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MOLECULAR BASIS OF GENETIC VARIATION OF VIRUSES: ERROR-PRONE REPLICATION

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ABBREVIATIONS

A	adenine
ADAR	adenosine deaminase acting on double-stranded RNA
AIDS	acquired immune deficiency syndrome
AMV-RT	avian myeloblastosis virus reverse transcriptase
APOBEC	apolipoprotein B mRNA editing complex
BHK	baby hamster kidney cells
bp	base pairs
C	cytosine
cDNA	complementary DNA

CVB3	coxsackievirus B3
DI	defective interfering
DI RNAs	defective-interfering RNAs
<i>E. coli</i>	<i>Escherichia coli</i>
FMDV	foot-and-mouth disease virus
G	guanine
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus type 1
HIV-RT	human immunodeficiency virus reverse transcriptase
IFN	interferon
I	inosine
IRES	internal ribosome entry site
Kb	kilobase
miRNA	micro-RNA
mRNA	messenger RNA
NGS	next generation sequencing
NTP-dependent	nucleoside triphosphate-dependent
OPV	oral poliovirus vaccine
PCR	polymerase chain reaction
PV	poliovirus
RdRp	RNA-dependent RNA polymerase
rRNA	ribosomal RNA
RT	reverse transcriptase
siRNA	small interfering RNA
T	thymine
U	uracil
VSV	vesicular stomatitis virus

2.1 UNIVERSAL NEED OF GENETIC VARIATION

Genetic change was a prerequisite for the early life forms to be generated and maintained (Chapter 1), and it is also a requirement for the evolution of present-day life. We may willingly or inadvertently modify selective pressures, but genetic change is rooted in all replication machineries. Viruses use the same molecular mechanisms of genetic variation than any other form of life: mutation, hypermutation, several types of recombination, and genome segment reassortment. Mutation is observed in all viruses, with no known exceptions. Recombination is also widespread, but its role in the generation of diversity appears to vary among viruses. Its occurrence was soon accepted for DNA viruses, but it was considered uncertain for the RNA viruses. Pioneer studies by P. D. Cooper and colleagues with poliovirus (PV) and by A.M. King and colleagues with foot-and-mouth disease virus (FMDV) provided the first evidence of recombination in RNA. The present perception is that recombination is more widespread than thought only a few decades ago, but that its frequency and the types of genomic forms it generates vary among viruses. For example, as a general rule, it appears that positive strand RNA viruses recombine more easily than negative strand RNA viruses to give rise to mosaic genomes of standard length. Several negative strand RNA viruses, however, can yield defective genomes through recombination, frequently characterized by deletions in their RNA. Genome segment reassortment, the

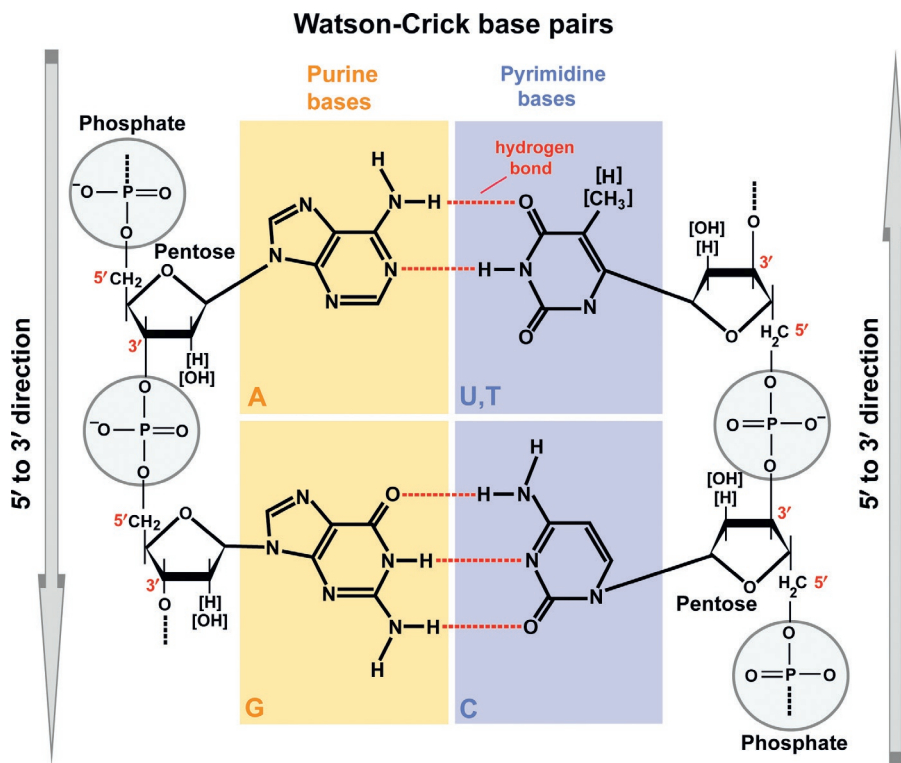
variation event in viruses closest to chromosomal exchanges in sexual reproduction, is obviously privative of segmented viral genomes. The three modes of virus genome variation are not incompatible, and reassortant-recombinant-mutant genomes have been described. The potential for genetic variation of RNA and DNA viral genomes is remarkable, and it is the ultimate molecular mechanism that lies at the origin of the virus diversity delineated in Chapter 1.

2.2 MOLECULAR BASIS OF MUTATION

Mutation is a localized alteration of a nucleotide residue in a nucleic acid. It generally refers to an inheritable modification of the genetic material. In the case of viral genomes, mutations can result from different mechanisms: (i) template miscopying (direct incorporation of an incorrect nucleotide); (ii) primer-template misalignments that include miscoding followed by realignment, and misalignment of the template relative to the growing chain (polymerase “slippage” or “stuttering”); (iii) activity of cellular enzymes (i.e., deaminases), or (iv) chemical damage to the viral nucleic acids (deamination, depurination, depyrimidination, reactions with oxygen radicals, direct and indirect effects of ionizing radiation, photochemical reactions, etc.) (Naegeli, 1997; Bloomfield et al., 2000; Friedberg et al., 2006).

The basis of nucleotide misincorporation during template copying lies mainly in the electronic structure of the bases that make up DNA or RNA. Each base in DNA (adenine, A; guanine, G; cytosine, C; thymine, T) and RNA (with uracil, U instead of T) includes potential hydrogen-bonding donor sites (amino or amino protons) and hydrogen-bonding acceptor sites (carbonyl oxygens or aromatic nitrogens) that contribute to standard Watson-Crick base pairs (Figure 2.1) as well as wobble base pairs (nonstandard Watson-Crick, but fundamental for RNA secondary structure and mRNA translation) (Figure 2.2). The conformation of the purine and pyrimidine bases is highly dynamic. Amino and methyl groups rotate about the bonds that link them to the ring structure. In dilute solution hydrogen bonds are established with water, and they can be displaced by nucleotide or amino acid residues to give rise to nucleotide-nucleotide or nucleotide-amino acid interactions. The strength difference between hydrogen bonds established in a polynucleotide chain with water, and their strength between two bases in separate polynucleotide chains determines whether a double-stranded polynucleotide will be stably formed.

Purine and pyrimidine bases can acquire different charge distributions and ionization states. As a consequence, in addition to the standard Watson-Crick and wobble, other base pairs are found in naturally occurring nucleic acids (notably cellular rRNA and tRNA) and in synthetic oligonucleotides (A-U or A-T Hoogsteen, and A-G, C-U, G-G, and U-U pairs, as well as interactions involving ionized bases). One of the types of electronic redistribution leads to tautomeric changes, such as the keto-enol and amino-imino transitions, which modify the hydrogen-bonding properties of the base; tautomeric imino and enol forms of the standard bases can produce non-Watson-Crick pairs. The proportion of the alternative tautomeric forms can be influenced by modifications in the purine and pyrimidine rings, which in turn can favor either the *syn* or *anti* conformation of a nucleoside, which is defined by the torsion angle of the bond between the 1' carbon of the ribose and either N1 in pyrimidines or N9 in purines (Figure 2.1). The *anti* conformation is usually the more stable in standard nucleotides and polynucleotides. The transition from the *anti* to the *syn* conformation may alter the hydrogen-bonding properties of the base thereby inducing mutagenesis (Bloomfield et al., 2000; Suzuki et al., 2006). The understanding of conformational effects on the base-pairing tendencies of nucleoside analogs in the

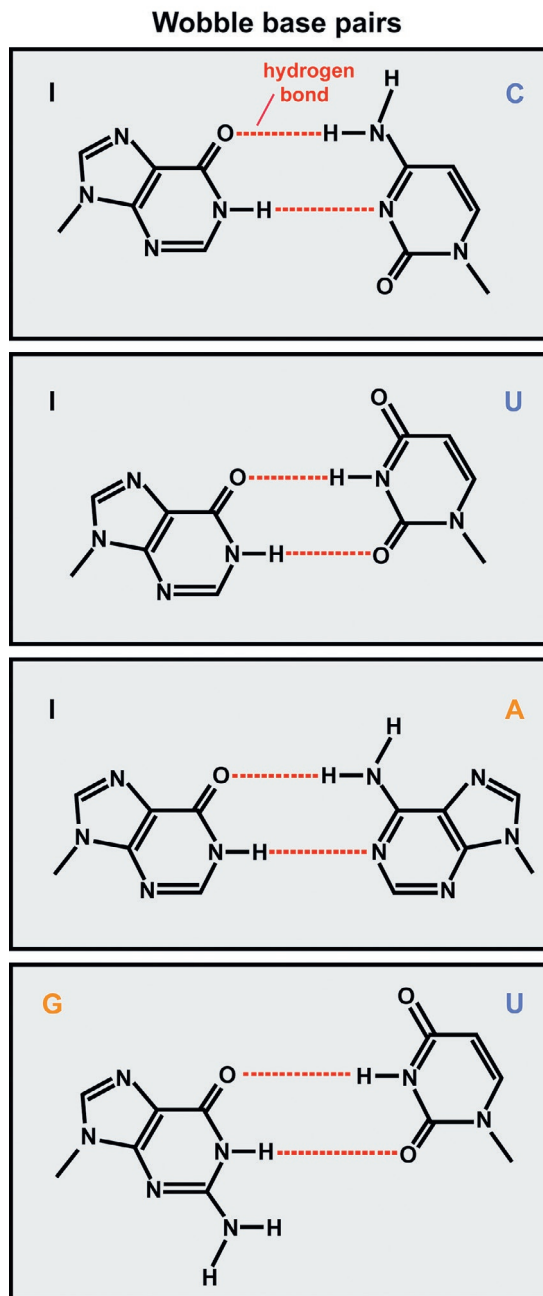
**FIGURE 2.1**

The standard Watson-Crick base pairs in DNA (A-T and G-C, with deoxyribose as pentose) and RNA (A-U and G-C, with ribose as pentose). Phosphodiester bonds of two potential polynucleotide chains of different polarity (outer arrows) are indicated.

context of the active site of a polymerase is very relevant to the design of specific mutagenic analogs for viral polymerases (Chapter 9).

Base-base interactions are not only responsible for part of the mutations that occur during genome replication, but also for the formation of double-stranded nucleic acids, either within the same polynucleotide chain or between two different chains. Transitions from a coil-like into an organized double-stranded (or other) structure are functionally relevant for both RNA and DNA. In the case of RNA, double-stranded regions in the adequate alternacy with single-stranded regions, determine key catalytic or macromolecule-attracting activities, as for example, the ribozyme activities mentioned in Chapter 1, the internal ribosome entry site (IRES) of several viral and cellular mRNAs, or multitudes of functional RNA-protein interactions currently being unveiled.

Stacking interactions due to electronic interactions (rather than hydrophobic bonds, as once thought) between adjacent bases in the same polynucleotide chain contribute to the stability and conformation of single-stranded nucleic acids, and also partially to duplex stability. Structural transitions due to alternative stacking conformations, particularly within polypurine or polypyrimidine tracts, can affect nucleic acid-protein interactions. In turn, replication machineries (typically including viral and host proteins

**FIGURE 2.2**

Examples of a class of non-Watson-Crick base pairs termed wobble base pairs. The drawing is similar to that of Figure 2.1 except that the sugar residues and phosphodiester bonds have been omitted. Hydrogen bonds (discontinuous lines in red) are shown between I (inosine) and C, U and A, and between G and U. Wobble base pairs are important for codon-anticodon interactions, as described in the text.

gathered in membrane structures) would also be expected to be affected by nucleic acid conformations; such effects are important in virology regarding consequences for mutant generation in a given template sequence context. These considerations on structural transitions are relevant to the nonneutral character of silent (also termed synonymous) mutations to be addressed in the next section. Transitions from a single-stranded into a double-stranded nucleic acid structure and the relative stability of the two forms depend on multiple factors that include the nucleotide sequence of the nucleic acid, its being a ribo- or a deoxyribo-polynucleotide, temperature and ionic composition, and ionic strength. Positively charged counterions neutralize negatively charged phosphates, and favor duplex stability [as an overview of physical and chemical properties of nucleic acids and their nucleotide components, see (Bloomfield et al., 2000)].

2.3 TYPES AND EFFECTS OF MUTATIONS

Mutations resulting from any of the mechanism just summarized can be divided in transitions, transversions (both referred to as point mutations), and insertions and deletions (referred to as *indels*) (Figure 2.3). The latter occur preferentially at homopolymeric tracts and also at short, repeated sequences which are prone to misalignment mutagenesis (Figure 2.4). An example is an editing mechanism for some viral mRNAs such as the phosphoprotein mRNA of the *Paramyxovirinae* [(Kolakofsky et al., 2005) and references therein]. Other examples *in vivo* are hot spots for variation in reiterated sequences in complex DNA genomes (Yamaguchi et al., 1998; Barrett and McFadden, 2008; McGeoch et al., 2008), or the insertion of two amino acids (often Ser-Ser, Ser-Gly, or Ser-Ala between residues

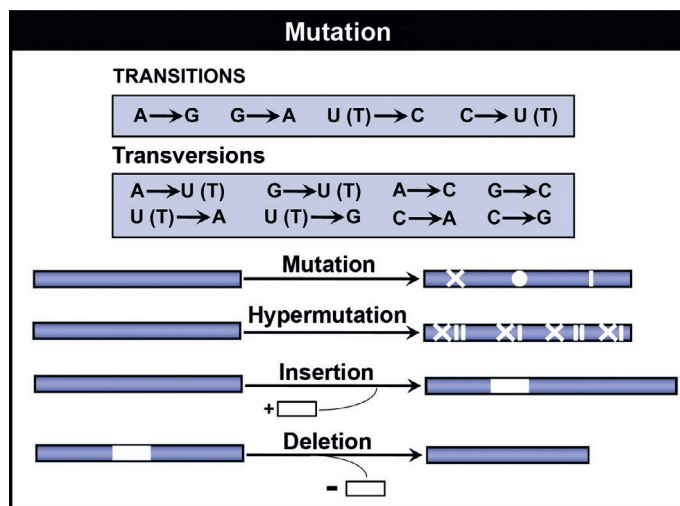


FIGURE 2.3

Major types of mutations in RNA (U) and DNA (T): four transitions and eight transversions. Below, a means to indicate point mutations, insertion or deletions (known as *indels*). A genome is depicted as an elongated rod. Symbols on the rod (cross, circle and line) represent mutations. Hypermutation is generally associated with high frequency of specific mutation types (crosses and lines). A region inserted or deleted from the genome is depicted as an empty rod.

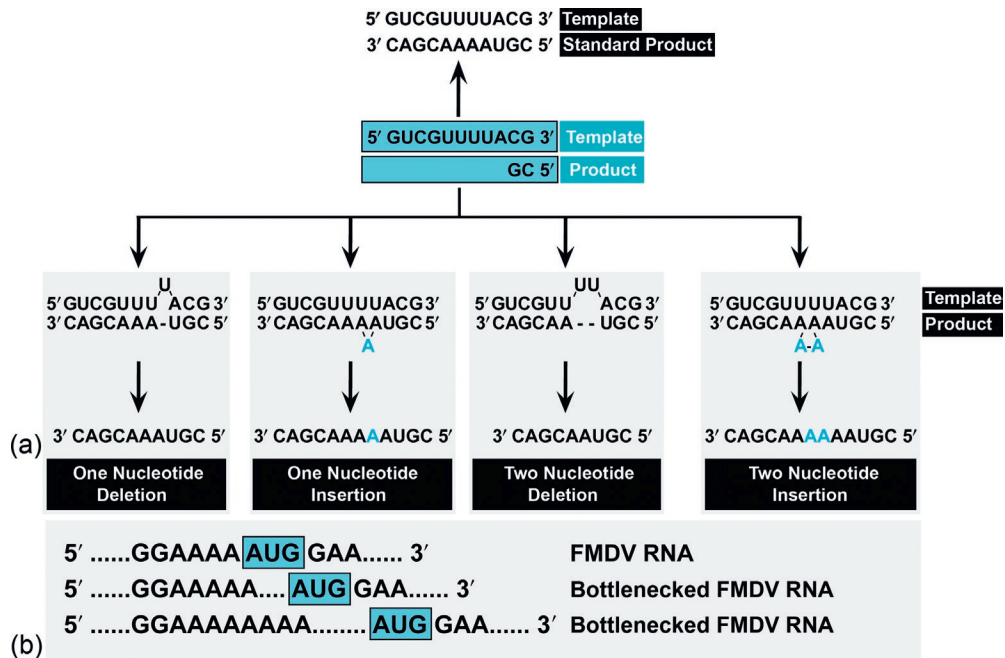


FIGURE 2.4

Misalignment mutagenesis. (a) Production of one or two nucleotide deletion or insertion at a homopolymeric tract in an RNA template. The basis of these events is a displacement of template or product residues during RNA synthesis. (b) A specific example of an internal oligoadenylated extension in the genome of FMDV subjected to plaque-to-plaque transfers. See text for biological implications and references.

69 and 70 of the HIV-1 reverse transcriptase), in concert with HIV-1 resistance to nucleoside inhibitors (Winters and Merigan, 2005) (Chapter 8). The molecular mechanisms involved in the generation of point mutations and *indels* are subject to thermodynamic and quantum-mechanical uncertainty inherent to atomic fluctuations, rendering mutagenesis a highly unpredictable event (Domingo et al., 1995; Eigen, 2013). This fact is relevant to virus evolution because it introduces an element of stochasticity (randomness) in a key motor of evolution: the generation of diversity at the molecular level.

Transition mutations occur more frequently than either transversions or *indels* during virus replication. Nucleotide discrimination at the catalytic site of viral polymerases fits this observation because of the more likely replacement of a purine or pyrimidine nucleotide by its structurally more similar nucleotide. In some cases, however, abundance of *indels* and similar numbers of transitions and transversions have been recorded (Cheynier et al., 2001; Malpica et al., 2002). The molecular bases of such unexpected behavior regarding mutational spectra are not well understood.

The effect of mutations on the structure and function of proteins is extremely relevant to penetrate into the mechanisms that drive virus evolution. Silent or synonymous mutations are those that do not give rise to an amino acid substitution despite being located in an open reading frame (protein-coding region) of a genome. The absence of amino acid substitution is due to the degeneracy of the genetic code: the same amino acid can be coded for by two or more triplets (codons), with the exception of AUG

for methionine and UGG for tryptophan. Synonymous mutations are not necessarily selectively neutral, neutral meaning that they have no discernible consequence for any viral function. The assumption that synonymous mutations are selectively neutral, and the fact that the early comparison of nucleotide sequences of homologous genes showed a dominance of synonymous over nonsynonymous mutations, contributed to the foundations of the neutral theory of molecular evolution. This theory attributed the evolution of organisms at the molecular level mainly to the random drift of genomes carrying neutral or quasi (or nearly) neutral mutations (King and Jukes, 1969; Kimura, 1983, 1989). The terms quasi neutral or nearly neutral may seem ambiguous to molecular biologists. However, in the formulation of the neutral theory they had a precise meaning of the selection coefficient being lower than the inverse of the effective population size, with minor variations in the equations of some formulations (Kimura, 1983).

Despite random drift of genomes playing an important role in molecular evolution, evidence gathered over the last decades renders untenable the assumption that synonymous mutations are neutral. Evidence to the contrary has been obtained with viruses and cells, including mutations in the human genome (Novella, 2004; Novella et al., 2004; Parmley et al., 2006; Hamano et al., 2007; Resch et al., 2007; Lafforgue et al., 2011; Nevot et al., 2011). There are several mechanisms by which synonymous mutations can affect virus behavior. They may alter *cis*-acting regulatory elements in viral genomes, decrease the stability of duplex structures stabilized between viral sequences and miRNAs or siRNAs, and promote alterations of viral gene expression or escape from RNA interference (Lafforgue et al., 2011; Nevot et al., 2011). Silent mutation-dependent modification of secondary or higher order structures of genomic RNA or messenger RNAs may have several effects including splicing precision or translation fidelity through the modification of RNA-RNA or RNA-protein interactions. Synonymous codons use different tRNAs for protein synthesis, and different tRNAs do not have the same relative abundance in different host cells. Thus, the rate of protein synthesis, an extremely important phenotypic trait for cells and viruses, can be affected by the frequency of alternative synonymous codons present in a mRNA (Richmond, 1970; Akashi, 2001). Not only does codon bias affect the level of gene expression, but rare codon distribution may regulate the folding of nascent proteins during translation (Makhoul and Trifonov, 2002; Rocha, 2004; Aragonés et al., 2010). As a consequence, generation of rare codons by mutation of abundant codons (or vice versa) can modify viral replicative capacity (or fitness, Chapter 5). Rare codons may also limit the fidelity of amino acid incorporation when the frequency of the required aminoacyl-tRNAs is low (Ling et al., 2009; Zaher and Green, 2009; Czech et al., 2010). The frequency of codon pairs in RNA genomes is also a fitness determinant currently under investigation to prepare attenuated viral vaccines. In Chapter 4, additional evolutionary events related to virus-host interactions that might have contributed to codon bias are discussed.

To complicate matters further, a synonymous mutation may be neutral or quasi neutral in one environment, but it may contribute to selection in a different environment, because of the phenotypic effects of RNA structure and codon usage.

Regarding the effects of mutations in viruses (Box 2.1), the following general statements are applicable to both DNA and RNA viruses:

- Truly neutral mutations (i.e., with no influence on the behavior of a virus in any environment) are probably very rare. This applies to synonymous as well as to nonsynonymous mutations.
- Of the nonsynonymous mutations, those leading to chemically conservative amino acid substitutions are more likely to be tolerated than those leading to chemically different amino acids. Tolerance must be distinguished from neutrality. A tolerated mutation may cause a reduction of fitness which is nevertheless compatible with virus replication.

BOX 2.1 THE EFFECTS OF MUTATIONS ON VIRUSES**In Noncoding Regions**

Mutations may affect stem-loop or other secondary and higher order structures involved in regulatory processes through nucleic acid-nucleic acid or nucleic acid-protein interactions. The primary sequence in nonstructured, noncoding regions may be also functionally relevant.

In Coding Regions

- Synonymous or silent mutations do not affect the amino acid sequence of the encoded protein.
- Nonsynonymous mutations give rise to an amino acid substitutions in the encoded protein.
- Some mutations may generate a stop codon, leading to a truncated protein.

Regarding Functional Effects

- Neutral mutations are those that have no functional effects.
- Nonneutral mutations can have a broad range of fitness effects: from nearly complete tolerability to lethality.
- Most proteins are multifunctional. A nonsynonymous mutation can affect one but not other functions performed by the same protein.

Context Dependence

The effect of a mutation may be context dependent in two manners: it may be affected by other mutations in the same genome (epistasis) or by other genomes of the surrounding mutant spectrum.

- A conservative amino acid substitution may have important biological effects.
- The effect of any individual mutation is context dependent in two ways: it may depend on other mutations in the same genome (epistasis, see also [Section 2.8](#) and Chapter 5) or on the mutant cloud that surrounds the genome harboring the mutation (complementing or interfering activity).
- The previous points do not deny the influence of random drift of genomes on intrahost- and interhost evolution. The currently most accepted view is that selection and drift occur continuously during virus evolution (Chapter 3).

2.4 INFERENCES ON EVOLUTION DRAWN FROM MUTATION TYPES

The proportion of transition versus transversion mutations may depend initially of the specific replication machinery of a virus that may tend to produce some mutation types preferentially over others. For a given virus, short-term evolution is often reflected in a dominance of transitions which is less apparent when distantly related sequences of the same virus are compared. The effect of evolutionary distance on the transition to transversion ratio was observed in the FMDV sequence comparison carried out in our laboratory over several decades, that ranged from analyses of mutant spectra relative to their corresponding consensus sequence to the comparison of independent viral isolates from a disease outbreak [reviews of the work on FMDV evolution in ([Domingo et al., 1990, 2003](#))]. These two levels of sequence analyses (quasispecies vs. independent isolates) can be further understood by comparing Chapters 3 and 7.

The proportion of synonymous and nonsynonymous mutations that have mediated the diversification of viral genomic sequences that belong to the same phylogenetic lineage is often considered informative of the underlying evolutionary forces. Probably because of the rooted (albeit uncertain)

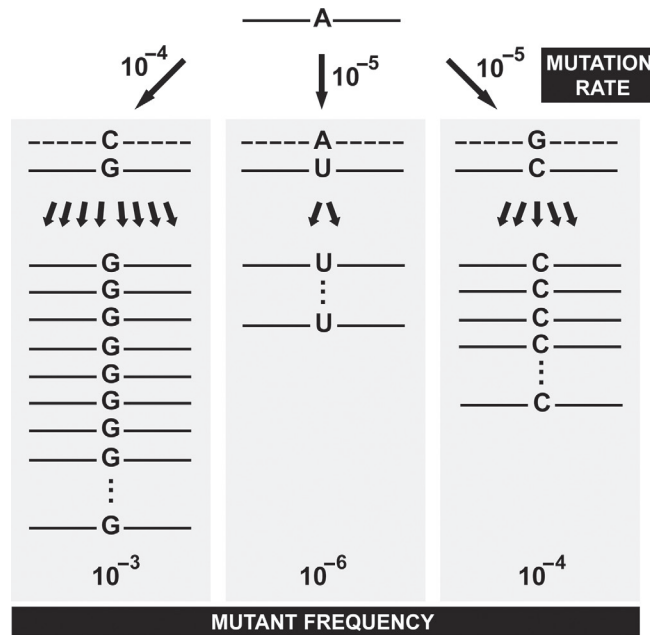
notion that biological function is more likely to reside in protein than in DNA or RNA, the ratio of nonsynonymous substitutions (corrected per nonsynonymous site in the sequence under study) (termed d_n), to the number of synonymous substitutions per synonymous site (termed d_s), ($\omega = d_n/d_s$) is calculated to infer the dominant mode of evolution (Nei and Gojobori, 1986). When $\omega = 1$ the evolution is considered neutral, when $\omega < 1$ purifying (or negative) selection is dominant, and when $\omega > 1$ positive (or directional) selection prevails (Yang and Bielawski, 2000). The types of selection undergone by viruses are discussed in Section 3.4 of Chapter 3.

There are several reasons to be cautious about the significance of ω : (i) synonymous mutations need not be neutral, for reasons discussed in the previous Section 2.3. (ii) In the course of evolution, important but transient events of positive selection (termed episodic positive selection) due to one or a few amino acid substitutions may be accompanied by a larger number of synonymous, tolerated mutations. In this situation, ω will indicate purifying selection despite a critical role of positive selection triggered by one or few nonsynonymous mutations in the evolutionary outcome which are insufficient to compute as $\omega > 1$ (Crandall et al., 1999). (iii) In a striking proof of the above arguments, statistically significant mutational biases led to a value of ω indicative of positive selection in an *in vitro* evolution experiment simulating pseudogene evolution in which positive selection was not possible (Vartanian et al., 2001). (iv) A synonymous change may permit a codon to acquire a relevant nonsynonymous change through a point mutation. The term *quasisynonymous* has been used to describe codons that encode the same amino acid, but that have a different evolutionary potential regarding the accessible amino acids in the encoded protein. Alternative codons for a given amino acid approximate a replicative system to points of sequence space from which a phenotypically relevant change has a different probability (Chapters 3 and 4). (v) Finally, it has to be considered that ω was initially proposed to compare distantly related rather than closely related genomes as is often the case in short-term evolution of viruses (Kryazhimskiy and Plotkin, 2008).

For all these reasons, ω values as a diagnostic test of forces mediating DNA and RNA virus evolution must be regarded only as indirect and suggestive, not as a definitive parameter. Despite these arguments, use of ω to propose a mode of virus evolution continues being surprisingly unchallenged in the literature of virus evolution. We use ω only in a limited way in subsequent chapters, because it does not help in the interpretation of critical evolutionary events regarding viruses. Related shortcomings apply to other tests of neutrality developed to interpret the origin of DNA polymorphisms in the years following the summit of the neutralist-selectionist controversy (Fu, 1997; Achaz, 2009).

2.5 MUTATION RATES AND FREQUENCIES FOR DNA AND RNA GENOMES

Mutation rates quantify the number of misincorporations per nucleotide copied, irrespective of the fate (increase or decrease in frequency) of the error copy produced. A mutation rate for a genomic site measures a biochemical event dictated by the replication machinery and environmental parameters. In contrast, a mutant or mutation frequency describes the proportion of a mutant or a set of mutants in a genome population. The frequency of a mutant will depend on the rate at which it is generated (given by the mutation rate) and on its replication capacity relative to other genomes in the population (Drake and Holland, 1999) (Figure 2.5). A specific mutation may be produced at a modest rate, but then be found at high frequency because the mutation is advantageous for replication in that environment. The converse situation may also occur. Some mutational hot spots (in the sense of genomic sites where mutations tend to occur with high probability) may never be reflected among the repertoire of

**FIGURE 2.5**

Scheme that illustrates the difference between mutation rate and mutant frequency. Residue A in a template residue (top) can be misread to incorporate a C, A, or G into the complementary strand (discontinuous lines), at a rate of 10^{-4} , 10^{-5} , and 10^{-5} substitutions per nucleotide, respectively. The replicative capacity of the newly generated templates (with G, U and C, continuous lines) will determine widely different mutant frequencies with $G > C > U$.

mutations found in a genome population because of the selective disadvantage they inflict upon the genome harboring them.

A very significant example is the elongation of an internal oligoadenylate tract located between the two functional AUG initiation codons in the FMDV genome. The homopolymeric tract constitutes a hot spot for variation due to polymerase slippage (Figure 2.4b). The elongation of the internal oligoadenylate was dramatic, but it was only observed when FMDV was subjected to repeated plaque-to-plaque (bottleneck) transfers, not to large population passages. In fact, this drastic genetic modification has not been recorded among natural isolates of the virus. The molecular instruction to elongate the oligoadenylate was very strong because it was observed in many independent biological clones subjected to bottleneck transfers (Escarmís et al., 1996). Despite qualifying as a hot spot for variation, the first event in fitness recovery when the clones were subjected to large population passages was the reversion of the elongated tract to its original size (Escarmís et al., 1999). The interpretation of these findings, to be further analyzed in Chapter 6, is that during plaque-to-plaque transfers the negative selection to eliminate unfit genomes is less intense than during large, highly competitive population passages. Again, a clear molecular instruction to elongate a homopolymeric track may not be reflected in a high frequency of the affected genomes. Therefore, although mutation rates and frequencies for viruses bear some relationship, rates cannot be inferred from frequencies and vice versa (Figure 2.5).

The first calculations of mutation rates for cellular organisms and for some DNA bacteriophages were carried out by J.W. Drake, who pursued comparative measurements that have generally supported a difference between mutation rates for DNA and RNA viruses. The rates estimated for bacteriophages λ and T4 were about 100 times higher than those of their host *E. coli*. An approximately constant rate of 0.003 mutations per genome per replication round was calculated for a number DNA-based microbes (Drake, 1991), an observation sometimes referred to as “Drake’s rule.” This rather surprising constancy suggests that different DNA organisms have accommodated the template-copying fidelity of their replication machineries to achieve a narrow window in the mutational load measured as mutations fixed per genome, a remarkable fitting of biochemistry with evolutionary needs. The basal mutation rate in mammalian cells has been estimated at about 10^{-10} substitutions per nucleotide and cell generation [reviewed in (Naegeli, 1997; Domingo et al., 2001; Friedberg et al., 2006)] (Table 2.1).

The synonymous mutation rate measured with experimental populations of bacteria has been assumed to reflect the neutral mutation rate (despite limitations explained in Section 2.3). Values for *E. coli* have ranged from 2×10^{-11} up to 5×10^{-9} substitutions per synonymous site per generation (Ochman et al., 1999) with 5×10^{-10} as the most likely estimate (Lenski et al., 2003). The latter value is in agreement with a rate of 3×10^{-10} to 4×10^{-10} substitutions per base pair and generation based on whole genome pyrosequencing of an experimentally evolved lineage of *Myxococcus xanthus* (Velicer et al., 2006). Mutation rates in cells and viruses depend on the replicative machinery (generally a multiprotein complex that includes the relevant viral polymerase with additional viral and host proteins and membrane structures) and on multiple environmental parameters. Whether bacteria are in the exponential or stationary phase of growth can affect intracellular metabolites and proton exchange rates which, in turn, may alter the proportion of tautomeric forms in nucleotides and misincorporation tendencies (Friedberg et al., 2006). The sequence context of the template nucleic acids (presence of repeated sequences that can induce misalignment mutagenesis or G-C vs. A-T rich regions in relation to relative nucleotide substrate abundances, etc.) may impel or attenuate mutability. Insertion elements may enhance mutation rates at neighboring sites in a bacterial genome (Miller and Day, 2004). Despite these influences, vesicular stomatitis virus (VSV) displayed comparable mutation rates in several host cells (Combe and Sanjuan, 2014) suggesting that there is a limited range of average error rates needed for a virus to maintain fitness (Chapters 5 and 9).

In addition to the general environmental and sequence context consequences for template-copying fidelity that may affect any genome type, mutation rates for DNA viruses will be influenced also by: (i) whether the DNA polymerase that catalyzes viral DNA synthesis includes or lacks a functional proofreading-repair activity. High copying fidelity is typical of DNA polymerases involved in cellular

Table 2.1 Mutation Rates and Frequencies for RNA and DNA Genomes

Viriods	2×10^{-3}
RNA viruses	10^{-5} to 10^{-3}
Retroviruses	10^{-6} to 10^{-4}
DNA viruses	10^{-8} to 10^{-3}
Cellular DNA	10^{-9} to 10^{-11}

Values are expressed as substitutions per nucleotide. The range of values is the most likely according to several independent studies. No distinction is made between mutation rates and frequencies. See text for comments and references.

DNA replication, and low copying fidelity is generally a feature of DNA polymerases involved in DNA repair (Friedberg et al., 2006). Thus, repair of lesions that by themselves might not be mutagenic may lead to the introduction of mutations during the error-prone repair process. (ii) Expression of proteins active in repair encoded in the viral genome, such as uracil-DNA glycosylase, DNA repair endonucleases, etc. (iii) The mechanism of viral DNA replication, particularly the occurrence of double-stranded versus single-stranded DNA in replicative intermediates. (iv) The intracellular site of replication and the availability of postreplicative DNA repair proteins (regarding both intracellular location and concentration) to the viral replication factories. Little is known of the spatial relationships and relative affinities of cellular and viral proteins and structures that may critically affect polymerase fidelity. Comparative measurements of mutation rates at specific genome sites of DNA viruses are needed, as a first step to define the cellular and biochemical influences on the fidelity of DNA virus genome replication.

General genetic variability affecting the entire virus genome should be distinguished from localized variability at hot spots in a genome. Even the extremely complex human genome shows genetic instability at specific loci, some associated with genetic disease (Domingo et al., 2001; Alberts et al., 2002; Bushman, 2002). Genome size is a parameter pertinent to biological behavior, not only because it imposes a commensurate copying fidelity, but also because it affects the impact of genetic heterogeneity within infected organisms, and upon invasion of new hosts (Chapter 3).

Mutation frequencies measured by subjecting virus to a specific selective agent (e.g., mutants that escape the neutralizing activity of a monoclonal antibody or mutants that escape inhibition by a drug) span a broad range of values (10^{-3} to 10^{-8}) for DNA and RNA viruses (Smith and Inglis, 1987; Sarisky et al., 2000; Domingo et al., 2001) (Table 2.1). The technical details of any procedure used to calculate a mutation frequency should be carefully evaluated to translate its meaning to the genome level. Important variables are the efficacy of the antibody or drug (which will be concentration dependent) or the possibility of phenotypic hiding-mixing in the escape mutants to be quantified (Holland et al., 1989; Valcarcel and Ortin, 1989). Unexpected low levels of escape mutants (that would imply $<10^{-6}$ substitutions per nucleotide) for an RNA virus can mean either a general or site-specific high polymerase fidelity, a selective disadvantage of the genome that harbors the mutation or, when a phenotypic alteration is measured, the requirement of two or more mutations to produce the alteration. Conversely, a high mutation frequency for a DNA virus whose replication is catalyzed by a high fidelity DNA polymerase may mean that either repair activities were not functional or that the mutant displayed a selective advantage and overgrew the wild type prior to the measurement of its frequency. Mathematical treatments that take into account reversion of a low fitness mutant and its competition with wild-type virus have been used to calculate mutation rates (Batschelet et al., 1976; Coffin, 1990).

Despite difficulties and limitations in the calculations, independent genetic and biochemical methods with different viruses support mutation rates for RNA viruses in the range of 10^{-3} to 10^{-5} substitutions per nucleotide copied [as representative articles and reviews see (Batschelet et al., 1976; Domingo et al., 1978, 2001; Steinhauer and Holland, 1986; Eigen and Biebricher, 1988; Varela-Echavarria et al., 1992; Ward and Flanagan, 1992; Mansky and Temin, 1995; Preston and Dougherty, 1996; Drake and Holland, 1999; Sanjuan et al., 2010)] (Table 2.1). Historically, a few early studies indicated unusual low mutation rates or frequencies for some RNA viruses. As discussed in some of the reviews listed above, there are technical reasons that suggest that such values were probably underestimates of the true average mutations rates or frequencies. Obviously, it cannot be excluded that some genomic sites or viruses under a given environment are unusually refractory to mutations, but most evidence supports the range of values listed in Table 2.1. The near million-fold higher mutation rates for RNA viruses than

cellular DNA, whose biological implications were presciently anticipated by J. Holland and colleagues (Holland et al., 1982), have been confirmed. That is, for RNA viruses of genome length between 3Kb and 32 Kb, an average of 0.1-1 mutations are introduced per template molecule copied in the replicating population. Unless most mutations impeded viral replication, a continuous input of mutant genomes is expected, as indeed found experimentally (Chapter 3).

High mutation rates for RNA genomes are also supported by measurements of template-copying fidelity by RNA polymerases, reverse transcriptases, and DNA polymerases devoid of 3'-5' proofreading exonuclease (or under conditions in which such exonuclease is not functional) [(Steinhauer et al., 1992; Varela-Echavarría et al., 1992; Mansky and Temin, 1995; Domingo et al., 2001; Friedberg et al., 2002, 2006; Menéndez-Arias, 2002), and references therein]. *In vitro* fidelity tests may be based on genetic or biochemical assays using homopolymeric or heteropolymeric template-primers. Measurements include the kinetics of incorporation of an incorrect versus the correct nucleotide directed by a specific position of a template or the capacity of a polymerase to elongate a mismatched template-primer 3' end, [these and other assays have been reviewed (Menéndez-Arias, 2002)] (see also Section 2.6). Differences between related enzymes (i.e., AMV RT is more accurate than HIV-1 RT), and the fact that amino acid substitutions in the polymerases affect nucleotide discrimination, demonstrate that proofreading-repair activities together with the structure of the polymerase and replication complexes are determinants of template-copying fidelity.

2.6 EVOLUTIONARY ORIGINS, EVolvABILITY, AND CONSEQUENCES OF HIGH MUTATION RATES: FIDELITY MUTANTS

The amino acid substitutions in the core polymerase that affect fidelity can be located either close to or away from the active site of the enzyme. The change in fidelity can reach almost one order of magnitude, but virus viability is not compromised. Thus, error rates themselves can be subjected to selection, as supported by theoretical studies on evolvability (Earl and Deem, 2004). It is not clear whether mutation rates of viruses have evolved to procure a balance between adaptability and genetic stability, or whether other selective constraints have imposed the observed values. It has been suggested that because of the generally deleterious nature of most mutations, the adaptive value of the high mutation rates for RNA viruses is debatable, and that there might have been a trade-off between replication rate and copying fidelity (Elena and Sanjuan, 2005). Mutation rates would be a consequence of rapid RNA replication, and an increase in copying fidelity would come at a cost, resulting in a lower replication rate. A connection between elongation and error rate has been suggested by results with some viral and cellular polymerases [review (Kunkel and Erie, 2005)]. In an early study with the poliovirus RdRp *in vitro*, an increase in the error frequency was observed when the pH-Mg²⁺ ion conditions were modified, and the decreased fidelity correlated with increased RNA elongation rate (Ward et al., 1988). A possible connection between elongation rate and copying fidelity cannot be ruled out, but current evidence points to template-copying fidelity as being the result of multiple factors, not necessarily linked to the rate of genome replication (Vignuzzi and Andino, 2010; Campagnola et al. 2015).

Many lines of evidence support an adaptive value of high mutation rates for RNA viruses *per se*, independently of their biochemical origins. A poliovirus mutant, whose RdRp displayed a three- to five-fold higher fidelity than the wild-type enzyme, replicated at a slightly lower rate than wild-type virus in cell culture, but displayed a strong selective disadvantage regarding invasion of the brain of susceptible

mice (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). The impediment to cause neuropathology was due to the limited complexity of the mutant spectrum since its broadening through mutagenesis restored the capacity to produce neuropathology. These and other studies have provided evidence that mutant spectrum complexity, by virtue of its impact on fitness, can be a virulence determinant. The work by M. Vignuzzi, J. Pfeiffer, R. Andino, C. Cameron, K. Kirkegaard, and their colleagues on poliovirus fidelity mutants opened a much needed branch of research in virus evolution and quasispecies implications. As a proof of this statement, the field of fidelity mutants is rapidly expanding, and references to the information they provide will be made in several chapters.

Theoretical models and experimental observations suggest that mechanisms for error correction had to evolve to maintain functionality of increasingly complex genomes (Swetina and Schuster, 1982; Eigen and Biebricher, 1988; Domingo et al., 2001; Eigen, 2002, 2013) (here complexity means genome size, provided no redundant information is encoded). The coronaviruses have the largest genomes among the known RNA viruses, with 30-33 kb. This is about 10-fold more genetic information than encoded in the simple RNA bacteriophages such as MS2 or Q β . Coronaviruses are replicated by complex RNA-dependent RNA polymerases which include a domain that corresponds to a 3'-5' exonuclease, proofreading-repair activity. The protein displays exonuclease activity *in vitro*, and its inactivation affects viral RNA synthesis (Minskaia et al., 2006), and results in increases of about 15-fold in the average mutation frequency (Eckerle et al., 2007, 2010). A coronavirus mutant devoid of this repair function is more susceptible to lethal mutagenesis (Smith et al., 2013; Smith and Denison, 2013), as expected from a connection between replication accuracy and proximity to an error threshold for the maintenance of genetic information (Chapter 9). Thus, it is likely that a proofreading activity evolved (or was captured from a cellular counterpart) in RNA genomes whose genomic complexity was in the limit compatible with the fidelity achievable by standard RNA replicases. It would be interesting to discover new RNA viruses with a single RNA molecule longer than 30 Kb as genome to analyze whether they have evolved more accurate core polymerases or exhibit a proofreading-repair function during replication. Toward the other end of the RNA size scale, viroid RNAs display a mutation rate higher than (or close to the highest) recorded for RNA viruses, consistent with the correlation between genome size and template-copying accuracy (Gago et al., 2009).

Studies with bacteria have identified some of the factors that successively increase copying fidelity. It has been estimated that during *E. coli* DNA replication the error rate would be 10^{-1} to 10^{-2} mutations per nucleotide copied if accuracy relied only upon the strength of interactions provided by base pairing (Section 2.2). The error rate would decrease to 10^{-5} to 10^{-6} with base selection and proofreading-repair, to about 10^{-7} with the contribution of additional proteins present in the replication complex, and to about 10^{-10} misincorporations per nucleotide with the participation of postreplicative mismatch correction mechanisms (Naegeli, 1997; Kunkel and Erie, 2005; Friedberg et al., 2006). The error rate of the bacteriophage ϕ 29 DNA polymerase is about 10^{-6} without the proofreading exonuclease activity, and it decreases to 10^{-8} with the correcting activity [(de Vega et al., 2010) and references therein]. Postreplicative repair pathways act on double-stranded DNA, but not (or very inefficiently) on RNA or DNA-RNA hybrids. Therefore, the known postreplicative repair systems that operate in cellular DNA do not make a significant contribution to error correction in RNA viruses.

The importance of copying fidelity for complex genomes is reflected in the fact that more than 100 proteins are directly or indirectly involved in repair of the human genome. Elevated mutation rates in the range of those operating for RNA viruses would be lethal for mammalian genomes. Localized genetic modification occur physiologically in processes such as somatic hypermutation

and class-switch recombination in B cells of the germinal centers, as mechanisms of diversification of immunoglobulin genes (Upton et al., 2011). Chromosomal instability has long been associated with cancer (Gatenby and Frieden, 2004; Stratton et al., 2009). Surveys have been (and are currently being) used to identify genes associated with chromosomal instability and their role in aging and disease (Aguilera and Garcia-Muse, 2013) (see also Chapter 10). While uncontrolled high mutability is deleterious for differentiated cellular organisms, it constitutes a *modus vivendi* for a great majority of viruses.

Despite its attractiveness, definitive proof of the hypothesis of a direct relationship between error rate and limited genome complexity will require additional functional and biochemical studies. Exceptions to the absence of repair activities in simple genetic elements have been described. A satellite RNA of the plant virus turnip crinkle carmovirus evolved a 3'-end RNA repair mechanism. It implicates the synthesis of short oligoribonucleotides by the viral replicase using the 3'-end of the viral genome as template. The mechanism consists probably of template-independent priming at the 3'-end of the damaged RNA to generate wild type, negative strand, and satellite RNA (Nagy and Simon, 1997). A reversible, NTP-dependent excision of the 3' residue of the nascent nucleic acid product has been described in some retroviruses and hepatitis C virus (Meyer et al., 1998; Jin et al., 2013). This activity is important for drug resistance and it may also modulate the overall fidelity of some polymerases. It cannot be excluded that some type of point mutation correction may operate in RNA genetic elements of less than 30 Kb. Such putative mechanisms may even diminish mutation rates that would otherwise be prohibitively deleterious, and they do not overshadow high mutation rates as a feature of RNA and some DNA genomes (Table 2.1).

Limited copying fidelity in the absence of proofreading-correction mechanisms can be regarded as an unavoidable consequence of the molecular mechanisms involved in template copying by viral polymerases. Most nucleic acid polymerases share a structure that resembles a right hand, with fingers, palm, and thumb domains (Figure 2.6a). Three-dimensional structures of viral RdRps and RTs indicate that interactions between the incoming nucleotide or residues of the template-primer with amino acids of the polymerase must permit displacement of the growing polymerase chain along the channel located at the palm domain of the polymerase (Steitz, 1999; Ferrer-Orta et al., 2006) (Figure 2.6b). If interactions around the catalytic site to ensure the correct nucleotide incorporation were so strong as to preclude misincorporations, the movement of the growing polynucleotide chain would be hampered. Again, there is a match between biochemical and evolutionary needs.

The orientation of the triphosphate moiety of the incoming nucleotide substrate is important for nucleotide incorporation (Menéndez-Arias, 2002; Graci and Cameron, 2004; Ferrer-Orta et al., 2009). One of the several steps involved in nucleotide incorporation is the formation of a ternary complex (polymerase with template-primer and the incoming nucleotide) that undergoes a conformational change (reorientation of the divalent ion-complexed triphosphate moiety of the incoming nucleotide). This conformational change activates the complex for phosphoryl transfer, to link the nucleoside-monophosphate to the 3'-terminus of the primer (or growing chain). Steps involved in the nucleotide incorporation are represented in Figure 2.6c. Both the conformational change and the relative rate of phosphoryl transfer for an incorrect nucleotide versus the correct nucleotide influence the error rate at each site of the growing chain. Critical kinetic constants in Figure 2.6c that are determined experimentally to quantify relative nucleotide incorporations and misincorporations are $K_{d,app}$ (expressed as μM), k_{pol} (expressed as s^{-1}), and the ratio $k_{pol}/K_{d,app}$ ($\mu\text{M}^{-1}\text{s}^{-1}$) termed the catalytic efficiency. The ratio of $k_{pol}/K_{d,app}$ for the incorporation of an incorrect nucleotide to $k_{pol}/K_{d,app}$ for a correct nucleotide gives

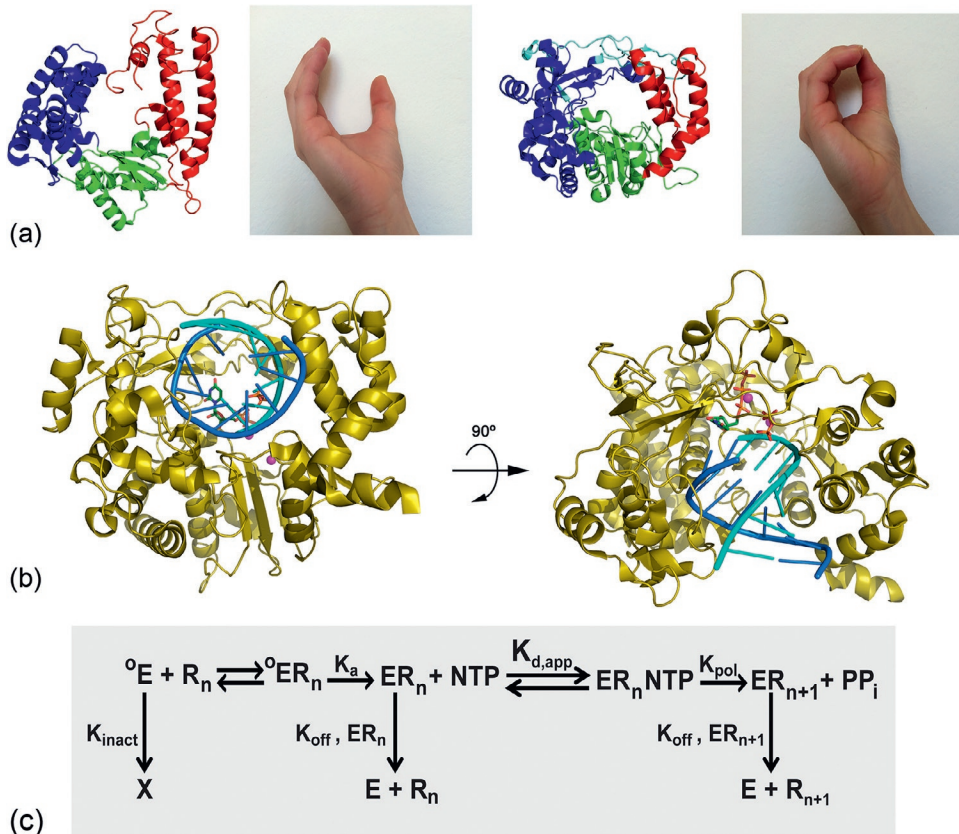


FIGURE 2.6

Polymerization by viral polymerases. (a) The structure of nucleic acid polymerases resembles that of a right hand. This was first evidenced with the structure of the Klenow fragment of the *E. coli* DNA polymerase I by T.A. Steitz and colleagues in 1985. On the left the Klenow fragment is represented with colored fingers, palm and thumb domains, next to an open right hand. The structure on the right is that of PV RdRp, next to a closed right hand. Courtesy of N. Verdaguer and L. Vives-Adrián (the hand is that of L. Vives-Adrián). (b) The structure of ternary complex between FMDV 3D, an RNA molecule and UTP as the substrate (PDB id. 2E9Z). The left panel is a front view of the complex, depicting the polymerase chain as a yellow ribbon, the RNA in dark blue (template) and cyan (primer). The incoming UTP and the pyrophosphate product are shown in atom type, and two Mg^{2+} ions as magenta balls. The right panel is the same complex in a top-down orientation. (Figure courtesy of C. Ferrer-Orta and N. Verdaguer). (c) Scheme of minimum number of steps involved in nucleotide incorporation. The first step consists of the binding of polymerase ${}^{\circ}E$ to the template-primer R_n (elongated up to nucleotide n) to form a complex ${}^{\circ}ER_n$. Formation of the activated complex ER_n is governed by the rate constant $k_{assembly}$ (k_a). The activated ER_n complex binds a nucleotide NTP with an apparent binding affinity given by $K_{d,app}$ to form the $ER_n NTP$ complex. Catalysis to incorporate covalently the NTP to the growing primer chain to yield ER_{n+1} and pyrophosphate (PP_i) is governed by the rate constant k_{pol} . Other constants depicted in the scheme are the inactivation rate constant (k_{inact}) of ${}^{\circ}E$, and dissociation of E from RNA (k_{off, ER_n} and $k_{off, ER_{n+1}}$). Based on (Arias et al., 2008), and previous studies with PV polymerase 3D by C. E. Cameron and his colleagues.

the frequency of that particular misincorporation, and an assessment of polymerase fidelity [(Castro et al., 2005) and references therein]. Modifications of polymerase residues by site-directed mutagenesis, combined with comparisons of the relevant structures, have identified critical amino acid residues involved in template-copying fidelity.

Viruses displaying higher than average template-copying fidelity have been frequently obtained by selecting viruses resistant to mutagenic nucleotide analogs such as the antiviral agent ribavirin (Beaucourt and Vignuzzi, 2014). Limited incorporation of a deleterious nucleotide can be attained either through a specific discrimination against the analog (ribavirin or other) or through a general decrease of all types of misincorporations, that is, a high fidelity phenotype. Structural modifications of viral polymerases that lead to high fidelity have inspired the design and testing of additional substitutions. In this manner, mutant viral polymerases showing either an increase or decrease of copying fidelity achieved through a single amino acid substitution have been characterized (Wainberg et al., 1996; Menéndez-Arias, 2002; Mansky et al., 2003; Pfeiffer and Kirkegaard, 2003; Arnold et al., 2005; Domingo, 2005; Vignuzzi et al., 2006; Coffey et al., 2011; Gnädig et al., 2012; Meng and Kwang, 2014; Rozen-Gagnon et al., 2014; Borderia et al., 2016). These results imply that the capacity of virus to evolve at higher or lower rates than their ancestors is easily achievable through modest numbers of mutations (limited movements in sequence space, Chapter 3), again emphasizing the evolvability of mutation rates.

2.7 HYPERMUTAGENESIS AND ITS APPLICATION TO GENERATE VARIATION: APOBEC AND ADAR ACTIVITIES

Some viral genomes either isolated from biological samples or evolved in cell culture show biased mutation types (e.g., monotonous G → A or C → U substitutions in the same genome), generally at frequencies of around 10⁻² substitutions per nucleotide (10- to 1000-fold higher than standard mutation rates and frequencies) (Table 2.1). Biased hypermutation was first observed in some defective-interfering (DI) RNAs of vesicular stomatitis virus (VSV) (Holland et al., 1982), and in variant forms of measles virus, associated with postmeasles neurological disease such as subacute sclerosing pan-encephalitis (Cattaneo and Billetter, 1992). Hypermutation is mainly due to the activity of cellular deaminases such as, the apolipoprotein B mRNA and the editing complex (APOBEC), or the adenosine deaminase acting on double-stranded RNA (ADAR) families, that are involved in cellular editing and regulatory functions (Sheehy et al., 2002; Santiago and Greene, 2008; Nishikura, 2010; Stavrou et al., 2014). In the event of a viral infection, such cellular functions can become part of an innate defense mechanism against the invading virus. Viral proteins (i.e., Vif in HIV-1) bind some APOBEC proteins thus inhibiting mutagenesis and permitting virus survival (Sheehy et al., 2002). In oncoretroviruses, retroviruses, and hepatitis B virus (HBV), the APOBEC-3 cytidine deaminase acts on single-stranded DNA and results mainly in G → A and C → U hypermutation, that may affect 40% to 100% of the G residues. The preferred sequence context for G hypermutation in HIV-1 observed *in vivo* is GpA > GpG > GpT ≈ GpC. The specific dinucleotide context of the hypermutated sites provides a means to distinguish genomes that have undergone hypermutation by cellular activities from those that are heavily mutated by other mechanism such as the action of mutagenic agents (Chapter 9).

The ADAR-associated hypermutation was identified in negative strand RNA riboviruses, and results mainly in A → G and U → C hypermutation. It is originated by A → I (inosine) modification in double-stranded viral RNA catalyzed by ADAR-1 L, one of more than one hundred proteins inducible

by type I IFN (Maas et al., 2003). Inosine can be recognized as G by the replication machinery (Valente and Nishikura, 2005), although it can form wobble base pairs also with A and U (Figure 2.2).

There are additional mechanisms of hypermutagenesis. Higher than average mutation frequencies can occur as a result of replication in the presence of biased concentrations of the standard nucleotide substrates; this has been applied to the *in vitro* generation of genes mutated at frequencies of 10^{-1} to 10^{-2} (mutagenic PCR), as a powerful tool to study sequence-function relationships and functional robustness of nucleic acids and proteins (Meyerhans and Vartanian, 1999). Error-prone PCR has been used in experiments of *in vitro* evolution of nucleic acid enzymes to generate heterogeneous collections of nucleic acid sequences to select for molecules capable of catalyzing specific reactions (Joyce, 2004) (Chapter 1).

2.8 ERROR-PRONE REPLICATION AND MAINTENANCE OF GENETIC INFORMATION: INSTABILITY OF LABORATORY VIRAL CONSTRUCTS

High mutation rates have practical implications in laboratory studies on the behavior of virus mutants obtained by molecular cloning of a biological sample, or constructed by site-directed mutagenesis. A transition mutation that causes a strong fitness decrease but that still allows residual RNA genome replication, will most likely revert following infection or transfection of cells with the mutant construct. Double or triple mutants (preferentially including transversions) should be engineered (when possible according to the genetic code) to study the behavior of a viral mutant with an amino acid replacement of interest that may produce a fitness decrease. As an example, a C → U transition found in an open-reading frame of an RNA virus may convert a Pro into a Ser (CCG → UCG). Since Ser will revert to Pro through a U → C transition in the triplet (a common type of misincorporation by most polymerases), Ser should be engineered to be encoded by AGU; in the course of replication, reversion to Pro would require at least two transversions since the codons for Pro are CCU, CCC, CCA, or CCG. Thus, if effects derived from the difference in primary sequence of the RNA or codon bias do not intervene in the behavior of the viral genome, codons with a high genetic barrier to reversion should be engineered for studies involving viral replication.

In general, deletions revert at a much lower frequency than point mutations, and, when appropriate for the question under study, a deletion should be introduced within the gene of interest to probe gene function in reverse genetic studies. High mutation rates also imply that infection or transfection with debilitated mutant viruses may result in progeny with sequences that differ from the input. V. I. Agol and colleagues have coined the term *quasi-infectious* to refer to mutant viruses that are capable of yielding progeny, but the progeny differs from the initial genome (pseudorevertants) (Gmyl et al., 1993). The difference between the input mutant and the rescued progeny virus will depend on the type of genetic lesion in the input virus and its consequences for replication. A single point mutation that decreases replication is likely to lead to a true revertant (return to the original sequence). If the same reversion depends on two or more mutations, a true revertant will be generated only after extended replication to achieve the required exploration of sequence space (Chapter 3). In this case, selection of compensatory mutations elsewhere in the genome (sometimes referred to as second site revertants) is likely. The term compensatory is often used for mutations that compensate the deleterious effects of other mutations. A typical example is a mutation that decreases the stability of a stem in an RNA stem-loop that functions as a *cis*-acting element. Frequently, a compensatory mutation occurs that restores

a stable stem. Thus, an engineered virus may produce progeny which differs from the parent. There is an uncertainty regarding the representativeness of a virus mutant or construct when the objective is to infer the behavior of genetic lesions in the natural context. If a substantial loss of replicative capacity is produced by a drastic genetic change (an *indel*, loss of a stem-loop structure, etc.) selection of a true revertant becomes extremely unlikely. The compensatory generation of alternative structures (or constellations of point mutations) that restore replication (partially or completely) becomes an interesting and informative possibility.

Procedures to copy an entire viral RNA genome into a cDNA for reverse genetics studies are now available (Fan and Di Bisceglie, 2010). If for technical reasons an infectious cDNA clone is constructed from several molecules which were copied from different genomes present in the mutant spectrum, the ligation product may be transcribed into an RNA which is not infectious. This is because while some constellations of mutations may be compatible with infectivity, others may not, or may allow limited, suboptimal replication, thus favoring the selection of additional mutations or reversions. The effect of different mutations in the same genome is often referred to as epistasis. Mutations that reinforce each other with regard to a viral function are said to produce positive epistasis and those that interfere with each other produce negative epistasis. Thus, a mutation may have an effect on its own, and an effect that depends on accompanying mutations in the same genome (also mentioned regarding the context-dependent effect of mutations in Section 2.3). Epistasis in RNA viruses may be blurred by the considerable weight of mutant spectra in determining viral behavior through intergenome interactions (Chapter 3).

An interesting contrast that recapitulates concepts given in Sections 2.5 and 2.6 is the effect of an active proofreading-repair activity in maintaining the infectivity of a viral genome upon its extended replication *in vitro* (in a test tube, in the absence of cellular extracts). The 19,285 bp bacteriophage ϕ 29 DNA can be amplified at least 4000-fold without detectable loss of infectivity due to the fidelity of ϕ 29 DNA polymerase conferred by a 3'-5' proofreading-repair exonuclease activity (Bernad et al., 1989). Engineered ϕ 29 DNA polymerases provide a powerful amplification tool in genomics (de Vega et al., 2010). In contrast, the 4,220 nucleotides long Q β RNA rapidly loses its infectivity when replicated by Q β replicase *in vitro* due to accumulation of mutations and deletions in the viral RNA (Mills et al., 1967; Sabo et al., 1977). The error-prone Q β replicase is not adequate to amplify infectious viral RNA, but it was at the origin of the quasispecies concept to be discussed in Chapter 3.

2.9 RECOMBINATION IN DNA AND RNA VIRUSES

Recombination is the formation of a new genome by covalent linkage of genetic material from two or more different parental genomes (Figure 2.7). Recombination can also involve different sites of the same genome to yield insertions or deletions, such as in the formation of defective interfering (DI) genomes. It is a widespread mechanism of genetic variation in all biological systems, and in cells it underlies critical physiological and developmental processes (splicing, generation of diversity in immunoglobulin genes and T cell receptors, transposition events, phase variation in bacteria, repair pathways that promote postreplicative error correction, etc.). Cellular DNA recombination relates to replication, repair, and completion of DNA replication, operations that involve multiple proteins displaying a variety of activities (Smith and Jones, 1999; Alberts et al., 2002; Nimonkar and Boehmer, 2003; Friedberg et al., 2006).

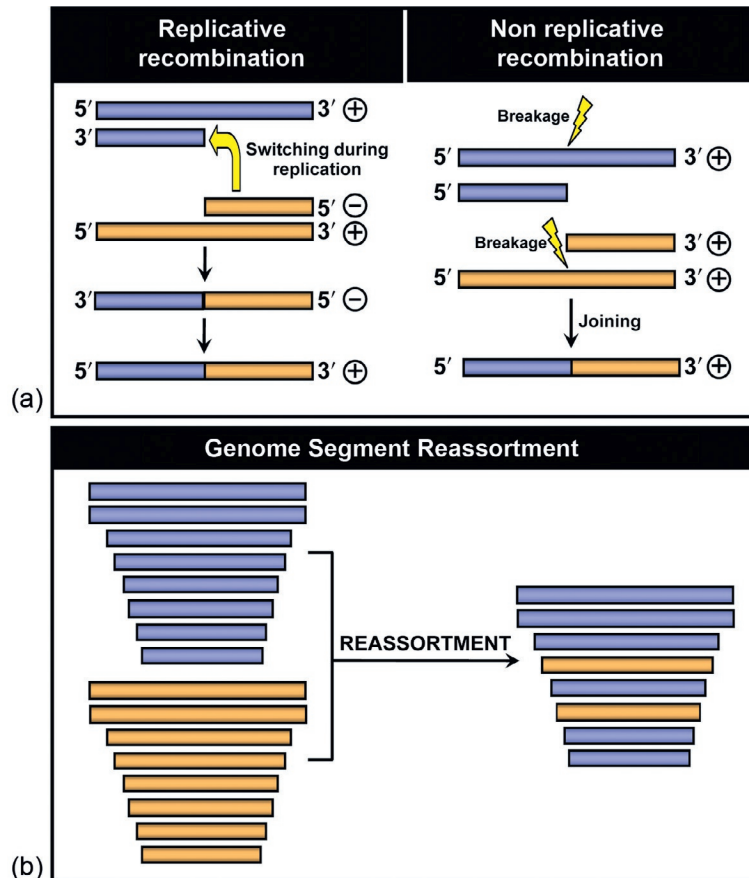


FIGURE 2.7

RNA recombination and segment reassortment. (a) Scheme of replicative and nonreplicative RNA recombination. RNA polarity is indicated by +, – symbols. Replicative recombination is displayed as the result of template switching during minus strand RNA synthesis. Nonreplicative recombination is depicted as the outcome of breakage and ligation (joining) of fragments of plus strand RNA. (b) An example of genome segment reassortment in which a new segment constellation is produced in which six genomic segments originate from one parent (blue) and two from the other (gold). Influenza virus is the best known example (see text).

Recombination occurs both with DNA and RNA viruses, often with participation of the virus replication machinery. Several types of recombination have been distinguished in viruses: homologous versus nonhomologous recombination, according to the extent of nucleotide sequence identity around the recombination (crossover) site, and replicative versus nonreplicative recombination, according to the requirement of viral genome replication for recombination to occur (Kirkegaard and Baltimore, 1986; King, 1988; Lai, 1992; Nagy and Simon, 1997; Plyusnin et al., 2002; Boehmer and Nimonkar, 2003; Gmyl et al., 2003; Chetverin et al., 2005; Agol, 2010; Simmonds, 2010; Bujarski, 2013).

As in the case of cells, homologous recombination in double-stranded DNA viruses is intimately connected with DNA replication and repair. It implicates multiple viral gene products (DNA polymerase, single-stranded DNA-binding proteins, processivity factors, helicase-primase, eukaryotic topoisomerase I, etc.), and a succession of protein-catalyzed steps. In the copy choice (or template switching) mechanism, the nascent DNA switches from one template molecule to another, resulting in synthesis of recombinant, daughter DNAs. In its basic form, recombination by breakage and rejoining starts with the introduction of a nick at one of the strands of each parental DNA, strand invasion of one parental DNA by the other, branch migration, ligation at the nicks (linking DNA strands from the two parents), and further isomerization and cleavage reactions. DNA recombination is responsible for the endonuclease-mediated isomerization of some herpesvirus genomes [four isomers defined by the orientation of the long (L) and short (S) regions of the viral genome]. During the late phase of herpes simplex virus-1 replication, the frequency of recombination has been estimated at 0.6% per Kb of genome (Boehmer and Nimonkar, 2003). Integration or excision of proviral DNA or temperate bacteriophage DNA, are examples of site-specific recombination that involves specific enzyme activities (i.e., retroviral integrases), and requires a short stretch of nucleotide sequence identity.

The copy choice mechanism of homologous RNA recombination is also associated with genome replication. An RNA polymerase molecule with its nascent RNA product jumps into the corresponding position of another template molecule, to complete synthesis of the RNA product (Figure 2.7). Given the large numbers of viral genomes often present in replication complexes in each infected cell, it is not surprising that this mechanism may give rise to frequent recombinant progeny genomes. Formation of mosaic genomes has long been recognized as an essential feature of the genetics of some retroviruses and plant RNA viruses. For HIV-1 and some plant RNA viruses recombination frequencies have been estimated at 2% to 10% of progeny per 100 nucleotides; for picornaviruses and coronaviruses the number of recombinants amount to 10% to 20% of the progeny (King, 1988; Lai, 1992; Nagy and Simon, 1997; Levy et al., 2004; Urbanowicz et al., 2005; Sztuba-Solinska et al., 2011). A phylogenetic approach estimated an average recombination rate of HIV-1 *in vivo* of 1.4×10^{-4} recombination events/site/generation, which is about fivefold greater than the average point mutation rate (Shriner et al., 2004). Recombination may be inefficient or absent in some negative strand RNA viruses. However, some of them undergo homologous recombination (Plyusnin et al., 2002), and certainly high frequency recombination in the generation of DI RNAs (Roux et al., 1991).

Recombination frequency may be altered by environmental factors that affect viral replication. A decrease of intracellular nucleotide levels as a result of treatment of cells with hydroxyurea may favor template switching reflected in an increase of intra- and intermolecular recombination (Pfeiffer et al., 1999; Svarovskaia et al., 2000).

Nonreplicative recombination does not require replication of the viral genome, and has been described upon cotransfection of cells with viral RNA fragments that could not replicate by themselves (Gmyl et al., 2003; Gallei et al., 2004; Agol, 2010). It appears to be a promiscuous event with a required 3'-phosphate in the 5' partner RNA and a 5'-hydroxyl residue in the 3' partner RNA. The cellular activities involved in nonreplicative RNA recombination have not been characterized.

2.9.1 MOLECULAR OCCURRENCE VERSUS OBSERVED RECOMBINATION

The emerging picture is that the frequency of recombination varies among viruses, and that as new tools for genome analyses have become available, recombination has been detected in an increasing number of viruses. Recognition of recombination in a viral system is facilitated when a cell culture

system is available. Controlled infection of cells with genetically marked parental viruses has been essential to estimate recombination frequencies, and to distinguish true recombination from mutation-reversion events that may mimic the formation of recombinants. As with the concept of high genetic variation in RNA viruses, recombination has often gone from being considered marginal to prominent and relevant; HCV is a typical example (Galli and Bukh, 2014).

Viral replicative machineries may be endowed with features that influence the occurrence of recombination. One such feature is processivity of the viral polymerase (capacity of continued copying of the same template molecule). Genome detachment of the polymerase complex from one genome to bind either to a different genome or to a distant site of the same genome is part of the standard replicative cycle of viruses such as retroviruses and coronaviruses. Reverse transcriptase participates in strand transfer during DNA synthesis, and coronavirus polymerase switches from one template site to another during discontinuous RNA synthesis. It may be significant that they belong to viral families displaying high recombination frequencies (Makino et al., 1986). Thus, here again we encounter a “molecular instruction” that evolved as an essential feature of viral genome replication and that can be exploited to generate variation, and permit new genomic forms to undergo the scrutiny of selection (Chapter 3).

The occurrence of recombination is diagnosed by discordant positions of different genes or genomic regions in phylogenetic trees, as a result of the transfer of part of a viral genome from one lineage to another (Chapter 7). A commonly used procedure measures similarity values between sequences using a sliding-window scanning method. The recombination crossover point (where the two parental sequences meet) is identified by the point (or region) where the similarity plot crosses from one sequence into another (Salemi and Vandamme, 2004; Martin et al., 2005; Kosakovsky Pond et al., 2006). Crossover points along a viral genome are not distributed at random, either because polymerase detachment from the template is sequence dependent or because many of the recombination events do not lead to viable progeny. Absence of recombinant viability introduces a parallel with the distinction we made in Section 2.5 between mutation rate and frequency; that is, a distinction between what does occur and what is observed. Many newly arising recombinants may be subjected to negative selection, and only a viable subset of all recombinants produced might be detectable in progeny virus (King, 1988; Lai, 1992). Thus, as in the case of mutations, many recombinant genomes are not detected because of their elimination by negative selection. An elegant study by D.J. Evans and colleagues documented “imprecise” enterovirus recombinant intermediates that were lost upon serial virus passage (Lowry et al., 2014). Recombination is viewed as a biphasic process consisting of initial imprecise events followed by a stage of resolution in favor of fit recombinants.

The distinction between generation and resolution events that applies both to mutants and recombinants has yet another implication for RNA virus genetics. Some mutants or recombinants that in isolation do not display sufficient replicative fitness to acquire dominance in a population may nevertheless persist as minority genomes. They may display low-level replication or be maintained by complementation by partner genomes (as in the case of two FMDV genome segments that are described in Section 2.11). As minority genomes, they may engage in modulatory activities such as those described in Chapter 3. When technically feasible, application of next generation sequencing (NGS) to characterize recombination intermediates should provide important information.

Homologous RNA recombination can be influenced also by amino acid substitutions in the polymerase, the primary sequence in the RNA (i.e., high frequency of template switching in AU-rich regions), the sequence identity between the nascent strand and acceptor template, and secondary structures at or around the crossover sites, among other influences [(Nagy and Simon, 1997; Alejska et al., 2005; Agol, 2010) and references therein]. At a cellular level, since recombination necessitates coinfections

of the same cell by at least two parental genomes, persistence of a viral genome in a cell increases the likelihood of sequential coinfections, unless some reinfection or superinfection exclusion mechanism operates [(Webster et al., 2013) and references therein]. Without such restrictions, persistently infected cells may be an environment with higher probability of recombination than transiently infected cells, assuming comparable genome loads at the sites of replication.

2.10 GENOME SEGMENT REASSORTMENT

In viruses whose genomes are composed of two or more RNA or DNA segments, genome segment reassortment consists in the formation of new constellations of viral genomic segments from two or more parental genomes (Figure 2.7). Reassortment can produce new phenotypic traits. It is the main mechanism of antigenic shift of influenza A viruses—often associated with new influenza pandemics (Webster et al., 1992; Morse, 1994; Gibbs et al., 1995; Domingo et al., 2001)—as opposed to antigenic drift which is mediated by amino acid substitutions in the surface proteins hemagglutinin and neuraminidase. Reassortments occur among the 9-12 double-stranded RNA segments of the widespread *Reoviridae* family (Tanaka et al., 2012). Fitness differences among all possible segment combinations (2^n , for two types of coinfecting particles with n genome segments) determine the types of genome segment groupings that dominate subsequent rounds of infection. In the laboratory, analysis of reassortant viruses has been applied to map a viral function into one segment or a combination of segments.

Genomic segments can be encapsidated either into a single virus particle (as in Orthomyxoviruses or Arenaviruses) or into separate particles (as in multipartite plant viruses). Multipartite virus can have either RNA or DNA as genetic material. The plant Nanoviruses have 6-8 molecules of single-stranded circular DNA of about 900 to 1000 nucleotides, and each segment encodes a single protein. In the case of the nanovirus Faba bean necrotic stunt virus, its 8 segments vary in frequency in a host-dependent manner (Sicard et al., 2013). This observation led the authors to propose a “setpoint genome formula” which may reflect a control of segment (gene) copy number that may provide some still unrecognized benefit to the multipartite phenotype (see next section). In principle, replication of multipartite viruses requires that each cell be coinfecting by at least one of each type or particle harboring a different genome type, which in fact represents a remarkable cost for replicative efficiency. The fact that unsegmented and segmented RNA viruses are well represented in our biosphere suggests that neither of the two organizations confers a definitive advantage for long-term survival.

2.11 TRANSITION TOWARD VIRAL GENOME SEGMENTATION: IMPLICATIONS FOR GENERAL EVOLUTION

The origin of viral genome segmentation is a debated issue, although there is general agreement that it may confer adaptive flexibility to viruses. Most proposals have been based on theoretical studies. Segmentation has been viewed as a form of sex that facilitates genomic exchanges, to counteract the effect of deleterious mutations (Chao, 1988; Szathmari, 1992). An alternative, not mutually exclusive model, is that genome segmentation may have an advantage because replication of shorter RNA

molecules is completed earlier than the unsegmented counterpart (Nee, 1987). Yet another possibility is that the life style of a virus (in particular, the particle yield in connection with the number of surrounding susceptible cells), established over long evolutionary periods, may favor segmentation over intactness of a genetic message or vice versa.

An experimental system of genome segmentation has become available with the picornavirus foot-and-mouth disease virus (FMDV). Its single-stranded RNA genome underwent a modification akin to genome segmentation when the standard virus was subjected to more than 200 passages in BHK-21 cells at high multiplicity of infection. The experiment was originally intended to investigate the limits of fitness gain during virus optimization in a defined environment, in this case BHK-21 cells in culture. The FMDV used was a biological clone obtained in BHK-21 cells; the virus had not adapted to the BHK-21 cell environment since it derived from a diseased swine during a disease outbreak. Upon extensive replication in BHK-21 cells, the virus evolved toward a bipartite genome (García-Arriaza et al., 2004) thus adding reassortment to mutation and recombination as potential mechanisms of genetic variation of this laboratory-adapted picornavirus.

Each of the two pieces of RNA that composed the bipartite (or segmented) genome version contained in-frame deletions affecting *trans*-acting proteins (Figure 2.8). Each segment in isolation could not infect cells productively, but, when present together, they were infectious by complementation, and killed cells in the absence of standard FMDV. Low multiplicity of infection rapidly selected the full length genome as a result of recombination of the two parental, defective segments (García-Arriaza et al., 2004). The particles containing the shortened RNA were thermally more stable than the standard particles (Ojosnegros et al., 2011), but this difference did not explain the initial trigger of the segmentation event. The solution to this question came with the demonstration that the transition toward genome segmentation was possible because of an extensive exploration of sequence space by the standard virus. Indeed, the mutations accumulated during serial passages enhanced fitness of the segmented genome version to a much higher extent than fitness of the standard genome, thus conferring a selective advantage to the segmented form over its unsegmented (monopartite) counterpart (Moreno et al., 2014) (Figure 2.8). Thus, in this segmentation event, gradual evolution (drift in sequence space) was a requirement for the major transition toward segmentation. This experimental result suggests that in evolution there is no unsurmountable barrier that allows the conversion between intact and split forms of the same genome, reflecting a remarkable genome flexibility that will be emphasized in Chapter 7 in the context of the relevance of virus variation in the emergence of viral pathogens.

2.12 MUTATION, RECOMBINATION, AND REASSORTMENT AS INDIVIDUAL AND COMBINED EVOLUTIONARY FORCES

Mutation, recombination, and segment reassortment contribute to the evolution of most DNA and RNA viruses. Sometimes one form of genetic change appears to be more prominent than another, and sometimes the concerted action of recombination or reassortment with mutation is apparent [i.e., antigenic drift in influenza virus, following the origin of a new antigenic type through reassortment (Ghedini et al., 2005)]. Mutation is a universal form of genetic change. It underlies numerous adaptive responses and critical biological transitions in viruses, and it is a prerequisite for recombination and reassortment to have a biological impact. If mutations were not present in different template molecules during replication, recombinants with the crossover point at equivalent positions

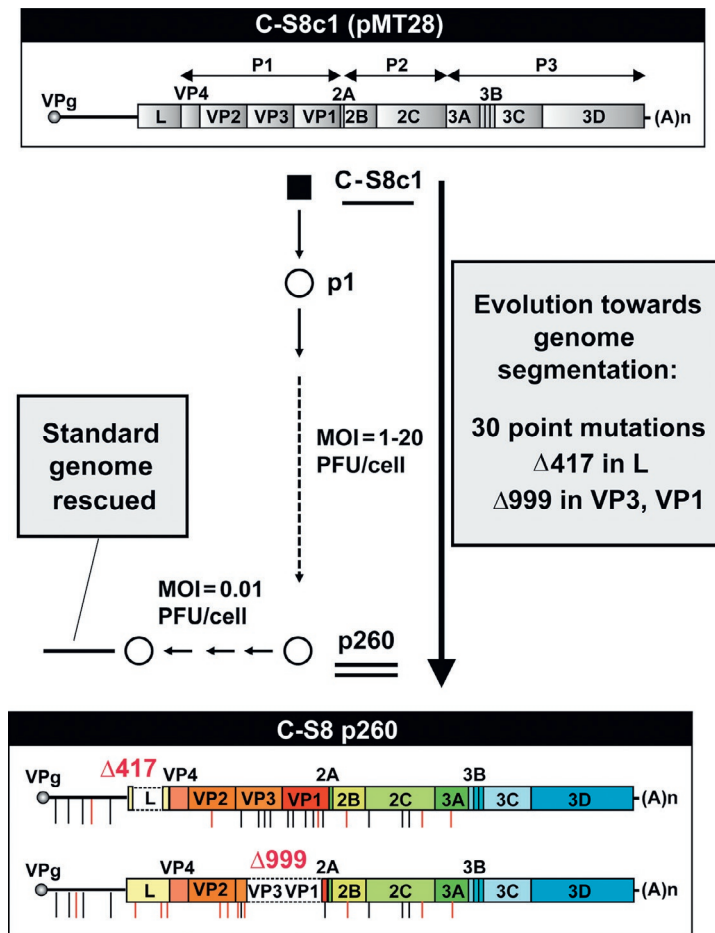


FIGURE 2.8

Evolution toward RNA genome segmentation in the laboratory. The monopartite, standard FMDV genome (clone C-S8c1 or pMT28, top) was subjected to 260 passages in BHK-21 cells. The resulting population p260 lacked detectable standard genome that could be rescued by low MOI passages. The evolved C-S8p260 accumulated 30 point mutations (depicted as vertical lines on the genome at the bottom) and consisted in two segments that were infections by complementation: $\Delta 417$, that lacked most of the L protease-coding region, and $\Delta 999$ that lacked most of the capsid proteins VP3, VP1-coding region. See text for further details and references.

of the parental genomes would be “silent,” and display the same behavior as the parental genomes. Apparently “silent” recombination events may take place within replicative units; even if some mutations distinguished individual genomes of the same quasispecies swarm, a recombinant would not be distinguished from a mutant genome. The frequency of recombination in HIV-1 was noticed only when the acquired immune deficiency syndrome (AIDS) pandemic had advanced and the virus had diversified through accumulation of mutations. Similar arguments apply to segment reassortment.

Genomes necessitate mutation-driven diversification for reassortment to provide a biological advantage (see also Chapter 10).

The evolutionary significance of recombination has been viewed in two opposite ways: as a means to rescue fit genomes from less fit parents (a conservative force that eliminates deleterious mutations), or as a means to explore new genomic forms for adaptive potential (a vast substrate for the exploration of sequence space; Chapter 3) [reviewed in (Zimmern, 1988; Lai, 1992; Worobey and Holmes, 1999; Simmonds, 2010)]. Recombination has been probably at the origin of new viruses that presently occupy a well-established niche, and it is also at play today to expand diversity during the spread of viruses. As a historical event, the coronavirus mouse hepatitis virus appears to have acquired its hemagglutinin-esterase gene by recombination with an influenza C virus. The alphavirus Western equine encephalitis virus originated probably by recombination between Sindbis-like and Eastern equine encephalitis-like viruses [reviewed in different chapters of (Domingo et al., 2008)].

Several recent poliomyelitis outbreaks have been associated with recombinants between oral poliovirus vaccine (OPV) viruses and other circulating enteroviruses (Gavrilin et al., 2000; Kew et al., 2002; Oberste et al., 2004). Intersubtype HIV-1 recombinants play a key role in current HIV-1 diversification, with an increasing number of circulating recombinant forms displaying complex mosaic structures (multiple crossover sites) (Thomson et al., 2002; Gerhardt et al., 2005). Positive selection of HIV-1 recombinants that unite different drug-resistant mutations in the same genome offers an example of the conservative force of recombination to rescue fit viruses in the face of a strong selective constraint (Menéndez-Arias, 2002). Recombination is expected to play an increasing role in the spread of drug resistance among viruses for which new antiviral agents are in use, such as HBV and HCV.

Some defective DNA and RNA genomes that include *indels*, notably DI RNAs, originate by recombination. DI RNAs can play an important role in the establishment and maintenance of persistent infections in cell culture, and can modulate viral infections *in vivo* (Holland and Villarreal, 1974; Roux et al., 1991). Detailed genetic and biochemical analyses by A. Huang, J.J. Holland and their colleagues on the generation of VSV DI's and their interplay with the standard, infectious VSV contributed to unveil a continuous dynamics of genetic variation, competition, and selection, observable within short time intervals, a hallmark of RNA genetics (Palma and Huang, 1974; Holland et al., 1982). Despite the continuous dynamics of escape of infectious virus to the interfering activities of DIs, some authors consider DIs as potential antiviral agents (Dimmock and Easton, 2014). DI RNAs can be regarded as the tip of the iceberg of many classes of defective genomes with a range of interfering or potentiating capacities that may coexist with standard animal, plant, insect, and bacterial viruses, and that may facilitate persistence and modulate disease symptoms (Holland et al., 1982; Vogt and Jackson, 1999; López-Ferber et al., 2003; Rosario et al., 2005; Sachs and Bull, 2005; Villarreal, 2005; Aaskov et al., 2006). Noncytopathic coxsackievirus B3 (CVB3) variants with deletions at the untranslated 5'-genomic region were isolated from hearts of mice inoculated with CVB3. The variants replicated *in vivo* and were associated with long-term viral persistence (Kim et al., 2005).

The presence of defective viruses may influence the behavior of viral populations by different means. If defective genomes are competent in RNA (or DNA) synthesis, or are complemented to replicate, they can act as dominant-negative swarms, provided they reach a sufficient load. In this manner, they may underlie suppressive effects of mutant spectra of viral quasispecies. Intramutant spectrum decrease of replicative capacity due to the presence of defective genomes is one of the mechanisms of virus extinction evoked by enhanced mutagenesis (Chapters 3 and 9).

Recombination events must have been the last step in ancestral processes of horizontal gene transfer that mediated the incorporation of host genes (or gene segments) into viral genomes, and vice versa. Host genes related to immune responses were probably captured by complex DNA viruses at early stages of their evolution (Alcami, 2003; McFadden, 2005). Mosaicism associated with nonhomologous recombination events is the norm among tailed bacteriophages (Canchaya et al., 2003). Nonhomologous recombination can give rise to genomic sequences with a viral and a nonviral moiety. They include DI RNAs of Sindbis virus containing cellular RNA sequences at their 5' ends, some cytopathic forms of bovine viral diarrhea virus, RNA of potato leaf roll virus containing tobacco chloroplast RNA, or an influenza virus with an insertion of ribosomal RNA into the hemagglutinin gene, mentioned in Chapter 5 regarding transient, high fitness levels [reviewed in (Domingo et al., 2001)].

Phylogenetic analyses have suggested that recombination between RNA and DNA viruses might have occurred to give rise to some present-day single-stranded DNA viruses (Stedman, 2013). However, the evidence for this attractive possibility is indirect and, to my knowledge, no experimental evidence of viral RNA-viral DNA recombination in cell culture or *in vivo* has been reported. Viability of mutant and recombinant viral genomes is severely constrained by the evolutionary history of the virus that has shaped viral genomes as coordinated sets of modules (Botstein, 1980, 1981; Zimmern, 1988; Koonin and Dolja, 2014). Experimental studies with engineered recombinant viruses have shown that modularity can restrict recombination (Martin et al., 2005).

It would be truly remarkable if a viral system could be proven to be totally devoid of one of the mechanisms of genetic variation to which it has access. It would imply that there are powerful molecular reasons to dispense with an effective adaptive mechanism. Absence of a mechanism is extremely difficult to demonstrate but, if we could, its basis would open a new chapter of molecular virology.

2.13 OVERVIEW AND CONCLUDING REMARKS

All forms of genetic variation of viruses must be viewed essentially as blind processes despite preferences of nucleotide sequences or structures for mutation and recombination events: *hot spots* with higher than average rate values, and *cold spots* with lower than average rate values. Mutation originates largely in fluctuations of electronic structure that modify base-pairing properties, and from features of polymerase-template interactions, not subjected to regulation. Recombination and reassortment are probably not regulated either. It is selection acting at the very center of replication and recombinant complexes that preserves some mutant and recombinant forms in detriment of others. Subsequent levels of selection occur when variant forms expand in multiple rounds of infection first within cells, then within an organism and then at the epidemiological level. The very nature of life in our planet has been built upon an inherent tendency to instruct variation in an incessant fashion, as necessary and unavoidable as the physical principles that dictate the behavior of our universe.

The net result of all mechanisms of genetic variation available to a virus is the generation of repertoires of variant genomes for random drift and selective forces to act upon. In other terms, genetic variation sets the scene for the actors of evolution to play their roles, and secure a continuous input of new forms despite subtle or catastrophic environmental perturbations. The same forces that drive general evolution have produced the dominant virus forms we see in nature, with all their nuances in the interaction with

cell components. The adaptation of viruses to participate in intracellular processes with cells dictates that genetic variation of viruses has its limits to prevent deleteriousness. This is currently exemplified by the effects of amino acid substitutions in viral polymerases that either increase or decrease template-copying fidelity. Viruses have reached a compromise between stability of a core information and flexibility for adaptability. Although not yet treated in this chapter, viral population numbers are a key parameter in the evolutionary events. Next chapters address some of these questions, not only in general conceptual terms but also in the way evolution affects our daily confrontation with viral disease (see Summary Box).

SUMMARY BOX

- Mutation, recombination and genome segment reassortment are the mechanisms of genetic variation used by DNA and RNA viruses. Mutations are due mainly to changes in the electronic distributions of the standard nucleotides, to damage of nucleotides by external influences, and by alignment alterations of template relative to product polynucleotide chains. The effect of a mutation can range from being well tolerated to highly detrimental or lethal.
- Mutation frequencies are only an indirect consequence of mutation rates. Their values for viruses whose replication is catalyzed by polymerases devoid of proofreading-repair activity are 10^5 to 10^6 -fold higher than those displayed by replicative cellular DNA polymerases. Error-prone replication is a hallmark of RNA viruses and some DNA viruses. The larger the amount of genetic information encoded in a viral genome, the lower the mutation rate must be to maintain the genetic message.
- Several mechanisms of genetic recombination have been described for DNA and RNA viruses. The best characterized is homologous recombination whose frequency of occurrence is dependent on the replicative machinery, in particular polymerase processivity. Genome segment reassortment is operative in segmented genomes and it gives rise to biologically relevant changes such as antigenic shift in the influenza type A viruses.
- Studies with foot-and-mouth disease virus have shown that extensive evolution of an unsegmented RNA genome has the potential to undergo a recombination-mediated transition akin to genome segmentation. Therefore, segmented and unsegmented forms of RNA viruses need not be considered as completely unrelated classes of genome organization.
- Recombination and genome segment reassortment have been viewed as conservative forces to rescue viable genomes from a damaged pool, and also as means to explore new genomic compositions that deviate from their parents. All forms of genetic variation give rise to repertoires of variant genomes on which selection and random drift act to produce the viral forms that we isolate and study.

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