consisted in the genetic and antigenic analysis of sequential FMDV samples extracted from cattle during a persistent infection established experimentally with a clonal virus population derived from a cattle isolate (Gebauer et al., 1988). The reason to bring this study here is not only because it is an interesting and informative example of experimental evolution *in vivo*, but also because its results were published at the time when the extensive genetic diversity of HIV-1 was being discovered. Many virologists regarded variation of HIV-1 as unusual, even unique, perhaps only paralleled by that of IV, with the concept of antigenic shift and drift of IV well established at the time (Chapter 7). H. Temin took the analysis of variation of FMDV reported by F. Gebauer and colleagues to emphasize to his retrovirologist colleagues that HIV-1 was not "unique but merely different" (Temin, 1989). In the study with FMDV, virus was recovered from the esophageal-pharyngeal area, the site of FMDV persistence in ruminants, and the virus was examined for up to 539 days postinfection. Despite the infection originating from a biological clone, the sequential samples displayed genetic heterogeneity and dominance of viral subpopulations. Moreover, the persistent virus evolved at rates as high as 0.9×10^{-2} to 7.4×10^{-2} substitutions per nucleotide and year (s/nt/y), which is as high or even higher than the rate calculated for HIV-1 [10^{-2} to 3.7×10^{-4} s/nt/y according to several studies (Hahn et al., 1986; Korber et al., 2000; Shankarappa et al., 1999)]. The mutations were certainly not neutral since several of them affected the reactivity of the virus with antibodies. This controlled experiment showed that FMDV underwent extensive genetic and antigenic variation during persistence in cattle. Thus, H. Temin could emphasize that HIV-1 is not unique concerning variation potential. If a virus is confronted with a focused selective pressure *in vivo*—as is the case of FMDV in the pharyngeal region where an active local mucosal immune response is triggered—it can reach remarkable rates of evolution that are 10^6- to 10^7-fold higher than average values for cellular genes (Holland et al., 1982) (Chapter 7).

In agreement with the model study by F. Gebauer and colleagues with FMDV, persistent viral infections in hosts that display an active immune response (albeit insufficient to clear the virus), may constitute a source of antigenic variants that may occasionally be transmitted to new hosts, or may remain essentially confined to the persistently infected individual. In the latter case, successive waves of variants may be selected to prevent virus clearance by the immune system thus contributing to persistence (Clements et al., 1988; Narayan et al., 1981; Pawlotsky, 2006; Richman et al., 2003; Sponseller et al., 2007, among other examples). Given the potential coevolution of antigenic sites and receptor-recognition domains (Section 4.5 in Chapter 4), persistent infections in animals are a potential threat for the zoonotic emergence of human pathogens, accentuated by the possibility of recombination between the persistent and a related virus from an external source.

6.3 VIRAL DYNAMICS IN CONTROLLED ENVIRONMENTS: ALTERATIONS OF VIRAL SUBPOPULATIONS

Experiments that revealed a sustained heterogeneity in replicating viral populations have spanned four decades and have been based on widely different technologies: the very early RNA T1 oligonucleotide

fingerprint used to analyze biological clones of Qβ RNA (Domingo et al., 1978) and the CirSeq design for next generation sequencing (NGS) applied to poliovirus (PV) populations (Acevedo et al., 2014), with many studies in between (Andino and Domingo, 2015).

The initial findings with bacteriophage Qβ illustrate how difficult it is for a virus to reach a true population equilibrium. The phage was multiplied for many years in its host *Escherichia coli* since its isolation from Kyoto feces in 1961 by I. Watanabe and colleagues (Miyake et al., 1967). It was passaged in the laboratories of I. Watanabe, S. Spiegelman (in Urbana), and C. Weissmann (in New York, 1965-1967 and in Zürich, 1967-1974). There is no record that the virus had been biologically cloned during these multiple passages in *E. coli*. In 1974, a clone termed A.S. (from A. Shapira who was working with M. Billeter in Zürich) was isolated and its T1 oligonucleotide fingerprint was compared with that of a reconstructed stock of the uncloned Qβ population. The comparison revealed that the uncloned stock was heterogeneous since one oligonucleotide that was present in full molar amount in clone A.S. was present in submolar amounts in the uncloned stock, and had been replaced by a mutant oligonucleotide (explained in Domingo et al., 1978). The resolved oligonucleotides that represented about 10% of the genome had been sequenced and mapped by M. Billeter, although the results were published years later (Billeter, 1978). These very early comparisons reinforced a suspicion that was frequently commented in the discussions held in Zürich: that bacteriophage Qβ, and other RNA bacteriophages, were probably highly heterogeneous [see Weissmann et al. (1973) for statements about potential heterogeneity at the time that nucleotide sequencing was slow and cumbersome]. Earlier indirect evidence of genetic instability of RNA viruses (abundance of temperature sensitive mutants in virus stocks, frequent reversion of phenotypic markers, etc.) was reviewed (Domingo and Holland, 1988). The observations with bacteriophage Qβ prompted the discovery of viral quasispecies (as described in Chapter 3), but the reason to bring them here is to emphasize that even after extensive passage without cloning, no population equilibrium with one dominant genome type was reached in the Qβ population.

From the current knowledge of quasispecies dynamics we can interpret that one of the reasons why no equilibrium with a defined consensus sequence was produced lies in the multiple possibilities of exploration of sequence space, open to any virus during replication as a result of the stochastic generation of mutations. Nonequilibrium might have been favored by the mutual effects (positive or negative, sometimes termed epistatic effects) among mutations in the same genome, and interactions among different genomes, or sampling effects during passages among other factors. It is worth (albeit not easy) picturing that a tendency toward equilibrium (meaning a trend toward a steady distribution of mutant forms) has to be based on the number and types of mutants available to the viral population at a given time. When new mutants are generated stochastically, the pathway toward equilibrium may change. A very large population will generate a larger number of alternatives toward equilibrium to choose from, as compared to a small population. This is why deterministic features of quasispecies are more likely to be observed with large viral populations (Section 3.6.2 in Chapter 3). No equilibrium can be assumed (and much less so the absence of mutations!) even for a virus with a long history of multiplication in the same environment (Box 6.1).

The initial FMDV passage experiments in cell culture that provided evidence of quasispecies dynamics for an animal virus were carried out with a biological clone of a swine isolate (Sobrino et al., 1983). The viral genome displayed increases and decreases in the molar proportion of T1 oligonucleotides, with heterogeneity levels estimated in an average of 2-8 mutations per genome in the mutant spectrum, as compared with the corresponding consensus sequence. Passage resulted in adaptation of the virus to the culture cells, as documented by an increase of infectious progeny production. Many experimental studies by J.J. Holland and colleagues contributed greatly to what we know about viral quasispecies, starting with pioneer experiments on the generation of defective interfering (DI) particles

BOX 6.1 SOME TEACHINGS OF EXPERIMENTAL VIRUS EVOLUTION

- Mutant viruses are continuously arising in viral populations. Even prolonged passage in the same environment does not mean that a population equilibrium has been attained. An invariant consensus sequence does not imply absence of mutations. It means that the genomes are mutating continuously to yield the same consensus.
- Virus evolution consists in the replacement of some viral subpopulation by others, due to random events or in response to selective constraints.
- The reorganization of viral subpopulations is profoundly altered by bottleneck events.
- The model studies predict that the effect of a bottleneck depends on its size and the fitness of the parental population.
- Work conducted under the guidance of J.J. Holland has permitted testing experimentally several theoretical proposals, hypotheses, and principles of general genetics. They include:
  - Muller's ratchet
  - Competitive Exclusion principle
  - Red Queen hypothesis

and their competition with standard virus (Holland et al., 1979, 1982). The studies carried out in J.J. Holland's laboratory in San Diego are summarized in several chapters of this book (Novella, 2003).

Application of standard molecular cloning and sequencing, and NGS to the analysis of viral populations during experimental infections has supported the view that viral populations are composed of many genome subpopulations, and that their evolution is best described as the replacement of some viral subpopulations by others (Acevedo et al., 2014; Baccam et al., 2003; Sobrino et al., 1983). Because conditions are far from population equilibrium, such replacements occur even in the absence of an externally applied selective pressure. A selective pressure acts as a guiding force to decant viral subpopulations in favor of those that best respond to the constraint, often after many transient, abortive attempts, as also observed in natural infections (Cale et al., 2011; Fischer et al., 2010; Kortenhoeven et al., 2015; Tsibris et al., 2009) (Figure 6.3). In other terms, different areas of sequence space are dynamically occupied prior to the occurrence of selective constraints as well as in response to selective demands (Chapters 3 and 7).

6.4 PERSISTENT INFECTIONS IN CELL CULTURE: VIRUS-CELL COEVOLUTION

Persistent infections *in vivo* may result from failure of immune surveillance systems to clear a virus, from infection of cells that exert functions related to the immune response, or from other mechanisms that limit viral population numbers and cell killing. Persistent infections with or without pathology may involve integration of genetic material of the virus into host cells or maintenance of the virus replicating by its standard mechanisms, but with modulation of viral population numbers. Persistent infections are abundant in most biological phyla that have been examined [review of mechanisms and biological consequences of viral persistence in (Ahmed and Chen, 1999; Nash et al., 2015; Nathanson and Gonzalez-Scarano, 2007; Oldstone, 2006; Roossinck, 2014)]. A feature of persistent infections is that the population numbers of infectious particles remain limited and constrained to a specific

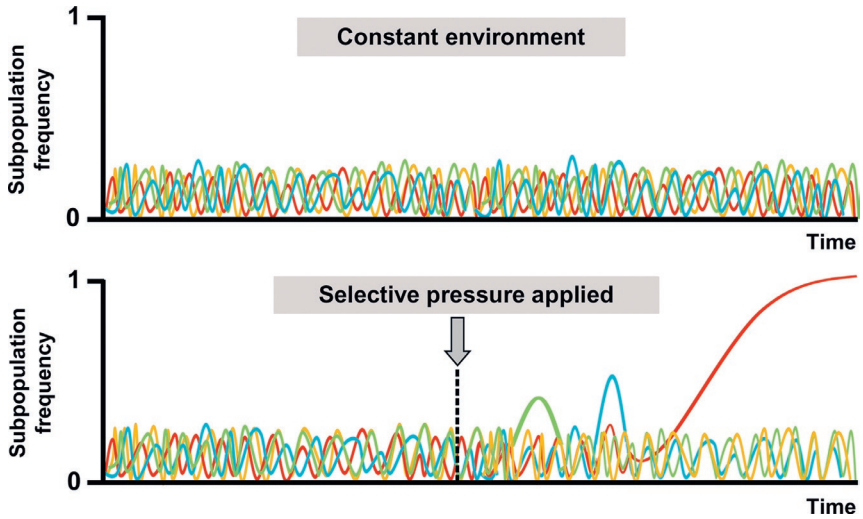

FIGURE 6.3

Diagram of dynamic genome subpopulations in the course of virus replication. Four different colors have been chosen to depict fluctuations of four genomic classes. In a real population thousands of genomes may be involved in each infected cell. When a selective pressure is applied, multiple transient selection events occur (green and blue outstanding waves) only to be finally displaced by a winning subset of genomes here represented in red. The time frame in the abscissa is left without units on purpose, because the diagram can represent events in a few infected cells during minutes or in a persistently infected organism during years. See text for examples and references.

environmental domain where viruses are enclosed (liver, kidney, brain, etc.). Under these circumstances the *r* strategy typical of overt virus infections (success based on rapid reproduction to cope with different environments) is transformed into one closer to the *K* strategy (limited progeny adapted to a specific environment) which is typical of large animals (compare with Section 4.1 in Chapter 4).

Cell culture systems have provided excellent tools to address the mechanisms of persistence of DNA and RNA viruses, including herpesviruses, polyomaviruses, parvoviruses, picornaviruses, reoviruses, flaviviruses, and retroviruses (Herrera et al., 2008; Imperiale and Jiang, 2015; Olivares et al., 2013, among other examples). Here we will analyze some of these systems that have provided new insights into the interaction of cells and viruses in a controlled environment, and in particular the evidence of virus-host coevolution during persistence. Takemoto and Habel reported the first evidence of virus-cell coevolution with the picornavirus Coxsackievirus A9 (Takemoto and Habel, 1959). R. Ahmed, B.N. Fields, and colleagues documented a role of the cells and coevolution of reovirus and L cells during a persistent infection in cell culture (Ahmed et al., 1981). These early studies were followed by experiments with the lymphotropic minute virus of mice (Ron and Tal, 1985, 1986), reovirus (Chiarini et al., 1983), poliomavirus (Delli Bovi et al., 1984), and FMDV (de la Torre et al., 1988) (several systems are summarized in Table 6.1).

Persistent infectious of FMDV were established in BHK-21 and IBRS-2 cells by growing the cells that survived a cytolytic infection (the frequency of survivors was 10^{-3} to 10^{-4}). During persistence the resident virus changed genetically and phenotypically: the heterogeneity of the resident viral genomes

Table 6.1 Examples of Virus-Cell Coevolution in Cell Culture

Virus Cell	Main Findings	References
Coxsackievirus A9 HeLa	Carrier cells showed increased resistance to virus. Virus underwent antigenic variation, plaque size reduction, increased virulence for HeLa cells and decreased virulence for suckling mice	Takemoto and Habel (1959)
Reovirus L	Mutant cells selected during persistence displayed partial resistance to reinfection with the parental virus, and readily reestablished persistence. Mutant virus selected during persistence overgrew the parental virus	Ahmed et al. (1981)
Bovine rotavirus AU-BEK	Persistence was dependent on the presence of fetal calf serum. Cells evolved to be highly resistant to the virus	Chiarini et al. (1983)
Polyomavirus (Py) Friend erythroleukemic (FL)	Viral genomes integrate at high temperature (large T-inactive). Viral genomes rescued at permissive temperature. Coevolution with selection of virus-free Py-resistant cells or cells shedding Py variants	Delli Bovi et al. (1984)
Lymphotropic minute virus of mice L	Host range virus mutants and virus-resistant cells were selected. Reconstruction of the persistently infected culture required mutant virus and mutant cells. Resistance to infection was due to an intracellular block that affected synthesis of viral single-stranded DNA	Ron and Tal (1985)
Herpes simplex virus type 1 Human lymphoblastoid CEM	Cured cells were partially resistant to parental virus. Virus isolated during persistence displayed increased virulence for the parental CEM cells	Cummings and Rinaldo (1989)
Reovirus L	Mutation in cells and virus affect an early step of the virus replication cycle. Amino acid substitutions that alter virus sigma 1 protein oligomerization mediate infection of virus-resistant L cells. The capsid is a determinant of reovirus adaptation	Dermody et al. (1993) and Wilson et al. (1996)
Poliovirus HEp-2c	Persistently infected cells cured of the resident poliovirus (PV) displayed selective permissivity to the parental PV (Mahoney strain) but partial resistance to several PV mutants. Restriction was mapped at an early phase (adsorption or uncoating) of the infection	Calvez et al. (1995)
Mouse hepatitis DBT	Murine astrocytoma (DBT) cells resistant to mouse hepatitis virus (MHV) were selected; resistance included diminished expression of the MHV receptor. MHVs with increased avidity for the receptor—and that recognized additional receptors—were selected	Chen and Baric (1996)
Rotavirus MA104	Virus rescued from persistently infected cells produced higher viral yield than the parental virus in persistently infected cells that were cured of the virus. Mutations in virus and cells affected virus entry	Mrukowicz et al. (1998)
Hepatitis C virus Human hepatoma cells	Virus variants with accelerated replication kinetics and higher peak titers were selected during persistence. The altered phenotype was associated with a substitution in the envelope E2 protein. Cells with decreased permissivity to the virus due to a block of virus entry, RNA replication, or both were selected	Zhong et al. (2006)

was estimated in 5×10^{-4} substitutions per nucleotide, and the virus displayed temperature sensitivity and small plaque morphology (de la Torre et al., 1985, 1988). During FMDV persistence in BHK-21 cells the virus became increasingly virulent for the parental BHK-21 cells, and, in turn, the cells became progressively more resistant to the parental FMDV (de la Torre et al., 1985, 1988). The resistance of the cells to the parental virus was not due to an impairment of virus attachment, penetration, or uncoating, but to some intracellular block as evidenced by virological and cell fusion experiments (de la Torre et al., 1988, 1989a).

The changes of FMDV and its host cells adhere to the concept of virus-cell coevolution, meaning a mutual influence in the evolution of two interacting biological entities (trait coevolution is discussed in Section 4.5 of Chapter 4). The coevolutionary process involved both viral and cellular heterogeneity. J.C. de la Torre and colleagues analyzed a total of 248 stable BHK-21 cell lineages established from individual cells isolated by limiting dilution from the persistently infected cultures (de la Torre et al., 1989a,b). They distinguished six cell categories based on morphology, duplication rate, and resistance to the FMDV that initiated persistence. The study indicated that the coexistence of the cell with the virus led to selection of cells with genetic modifications that resulted in altered phenotypes. In particular, increased cell transformation (evidenced by rapid cell duplication, loss of contact inhibition, and growth in semisolid agar medium) correlated with resistance to FMDV, although a possible mechanism that could link the two phenotypic traits was not investigated. Contrary to expectations, it was not the virus but the initial capacity of BHK-21 cells to vary that permitted the establishment of the persistent infection with FMDV (Martin Hernandez et al., 1994). Mutual influences that lead to both cell and virus modifications provide long-term stability to the system without either the cells or the virus eliminating its partner (except for documented "crises of cytopathology" during which high virus titers are reached, suggesting a transient imbalance in favor of the virus) (Herrera et al., 2008). The generality of these proposals are supported by observations with several virus-host systems (Table 6.1).

6.4.1 BACK AGAIN 4000 MILLION YEARS: CONTINGENCY IN EVOLUTION

In an excursion toward the question of contingency in evolution, M. Herrera and colleagues repeated again the entire process of establishment and maintenance of FMDV persistence 20 years later, starting from frozen virus and cell stocks (Herrera et al., 2008). The main conclusion with two new persistent BHK-21-FMDV lineages established in parallel was that the virus-cell coevolution displayed features very similar to those observed in the first persistent lineage, although there were differences in some of the mutations in FMDV. However, two amino acid substitutions which affected residues around a pore located at the capsid 5-fold axis were selected in the three persistent lineages. One of the substitutions (D9 → A in capsid protein VP3) was later selected in other FMDV serotypes that having originated in acute infections in animals, were passaged in evolved BHK-21 cells that displayed partial resistance to the virus (de la Torre et al., 1988; Díez et al., 1990; Escarmis et al., 1998). This substitution belongs to the category of "joker" substitutions in the sense of providing a selective advantage to the virus in a variety of sequence contexts and environments (Section 4.9 in Chapter 4).

The two newly established BHK-21-FMDV persistent lineages displayed strikingly parallel features in the variation of progeny production and imbalances in the ratio of positive versus negative strand viral RNA, that were found at the same passage number (Herrera et al., 2008). This parallel behavior reflects a deterministic feature of virus evolution, that has been encountered with other virus-host systems (see Section 6.7.1 and the general concept of deterministic vs. stochastic quasispecies in Section 3.6.2 of

Chapter 3). Interestingly, a deterministic behavior should be generally favored by large viral population sizes, but in this case a drastic decrease in population size preceded its manifestation. This unexpected association was interpreted as deterministic behavior being due to strict selective demands that guide the virus toward one (or very few) biological solutions to respond to the constraint (Herrera et al., 2008). The reproducibility of the main features of FMDV persistence in independent establishment events was suggested to mean that when a biological system is highly constrained, there may be limited room for contingency. Thus, we can now consider the question: If we could rewind the tape of evolution and play it again, would it turn out to be similar or different from what we know? According to S.J. Gould if we could rewind the tape to the remote past, evolution would turn out entirely different (Gould, 1989). The answer proposed by M. Herrera and colleagues was that the tape would turn out equally, differently or "in between" depending on the constraints operating at the time we start to retape. If the tape was situated in the RNA world around 3.6×10^9 to 4.2×10^9 years ago or perhaps prior to the Cambrian explosion, about 5×10^8 years ago (Conway Morris, 1998; Gould, 1989) there would be ample room for divergence due to equally valid alternative solutions. In contrast, once the constraints inherent to functional cells and viruses are in place, the system should be forced to a seemingly deterministic behavior (see Chapter 1 for the time frame in which the increasingly chemical and precellular complexity evolved on Earth).

It is tempting to make a connection between virus-cell coevolution in cell culture and the models of virus origins that contemplated vesicle-wrapped primitive cellular and viral entities (Section 1.5.5 in Chapter 1). What the experiments of viral persistence in cell culture show is that as part of coevolutionary mechanisms, a cell may diminish the expression of surface proteins that act as receptors for the coevolving virus or even alter fundamental properties such as the rate of cellular multiplication. Cells persistently infected with PV express mutated forms of the PV receptor CD155 (Gosselin et al., 2003; Pavio et al., 2000), and persistently infected BHK-21 cells increased their degree of cellular transformation (de la Torre et al., 1988, 1989b). [As review of picornavirus persistence, see Colbère-Garapin and Lipton (2010).] It has been proposed that in the primitive biosphere early vesicles evolved to limit their invasion by virus-like elements, and they did so by building protective walls that culminated in primitive receptor-dependent virus entry into increasingly autonomous precellular entities. The insights provided by current cell-virus coevolution models indicate that such kind of mutual influences occur with naked or enveloped viruses without the need for the virus to integrate its genome (or part of it) into the genetic material of the coevolving cell (Colbère-Garapin and Lipton, 2010; de la Torre et al., 1988). During persistent infectious in cell culture, virus titers change in ways partially dictated by their own quasispecies dynamics and coevolving carrier cells.

An area of experimental evolution that puts its emphasis on the virus rather than the host cells consists in subjecting the virus to repeated bottlenecks that are experimentally realized through plaque-to-plaque transfers (Figures 6.1 and 6.2). In this design, the cell obviously plays the essential role of hosting viral replication, but it cannot evolve since fresh (uninfected) cells are used for each virus transfer. We have learned a lot from this class of experiments.

6.5 TEACHINGS FROM PLAQUE-TO-PLAQUE TRANSFERS

Bottlenecks increase the stochasticity of evolutionary events and may modify the course of selection (Chapter 3). In the present section we review evidence that population bottlenecks may have additional and even more profound influences in the composition of viral quasispecies, by permitting hidden

minority genomes to surface in populations. This important line of investigation of viral genetics was initiated by L. Chao working with the tripartite double-stranded RNA bacteriophage φ6 (Chao, 1990). He demonstrated average fitness decrease in φ6 clones subjected to serial plaque transfers in its host bacterium *Pseudomonas phaseolicola*. The results constituted the first experimental support for the operation of Muller's ratchet, a concept from theoretical biology explained in Section 6.5.1. However, the molecular basis of fitness decrease was not investigated in this first study.

The experimental design of L. Chao was extended by J.J. Holland and his colleagues to the animal virus VSV growing in mammalian cells (Duarte et al., 1992). The results agreed with those of L. Chao and documented variable, but in some cases severe fitness drops by subjecting VSV to only 20 serial plaque-to-plaque transfers. A relevant observation was the large difference in the extent of fitness decrease among biological VSV clones (from a common parental population) subjected to the same passage regime, which is itself a reflection of the remarkable heterogeneity within a VSV population. A second highly significant observation was that VSV clones isolated from a population with a long history of passage in BHK-21 cells displayed a more pronounced fitness decrease when subjected to plaque-to-plaque transfers in the alternative hosts HeLa or MDCK cells than in BHK-21 cells to which the virus was better adapted due to its prior passage history. This difference probably reflects the fact that when the virus replicated in a less adequate environment provided by the unfamiliar host cell, a larger proportion of genetic variants occupied the most frequent class of genomes. The genomes forced to be more represented are those more likely to be picked at random from individual plaques to enter the following transfer. J.J. Holland and colleagues extended these studies with VSV to show fitness decreases at different plating temperatures, and contrasted the fitness loss associated with plaque transfers with fitness gain upon large population passages in the same host cells. Clones that had attained low fitness due to plaque transfers rapidly regained fitness when they were passaged as large populations (Clarke et al., 1993). A study of alternation between passage regimes evidenced that the fitness increase that occurs during two successive large population passages was not sufficient to overcome the decrease produced by a single bottleneck passage (Duarte et al., 1993).

The model studies of J.J. Holland, I.S. Novella, and colleagues with VSV represented great progress in the understanding of virus evolution, and some of the information provided by the results is not (even today!) sufficiently considered in interpreting the consequences of bottlenecks and population expansions *in vivo* (Chapter 5). A pertinent study was the quantification of the effect of the bottleneck size (how many infectious particles participate in the bottleneck passage) on viral fitness. This effect depends on the fitness of the population subjected to the bottleneck (Novella et al., 1995) (Figure 6.4). When the starting population has low fitness (point L in the middle and bottom schemes of Figure 6.4), few particles per transfer are sufficient for the passaged population to gain fitness. In contrast, when the initial population has high fitness (point H in the middle and bottom schemes of Figure 6.4), a large number of particles is needed just to maintain fitness. An even larger number is necessary to increase fitness above the initial level of point H.

If these observations on fitness dependence of fitness evolution were operative in the patchy environments provided by nature (still to be proven) they could contribute to maintaining equilibrium between population numbers of viruses and their hosts. Large fitness increases of viruses would be prevented since whenever a bottleneck of any size is reached, it will act to limit fitness to an extent which is commensurate with the fitness value already attained by the relevant population (see also Section 6.6). This modulating effect is credible in view of the evidence of the frequent occurrence of bottlenecks in nature (compare Figure 6.4 with the evidence of bottlenecks during the arbovirus life cycle described in Section 4.10 of Chapter 4). It is tempting to consider that the equilibrium between

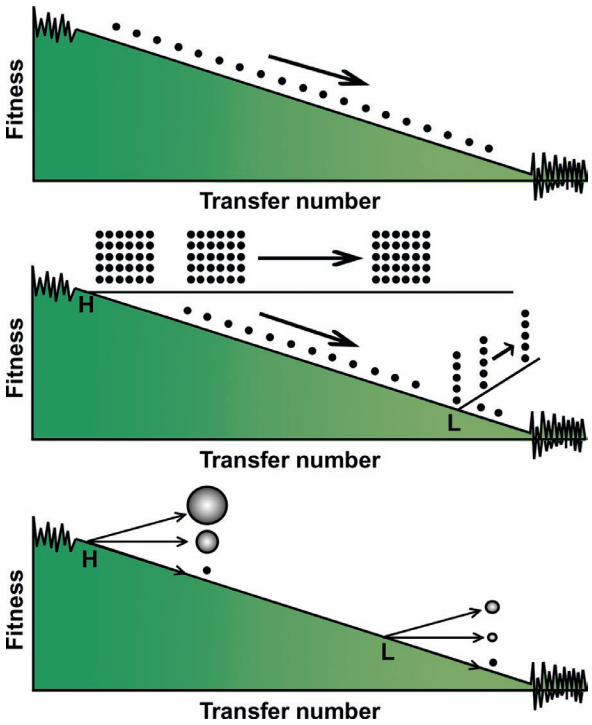

FIGURE 6.4

The effect of bottleneck size on fitness is dependent on the fitness of the population subjected to the bottleneck. At the top, plaque-to-plaque transfers (bottleneck size 1, black dots) lead to fitness decrease dependent on the number of transfers (downward arrow). The middle diagram shows that when the starting population has high fitness (point H in the graph) a bottleneck size of 30 (pool of virus from thirty plaques) is necessary to just maintain the fitness value (horizontal arrow). In contrast, when the starting point has low fitness (point L in the graph) a bottleneck size of 5 is sufficient to increase fitness (upward arrow). In the bottom diagram bottleneck sizes are depicted as spheres of different size to summarize schematically the bottleneck size requirements to maintain or increase fitness depending on the initial fitness (H, high; L, low). The concept expressed in this diagram is based on studies with VSV by I.S. Novella, J.J. Holland, and colleagues, as described in the text. The fluctuations in fitness values at very high and very low-fitness values have been observed experimentally, and their interpretation and implications are also discussed in the text.

viral and host populations numbers that had to exist from ancestral times (Chapter 1) is not only due to interaction and escape strategies between viruses and host immune systems (Chapter 4), but also due to in-built self-regulatory mechanisms inherent to viral genome dynamics that limit excessive replication capacity in the face of a far more inflexible cellular world. Bottleneck size may be an evolvable trait that has contributed to some balance between virus and host population numbers.

Repeated bottlenecks not only decrease fitness as summarized in previous paragraphs, but, in addition, they may have long-term detrimental effects on virus adaptability. Even when a bottlenecked VSV population had the same fitness than a virus maintained under large population passages, it displayed lower adaptability (Novella, 2004). This result means that it is not fitness *per se* that determines the

adaptive potential of a virus, but it is the part of sequence space that it occupies that does. Different areas of sequence space may provide a mutant cloud with comparable fitness, but with different capacity to respond to perturbations (see Section 6.7.1 in this chapter and Section 3.7 in Chapter 3). A similar, also rather counterintuitive conclusion is reached when comparing mutation frequencies (or other complexity measurements) of viral populations: the same high mutation frequency may either permit viral survival or drive the virus toward extinction. It is not the mutation frequency value *per se* that counts, but the context in which the mutations occur (the experimental values that justify such an assertion are discussed in Chapter 9).

Once more we come to the conclusion of the importance of viral population numbers in fitness variations and in guiding evolutionary episodes. It is obvious that some nuances must be introduced into the rather simplified view that bottlenecks lead to fitness loss (Chapter 5). There is no absolute population size value that guarantees avoidance of fitness decrease. The bottleneck size that leads to decrease, maintenance, or increase of fitness is dependent on the initial fitness and on the position of the virus in sequence space. It is quite clear that in this type of studies, experimental evolution has gone far ahead than field observations, partly due to the difficulties in following fitness evolution and in controlling bottleneck sizes *in vivo*. With these clarifications, we are now in a position to begin to approach the general question (or dilemma) between asexual and sexual modes of reproduction, as a precedent to approach clonality versus nonclonality in biological evolution to be addressed in the closing Chapter 10.

6.5.1 MULLER'S RATCHET AND THE ADVANTAGE OF SEX

Fitness loss due to bottleneck events (with all its participating parameters discussed in the previous section) provides experimental support to a theoretical proposal made by H.J. Muller, known as Muller's ratchet (Maynard-Smith, 1976; Muller, 1964; Nowak and Schuster, 1989). The proposal is that small populations of asexual organisms that display high mutation rates will tend to incorporate mutations (the majority deleterious) in an irreversible, ratchet-type mechanism, unless recombination can yield the initial type of genome devoid of mutations (often termed the zero mutation class of genomes). Fitness decrease due to accumulation of mutations proposed by H.J. Muller in general terms is expected to be accentuated in the case of viral populations with continuous generation of new mutant genomes. In mutant spectra of viruses the least mutated class of genomes will be the one displaying highest replicative fitness and will correspond to the master sequence that dominates the population (Chapter 3). With the plaque-to-plaque transfer design there is a probability that in each plating (a step or click in the ratchet) the least mutated class of genomes is lost. Therefore, the viral population is forced to regenerate a distribution from which again the least mutated class will be lost in the next ratchet click. The system is doomed to rapid deterioration and extinction, unless mechanisms to restore fitter genomes operate.

The operation of Muller's ratchet appears as rather general in viruses since, in addition to bacteriophage φ6 and the animal virus VSV (studies summarized in Section 6.5), other viral systems have been used to document fitness loss associated with serial bottlenecks (de la Iglesia and Elena, 2007; Escarmís et al., 1996; Jaramillo et al., 2013; Yuste et al., 1999). The studies by C. Escarmís and colleagues with FMDV contributed decisively to define the molecular basis of fitness loss in an RNA virus, and to unveil unusual genetic lesions (with phenotypic consequences) that hide in mutant spectra. For these reasons the studies by C. Escarmís and colleagues are detailed in Section 6.5.2. Muller's ratchet operates not only in viruses but also in bacteria, protozoa, plants, fish, and mitochondrial DNA (Allen et al., 2009; Andersson and Hughes, 1996; Bell, 1988; Coates, 1992; Engelstadter, 2008; Leslie and Vrïjenhoek, 1980; Loewe, 2006; Moran, 1996).

The concept that sex conferred an advantage to organisms by providing new gene combinations for adaptation dates back to A. Weismann (Bell, 1982; Weismann, 1889-1892). Avoidance of the detrimental effects of Muller's ratchet is believed to have been one of the driving forces to introduce genetic recombination and sex in the reproduction and evolution of living systems (Agrawal, 2006; Barton and Charlesworth, 1998; Maynard Smith and Szathmáry, 1999; Maynard-Smith, 1976). In the words of G. Bell: "Sex acts as an editor which detects serious copying errors and enables the genetic message to be transmitted without contamination" (Bell, 1988). It is worth comparing this sentence by G. Bell with the molecular evidence discussed in Chapters 2 and 3 that copying errors during virus replication are not corrected because of the absence of proofreading exonucleases (or related) activities in RNA viruses. Lack of correcting activities is remarkable from the point of view of an evolution rich in lateral gene transfers because proofreading-repair activities are available not only from DNA polymerases but also from the coronavirus polymerases (and likely other large RNA genomes to be discovered). This raises the issue of clonal evolution in viruses, an important feature of long-term virus evolution (Chapter 10). Now we return to plaque-to-plaque transfers because there is more to learn from them.

6.5.2 MOLECULAR BASIS OF FITNESS DECREASE: DEEP FLUCTUATIONS, MASSIVE EXTINCTIONS, AND RARE SURVIVORS

The genomic RNA of 19 biological clones of FMDV that had undergone fitness decrease as a consequence of 30 serial plaque-to-plaque transfers was sequenced and the sequences compared with those of the parental clones, and to the parental populations subjected to large population passages. Many mutations that had never been found in natural isolates or laboratory clones and populations of FMDV were found. A total of 69 mutations affecting the internal ribosome entry site (IRES), L-protease, P1- and polymerase-coding regions were identified (Escarmís et al., 1996). Notably, 9 out of 19 clones showed an extension of an internal tract of four adenylate residues that precedes the second functional AUG. Depending on the clone, the extension was of one, or several residues and up to 23 additional adenylates in one of the clones. This elongation affected translation, was one of the determinants of fitness decrease, and reverted when the clones were subjected to large population passages. It was proposed that the elongation, that constitutes a hot spot for variation, was prompted by slippage mutagenesis of the polymerase. This genetic lesion in the FMDV genome was given as an example of mutational instruction in Section 2.3 of Chapter 2, and it served as one of the genetic markers for quasispecies memory when it reverted upon large population passages of the altered clones (Section 5.5 in Chapter 5). The molecular instability associated with this oligoadenylate elongation is illustrated by the fact that genomes with different numbers of adenylate residues coexisted in the same viral plaque.

Another rare lesion was a point deletion located between the two functional AUG protein synthesis initiation codons, that led to a predicted stop codon for the proteins synthesized from the first AUG. A significant finding is that of the total number of mutations that had accumulated at the end of the 30 transfers, one-third were already present in the first transfer, although a rather steady accumulation of mutations was observed when the clones were subjected to many additional transfers. A possible limitation in the accumulation of mutations is examined again in connection with limited tolerance to mutations and the concept of contingent neutrality (Section 6.7.1).

FMDV clones were subjected to additional (up to 409) plaque-to-plaque transfers that unveiled additional rare genetic lesions, unusual phenotypes, and a remarkable resistance to extinction (Escarmís et al., 2002, 2008). The most salient phenotypic change is that after more than 100 transfers the virus

became noncytolytic, was unable to form visible plaques, and it could readily establish a persistent, noncytocidal infection that normally would be established only from the few cells that survived a cytolytic infection (see Section 6.4). Thus, strikingly, a fundamental property of the virus-host interaction such as the capacity to kill cells [a very marker of virus virulence! (see Section 5.6 in Chapter 5)], was totally altered as a result of repeated bottleneck passages. In fact, if infectivity is judged by the capacity to produce plaques, these multiply transferred clones underwent decreases of specific infectivity (the ratio between the amount of infectivity and that of viral RNA) of at least 140-fold relative to their corresponding parental biological clone. Such a reduction is enormous, and it should serve to compare the decrease of specific infectivity that accompanies virus extinction by lethal mutagenesis. (Decreases of specific infectivity is mentioned in Section 4.3 of Chapter 4 as one of the consequences of suboptimal codon usage, and are revisited in Chapter 9 as a diagnostic parameter of lethal mutagenesis.) A proof that pursuing molecular analysis of clones subjected to many bottleneck transfers may provide new information was documented by the discovery of an amino acid substitution in capsid protein VP1 that produced virus thermosensitivity, and exerted an effect at a distance in the processing of the FMDV polyprotein (Escarmís et al., 2009), unveiling a new feature of picornavirus protein processing (Martínez-Salas and Ryan, 2010). Unusual FMDVs isolated after plaque transfers are summarized in Box 6.2.

Several mechanisms have been proposed to explain why so many unusual, often unique, detrimental mutations can be rescued in clones subjected to many plaque transfers. One model emphasizes that negative selection is attenuated during plaque transfers in the sense that no truly competitive optimization of the mutant spectrum is allowed, except for intracellular competition in each individual cell. The competitive mixing of genomes is limited in the cell monolayer whose individual cells become infected by the wave of progeny virus that had its initial focus in the cell hit by the virus in the corresponding transfer. Even accepting that intraplaque MOI might be high, no competition among many of the newly arising genomes can take place in any of the cells. Therefore, the displacement of unfit genomes by fitter ones is limited. A necessity to explain the frequency and types of mutations observed is that the virus must be subjected to high mutational pressure. Although mutation rates for RNA viruses are discussed in Chapter 2, it must be stated here that many of the studies that allowed a quantification of high mutation rates came from experimental evolution designs. The fact that one of the clones at transfer 409 differed from its parental clone in 122 mutations, implies a mutation frequency of 1.5×10^{-2} s/nt (Escarmís et al., 2008). Remarkably when FMDV is subjected to mutagenesis, a tenfold lower frequency can drive the virus to extinction (Chapter 9). The observed mutability during plaque transfers renders perfectly understandable multiple reversion events scored in picornavirus genomes (de la Torre et al., 1992; Domingo et al., 2010).

Another, not mutually exclusive, mechanism is that many extinction events take place in the course of the transfers and that the low-fitness survivors acquire compensatory mutations that still allow them

BOX 6.2 UNUSUAL GENOTYPES AND PHENOTYPES IN FOOT-AND-MOUTH DISEASE VIRUS SUBJECTED TO PLAQUE-TO-PLAQUE TRANSFERS

- Mutations never found in other populations of the same virus subjected to other passage regimes, including a rare one nucleotide deletion.
- Noncytocidal mutants that can establish a persistent infection in cell culture without intervening cell killing.
- Amino acid substitutions in the capsid that can affect thermal stability and polyprotein processing.

to form a plaque, unless a noncytolytic phenotype that still allows intracellular RNA replication to take place is produced.

C. Carrillo, D.L. Rock, and colleagues applied an *in vivo* protocol of 20 serial swine-to-swine contact transmission that resembles the plaque-to-plaque transfer design (Carrillo et al., 2007). Interestingly, profound phenotypic changes occurred in the virus after several transfers, including reduction of virulence and establishment of a carrier state in pigs, previously thought to be typical of ruminants. Several mutations accumulated in the viral genome, suggestive of the operation of Muller's ratchet *in vivo*. This study proves the feasibility of serial bottleneck passages in animals, that may be highly informative of potential viral alterations associated with transmission events.

6.6 LIMITS TO FITNESS GAIN AND LOSS

A key issue in the studies of fitness evolution is whether fitness of a virus population can grow indefinitely or it has a limit, reaching a plateau value. This question is of theoretical and experimental interest. Limitations in the capacity to occupy sequence space suggest that there must be a limit to fitness gain and that, in the case of viruses this limit may be imposed by the viral population size. Several experimental studies of experimental evolution and theoretical predictions support that either increases or decreases of fitness reach a plateau, proposed to be the result of mutational effects and the ratio of beneficial to deleterious mutations (average mutations will increase fitness of a very low-fitness population but decrease fitness of a high-fitness population) (Silander et al., 2007).

The situation may not be so simple. I.S. Novella and colleagues demonstrated that exponential increase of VSV fitness has a limit but that when the limit is reached, stochastic fitness fluctuations occur (Novella et al., 1999b). Variations are represented by the zig zag lines at the upper left side of the fitness diagrams shown in Figure 6.4. Since the viral population size needed to increase viral fitness is larger the higher the fitness value, the results of I.S. Novella and colleagues suggest that VSV reached a fitness value whose further increase could not be guaranteed by the replicating population size attained. An area of sequence space is reached where the newly arising mutations are not steadily incorporated, but rather they produce unpredictable fitness jumps.

A similar fluctuation of fitness values was observed in the case of FMDV subjected to many plaque-to-plaque transfers (Lázaro et al., 2003). Variations are represented by the zig zag lines at the lower right side of the fitness diagrams shown in Figure 6.4. An example with four FMDV subclones is presented in Figure 6.5. The fluctuating pattern at low-fitness values followed a Weibull statistical distribution which was taken to mean that complex virus-cell interactions contribute to the level of progeny in each individual plaque. The results reinforce the concept of extreme resistance of viruses to extinction at the population level despite many extinctions at the individual level. The reason is that when fitness is very low the probability of stochastic occurrence of beneficial mutations can rescue subpopulations from their fate toward extinction (Escarmís et al., 2002).

Fitness instability might underlie the transition toward genome segmentation underwent by FMDV upon extended high MOI replication in cells, an experiment intended to see if FMDV reached a fitness plateau (García-Arriaza et al., 2004). In the course of hundreds of passages, the virus diversified in two distinct subpopulations that exhibited a competition-colonization dynamics previously shown by D. Tilman to operate in classical ecological systems (Tilman, 1994). The viruses diversified into colonizers which were efficient in killing cells, and competitors that modulated cell killing. Thus, internal quasispecies interactions modulated virus virulence (Ojosnegros et al., 2010). This balance was maintained

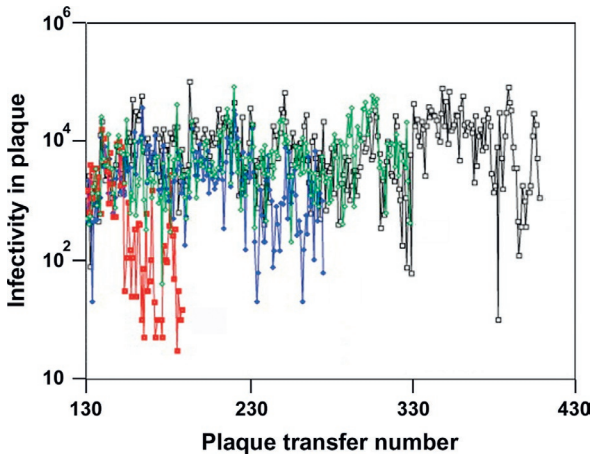

FIGURE 6.5

The fluctuation pattern of fitness values (infectivity in plaque) of FMDV subclones subjected to a maximum of 400 serial plaque-to-plaque transfers. Results for four different subclones (color-coded) for passage 130–400 are shown. Only one of the subclones (identified in black color) survived to produce plaques to the end of the transfer series. The other clones (coded red, blue, and green) became noncytopathic at different passages.

Modified from Escarmís et al. (2008), with permission of the authors.

until around passage 260 at which the viral genome became segmented: the monopartite genome was converted into two segments that complemented each other to replicate and kill cells (García-Arriaza et al., 2004, 2006; Moreno et al., 2014; Ojosnegros et al., 2011). This transition was discussed in Section 2.11 of Chapter 2 in connection with a mutation-driven genome transition involving RNA recombination. The transition toward segmentation might have been favored by fitness instability when fitness increase reached its population size-dependent limits.

6.7 COMPETITIVE EXCLUSION PRINCIPLE AND RED QUEEN HYPOTHESIS

J.J. Holland and colleagues examined the fate of two competing VSV clones that displayed approximately equal fitness. The two clones coexisted for several generations until one of the populations rapidly outgrew the other (Clarke et al., 1994). This observation is schematically depicted in Figure 6.5, and agrees with the Competitive Exclusion principle of population genetics (Gause, 1971). This principle states that one competing species will always outcompete the other provided no niche differentiation exists between them. In a related formulation, the resource-based competition theory asserts that two consumers that share a single, limiting resource cannot stably coexist in a spatially and temporally homogeneous habitat. It is assumed that there are no other intervening ecological factors. In testing the Competitive Exclusion principle experimentally, the precise definition of "limiting resource" is essential. In a series of classical experiments, F.J. Ayala showed that two species of *Drosophila* could coexist for many generations in competition for limited resources (Ayala, 1971). The genetic and phenotypic inflexibility of a differentiated organism such as *Drosophila* may explain its very different behavior as compared with two VSV quasispecies. The genetic and phenotypic constraints of *Drosophila* within the time frame of the experiment contrasts with the much more voluble and dynamic nature of two competing VSV mutant clouds. Indeed, the eventual exclusion of one VSV quasispecies by the other is expected as a consequence of the

random, infrequent occurrence of advantageous mutations in a genome of one of the populations and not in the other. During the competition process, both the winners and the losers gained fitness at similar rates, in agreement with the Red Queen hypothesis: "No species can ever win and new adversaries grinningly replace the losers" (Van Valen, 1973). Among competing viral quasispecies that have approached some population equilibrium in which the mutant spectrum can modulate the behavior of the ensemble, infrequently arising, superior mutants are likely to perturb the equilibrium in such a way as to exclude or maintain at low levels all other mutants present in the competing quasispecies. Until such exclusion occurs, most members of the quasispecies gain replicative fitness, including minority members present at "memory" levels (Section 5.5 in Chapter 5). As expressed by the Red Queen in Lewis Carrol's *Through the Looking Glass*: "It takes all the running you can do to stay in the same place." It appears as if the mutational background associated with some competitive optimization of quasispecies affected both competing populations in a similar way, while the stochastic occurrence of a saliently beneficial mutation disrupted coexistence. The results imply also that the frequency of mutations that are advantageous enough to upset the coexistence of the two populations is low. This is expected from the fact that detrimental and lethal mutations are far more frequent than advantageous mutations (Figure 6.6).

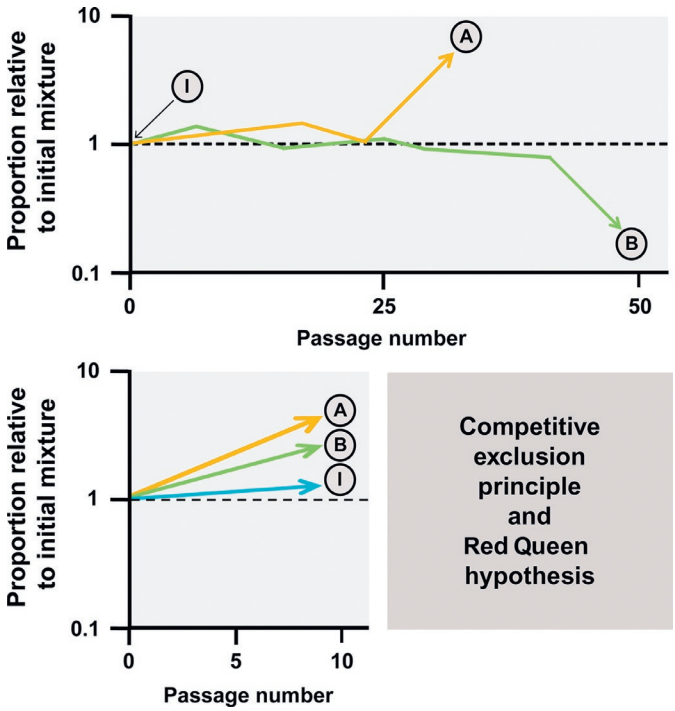

FIGURE 6.6

Schematic representation of the Competitive Exclusion principle and the Red Queen hypothesis applied to RNA viruses. At the top, two populations of fitness equal to a control I (initial population), were serially passaged in cell culture. After many passages in which both populations coexisted at about the same frequency, suddenly one of the populations (A, yellow arrow, or B, green arrow) displaced the other. This behavior is in agreement with the Competitive Exclusion principle of population genetics. Below, both populations A (winner) and B (looser) gained fitness relative to the control I. This observation agrees with the Red Queen hypothesis. See text for implications and references.

6.7.1 CONTINGENT NEUTRALITY IN VIRUS

The behavior of competing VSV populations was not always unpredictable. In parallel competitions of a wild-type VSV and a surrogate marked subclone of equal fitness, a predictable nonlinear behavior of the two populations was characterized (Quer et al., 1996). After nearly constant times, the two viruses competing in a constant cell culture environment followed different trajectories. There was a reproducible tendency of the wild type to gain fitness at a higher rate than the surrogate mutant. Thus, despite the stochastic occurrence of mutations, a nearly deterministic evolutionary behavior was observed (Tsimring et al., 1996). A number of environmental perturbations (presence of DIs, increased temperature during viral replication, or limited, enhanced mutagenesis) led to an accelerated dominance of the wild type over the mutant (Quer et al., 2001). Comparison of consensus nucleotide sequences of the entire VSV genomes showed that the mutant genome had a number of mutations with respect to the wild type, and suggested that neutrality of the mutant relative to the wild type was maintained provided the environment was not perturbed. The behavior of the mutant relative to the wild type was described as being of "contingent neutrality." The presence of mutations rendered the virus less robust to accept additional mutations. In terms of fitness landscapes, the results can be interpreted as if that the wild type lies on a relatively flat (or less rugged) fitness surface that preserves its replicative efficacy despite occurrence of mutations. In contrast, the mutant lies on a sharper fitness peak prone to fitness decreases upon mutation or environmental stress (Sections 5.3 and 5.7 in Chapter 5).

Thus, the behavior of competing viral subpopulations, a frequent occurrence in quasispecies dynamics may be affected by the mutational load in the competing genomes to the point of accelerating a selective advantage by virtue of limited tolerance to acquire new mutations.

6.8 STUDIES WITH RECONSTRUCTED QUASISPECIES

Experimental evolution offers the possibility—that has so far been exploited only minimally—of reconstructing complex quasispecies swarms with specific mutants or viral subpopulations, to examine their behavior during replication under different environmental conditions. These types of experiments in which population evolution can now be followed by NGS methodologies, would actually represent an important contribution to the study of complexity, a field of science in need of experimental approaches.

A FMDV quasispecies was reconstructed with 19 antigenic variants of the virus, each identified by an amino acid substitution at the major antigenic site that conferred resistance to a monoclonal antibody (MAb). The mutants were added to the mutant spectrum of a biological clone of FMDV, at a concentration typical of the antibody-resistant mutants found in FMDV. The reconstructed quasispecies was allowed to replicate in the absence and presence of the MAb, and the resulting populations analyzed. In the populations passaged in the presence of the antibody, but not in the control population, 10 out of the 19 mutant introduced became dominant, indicating selection of a mutant cloud that shared the required phenotype (Perales et al., 2005). In a subsequent study, the FMDV quasispecies was reconstructed with matched pairs of distinguishable MAb-escape mutants of the same antigenic site. Each mutant of a pair differed from the other in 11- or 33-fold in fitness. The analysis of the populations subjected to antibody selection revealed dominance of the corresponding high-fitness mutants. Thus, relative viral fitness can influence significantly the mutant repertoire selected by neutralizing antibody (Martin et al., 2006). Such fitness effects are likely to underlie the response to any selective constraint, and should be considered in the interpretation of the effect of selective forces acting on complex viral populations.

6.9 QUASISPECIES DYNAMICS IN CELL CULTURE AND *IN VIVO*

Some authors have argued that quasispecies dynamics is valid for viruses replicating in cell culture, but that its basic principles are not adequate to understand virus behavior *in vivo*. A reexamination of the recent evidence derived from application of NGS to *in vivo* systems renders the suggestion of a fundamental difference between cell culture and *in vivo* quasispecies dynamics untenable. One can argue that *in vivo* systems offer highly complex, compartmentalized environment that delays a possible approach to population equilibrium, as compared with a uniform and constant environment. What we have learnt, however, from the several model experimental studies summarized in this chapter, both using cell culture systems and animals, is that there are no fundamental differences in the quasispecies behavior of viruses in cell culture and *in vivo*. Perhaps a significant example is provided by the accumulation of mutations in the FMDV genomes and the profound phenotypic changes observed both in cell culture plaque-to-plaque transfers and in serial bottleneck transmission of the virus in swine (Section 6.5.2). Evidence in support of the value of quasispecies to interpret viral population dynamics *in vivo* is increasing as the new tools of NGS are applied to evolving viral population.

6.10 OVERVIEW AND CONCLUDING REMARKS

The possibilities of experimental evolution to gain new insights into the mechanisms of virus evolution are enormous, and they remain largely unexploited. In this chapter we have summarized cell culture and *in vivo* designs that have revealed fundamental features of virus evolution, including some that have provided experimental confirmation of some concepts of population biology.

Coevolution of cells and viruses appears to be quite common during persistent infections in cell culture. Several reproducible traits of virus and cell variation are shared by widely different viral pathogens and cell types. It is tempting to speculate that coevolutionary interactions reflect an inheritance of old-time relationships between primitive forms of viruses and cells.

Experimental evolution opens the possibility to examine the effects of extreme population regimes in fitness evolution, notably the result of massive infections versus the most profound bottleneck restriction: serial infections limited to one infectious particle per passage. Teachings of plaque-to-plaque transfers include the evidence of highly unusual mutations, and phenotypes that contradict textbook types of viral properties. In particular, the recognized cytopathic nature of FMDV is turned into noncytopathic in clones rescued from the low-frequency levels of viral quasispecies. In a similar note, repeated bottleneck passages of FMDV in swine produced viruses that established a carrier state in swine, a type of virus-host interaction previously thought to occur only in ruminants.

Muller's ratchet, Competitive Exclusion principle, Red Queen hypothesis, and contingent neutrality are concepts that have been established in experimental designs based on competitive, large population passages of marked mutant mixtures. These types of experiments, and those using reconstructed quasispecies, face a promising research time in which the application of NGS should clarify the mechanisms by which some variants overgrow others, opening new avenues for the understanding of viruses at the population level. The potential implications for the diagnosis of viral disease and considerations for treatment options are very evident, and they will be discussed in coming chapters (see Summary Box).

SUMMARY BOX

- Experimental evolution permits the establishment of many fundamental concepts of virus evolution that are facilitating the interpretation of observations in the far more complex natural scenarios.
- Designs intended to reproduce extreme passage regimes have unveiled many features of mutant spectra that are hidden to standard analyses based exclusively on consensus sequences. In particular, multiple plaque-to-plaque transfers have revealed that extremely unusual mutations can be found in low-fitness viruses that result in extreme phenotypes. Such unusual viral subpopulations replicate thanks to the presence of compensatory mutations that rescue a few genomes out of a great majority that are extinguished.
- The experimental studies have revealed the fitness dependence of fitness variation, a field of research still to be applied *in vivo*. Fitness increases and decreases have a limit imposed either by an insufficient population size or by extremely low replicative fitness. In such extreme scenarios, unexpected evolutionary transitions may be favored.
- Several concepts of population genetics have found experimental support in work with viruses, notably Muller's ratchet, Competitive Exclusion principle, and the Red Queen hypothesis.
- The dynamics of competition between viral populations has defined the concept of contingent neutrality in virus evolution.
- The capacity to reconstruct quasispecies with selected types of mutants opens new research avenues to understand viral population dynamics under controlled conditions.
- It will be extremely interesting to reexamine the population dynamics in the different designs described in this chapter with the new tool of next generation sequencing.

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