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Mechanisms and Concepts in RNA Virus Population Dynamics and Evolution

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Abstract

RNA viruses are unique in their evolutionary capacity, exhibiting high mutation rates and frequent recombination. They rapidly adapt to environmental changes, such as shifts in immune pressure or pharmacological challenge. The evolution of RNA viruses has been brought into new focus with the recent developments of genetic and experimental tools to explore and manipulate the evolutionary dynamics of viral populations. These studies have uncovered new mechanisms that enable viruses to overcome evolutionary challenges in the environment and have emphasized the intimate relationship of viral populations with evolution. Here, we review some of the emerging viral and host mechanisms that underlie the evolution of RNA viruses. We also discuss new studies that demonstrate that the relationship between evolutionary dynamics and virus biology spans many spatial and temporal scales, affecting transmission dynamics within- and between-hosts as well as pathogenesis.

INTRODUCTION

RNA viruses, those viruses that replicate via an RNA-dependent RNA polymerase (RdRp), exhibit the highest mutation rates in nature (1, 2). They constitute a diverse class of viruses that infect eukaryotic, prokaryotic, and archaeal hosts; RNA virus evolution therefore influences ecological dynamics in marine and terrestrial ecosystems in profound ways (3). They include important historical and emerging human pathogens, including influenza virus, poliovirus, rhabdovirus, and Zika virus (ZIKV). The rapid evolution of RNA viruses sets the tempo of host evolution by placing major selective pressures on their genomes. As the result of past epidemics, genes with antiviral and immune functions (4) and those targeted by viruses through physical interactions (5, 6) are the fastest evolving in host genomes. Here, we review the mechanisms that underlie the evolutionary dynamics of RNA viruses and the experimental tools employed to study them. We then focus specifically on new insights into the role of mutation, recombination, and population size in RNA virus evolution. We also explore how the emerging field of in vivo virus evolution is broadening our understanding of evolutionary dynamics in RNA viruses from single cells to global epidemiology.

MECHANISMS OF RNA VIRUS EVOLUTION

Viral populations evolve by the action of mutation and recombination and are subject to the same evolutionary forces as all organisms, including random genetic drift and natural selection. However, due to their high mutation rates and profound population size fluctuations, RNA virus populations encounter these forces in fundamentally different ways than cellular organisms do. The life histories of viruses are characterized by episodes of strong purifying selection that drive rapid adaptive evolution, and frequent repeated population bottlenecks that result in genetic drift. The relative influences of these forces on the evolutionary trajectory of viral populations, and the scales at which the populations are observed, vary spatially and temporally.

Mutation

Mutation is the source of genotypic diversity within an evolving population (**Figure 1**). The RdRps of RNA viruses are especially error prone, exhibiting the highest mutation rates (mutations per site per generation) in nature (7, 8). As a result, many RNA viruses evolve very rapidly (1). The high mutation rates of RNA viruses correspond to short genome lengths, usually on the order of 10 kbp, consistent with a general inverse correlation between genome length and mutation rate among viruses and noneukaryotic organisms (9, 10). In practical terms, by dictating the probability that a mutation will arise in a population, mutation rates serve as critical parameters in evolutionary models used in phylogenetic inference and population genetics. Therefore, their accurate measurement is critical to our understanding of the evolution of RNA viruses.

Mutation rates were first estimated using fluctuation tests, which relied on measuring the frequency of reversion of a mutant phenotype (11). Fluctuation tests in RNA viruses later revealed mutation rates near 1 mutation per genome per replication (7). RdRps exhibit different rates of mutation for individual nucleotide substitutions (e.g., A to G, A to U), which have recently been characterized using new techniques (12–14). Pauly et al. (14) reported a modified fluctuation test for estimating class-specific mutation rates of influenza A virus (IAV) that employs a library of green fluorescent protein mutants. The assay relies on the use of viruses encoding nonfluorescent mutants of green fluorescent protein that can be reverted by single mutations of each class. The viral population is distributed into multiple wells containing cultured cells at a low fixed multiplicity of infection. The rate of mutation is then estimated by counting the green fluorescent protein–positive wells (14).

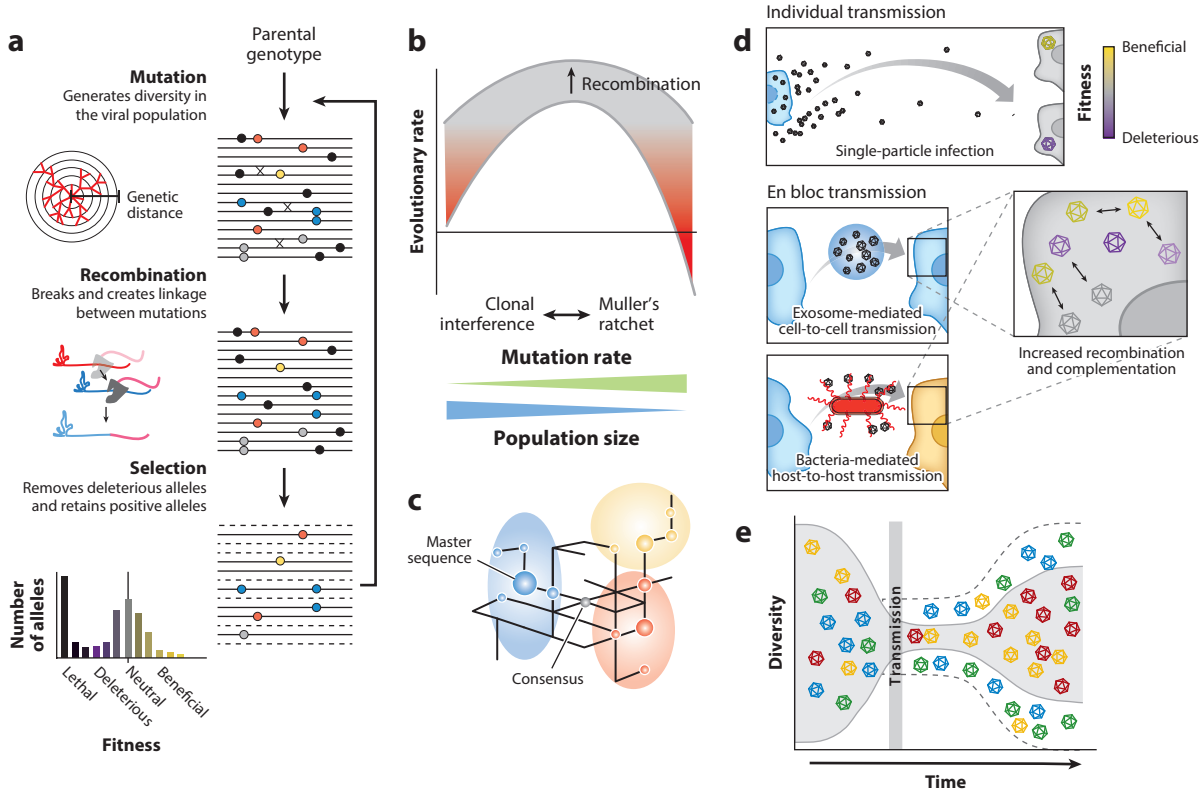


Figure 1

Mechanisms of viral evolution. (*a*) Evolution occurs through the process of mutation, selection, and drift. These processes generate and remove diversity in the population, leading to evolutionary change in populations. During replication, RNA-dependent RNA polymerase introduces mutations and mediates recombination between RNA genomes and/or antigenomes. Selection acts on the alleles on the basis of their fitness effects, represented for all alleles as a distribution of mutational fitness effects (*bottom left*). (*b*) Recombination is a common feature of many RNA viruses. It increases the adaptive rate by relieving the effect from drift at small population sizes and high mutation rates, and by combining cocirculating adaptive mutations at large population sizes and low mutation rates. (*c*) At large population sizes, viral populations form a swarm of mutant genotypes surrounding a modal master sequence, or sequences (*large nodes on network*), connected by a network of single mutations. Individual subpopulations (*groups of colored nodes*) can interact within the viral population through antagonistic and cooperative interactions. (*d*) Transmission of multiple viruses as a single infectious unit is more likely to result in coinfected cells. Coinfection provides the opportunity for viral particles (from a range of fitness values) to interact through complementation, cooperation, and/or recombination. (*e*) En bloc transmission also helps the influence of drift on the population during bottlenecks. As a viral population transmits through a host, or between hosts, it encounters multiple bottlenecks that reduce the population size (*gray area*). En bloc transmission (*broken lines*) counters the effect of bottlenecks by increasing the size and diversity of the population transmitted to the new host cell.

In the same study, the substitution-specific mutation rates of IAV were determined using a deep-sequencing approach known as a primer ID. This method uses barcoded complementary DNA (cDNA) primer sequences to trace the fate of single RNA templates after polymerase chain reaction amplification to control for spurious mutations introduced during amplification of the sequencing library (**Figure 2**) (14, 15). Another high-resolution deep-sequencing approach used to control such artifacts, known as circular sequencing (CirSeq), was developed by Acevedo et al. (13). They used the method to estimate the mutation rates associated with all nucleotide substitutions in poliovirus (13). This approach reduces the error associated with traditional short-read RNA

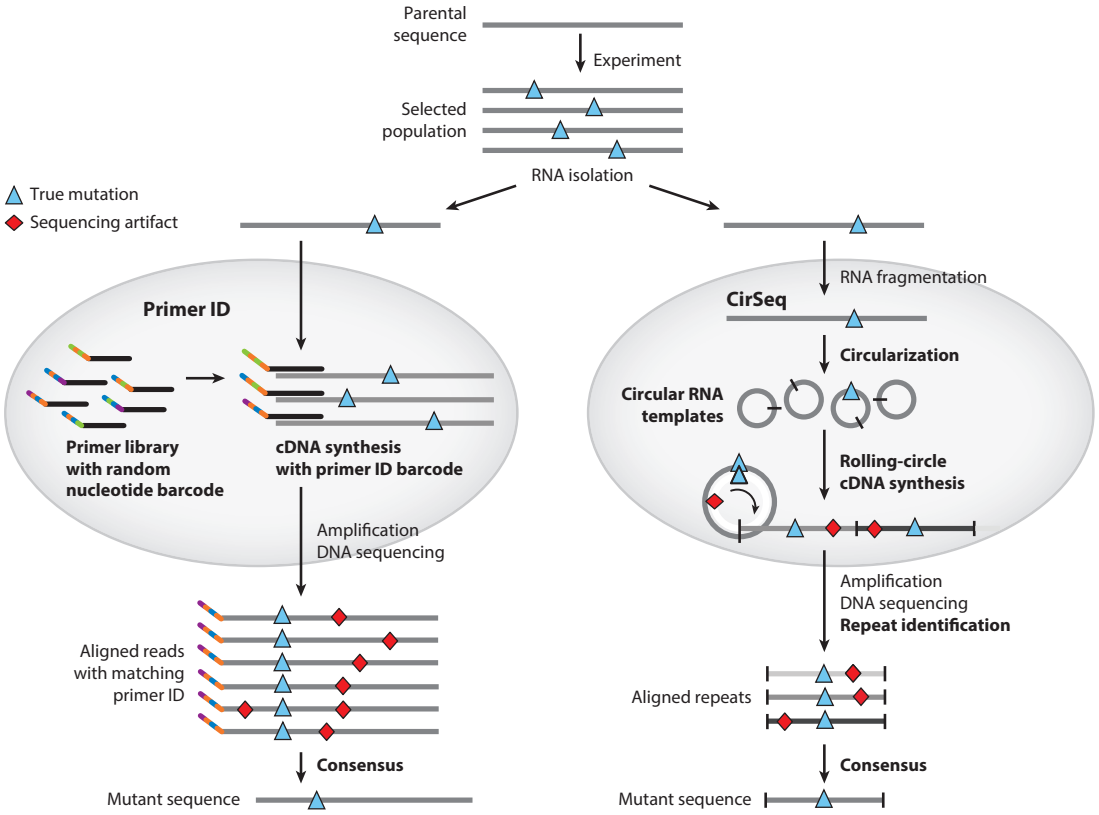


Figure 2

High-resolution population sequencing approaches. Two high-resolution RNA sequencing procedures have been developed to measure the frequency of low-frequency alleles in viral populations. Both approaches take advantage of alterations in traditional RNAseq (*gray ovals* and *bold text*) to reduce the error rates associated with library generation and amplification. Primer ID (*left*) uses a library of randomized reverse transcription primers during cDNA synthesis to barcode reads that originate from the same template molecule, allowing bioinformatic analysis to remove spurious mutations (*red diamonds*) and reveal true mutations present in the populations (*blue triangles*). In CirSeq, template RNA fragments are circularized, and the cDNA is generated using rolling-circle reverse transcription to generate repeated reads of a single molecule. The repeats are analyzed bioinformatically to remove spurious mutations.

sequencing by circularizing template RNA fragments and generating cDNA with tandem repeats prior to sequencing (16, 17).

In addition to mutations acquired through RdRp replication errors, virus mutation rates are influenced by host editing enzymes including apolipoprotein B editing complex (APOBEC) and adenosine deaminase, RNA-specific (ADAR). Although APOBEC proteins typically function as restriction factors to retroviruses and DNA viruses (reviewed in 18), proteins in the ADAR family act directly on regions of double-stranded RNA by deaminating adenosine to produce inosine, leading to A-to-G mutations. Signatures of ADAR activity have been found in a number of viruses (reviewed in 19), including influenza virus (20), measles virus, Rift Valley fever virus (21–23), and *Drosophila* sigma virus (24). Furthermore, both Ebola virus (EBOV), and ZIKV have recently been identified as potential ADAR targets (25–33). Human-derived samples of EBOV show clear signs of A-to-G (U-to-C in the EBOV positive coding strand) editing, including samples from EBOV survivors (32). ADAR proteins have functional consequences on viral growth (reviewed in 34), exhibiting both antiviral (22) and pro-viral activity (35, 36), depending on the system. The details

of ADAR activity and its effect on viral evolution are still emerging, but these data suggest ADAR is a likely source of diversity in RNA viruses. Elucidating functional consequences will further clarify how these exogenous sources of mutation affect the evolution of their target viruses.

Selection and Drift

Each mutation has a characteristic effect on the replicative success, or fitness, of the virus, known as a mutational fitness effect (**Figure 1*a***). In RNA viruses, the fitness effect (W) of most mutations is deleterious ($W < 1$) or lethal ($W = 0$) (13, 37–39). Mutations that exert no, or little, effect on the fitness of the viral population are referred to as neutral, or nearly neutral ($W \approx 1$). Beneficial mutations, those mutations conferring an adaptive advantage on the virus ($W > 1$), are comparatively rare. In aggregate, mutations present on a genome, its genotype, determine the fitness, or phenotype, of the genome.

Natural selection acts on the phenotypic diversity of mutant genomes in the population to drive populations toward increased fitness. Beneficial mutations, if unimpeded, will increase to fixation driven by positive selection. Deleterious mutants, which result in reduced fitness, are removed from the population by negative, or purifying, selection. As such, natural selection has a deterministic influence on the evolution of viral populations. The probability that an adaptive allele will go to fixation and replace the master sequence within the population is a function of its frequency, its fitness effect, and the effective population size.

In finite populations, the evolutionary process is driven not only by the deterministic influence of selection but also by the stochastic influence of random genetic drift (40). Genetic drift, the stochastic fluctuation of allele frequencies in the population, occurs in all finite populations as a result of sampling error from generation to generation. Drift alone can lead to the fixation of neutral and deleterious mutations in the population. Sampling error and, thus, the influence of genetic drift on evolutionary trajectories is greatest in small population sizes. During population bottlenecks, or hard selective sweeps, where the population size drops dramatically, neutral and deleterious mutations can be fixed, while rare adaptive alleles may be lost until they can be reintroduced by mutation in later generations. The population size could also be dramatically reduced during transmission of viruses to new hosts or especially to new species. Neutral and deleterious mutations can also be fixed in the population by genetic draft, a related phenomenon wherein these mutations are fixed through linkage to adaptive mutations (41).

Recombination and Reassortment

Mechanisms that break and create linkage between mutations in the genome are critical for populations to efficiently explore sequence space (**Figure 1*a*,*b***). In small populations, drift leads to the accumulation of neutral and deleterious "hitchhiker" mutations on adaptive lineages. Without a mechanism to shed the deleterious mutational load from adaptive lineages in the population, the population will be overcome by deleterious mutations. This results in a slowing of the evolutionary rate, an effect known as Müller's ratchet (42–44) (**Figure 1*b***). Competition between adaptive lineages also slows phenotypic evolution, through a process known as clonal interference (45). In large populations with sufficiently high mutation rates, many adaptive alleles cocirculate and compete. In such cases, recombination is advantageous because it combines advantageous alleles to generate novel genotypes with greater fitness.

Recombination. Recombination among RNA viruses occurs through a mechanism known as copy choice, wherein an RdRp associated with a nascent transcript dissociates from one template and associates with another (46). When this occurs at the same site, recombination is homologous.

When recombination occurs between different sites (or with cellular RNA), recombination is nonhomologous (and could be considered ectopic). Recombination rate determinants in the RdRp have recently been identified. Mutations that reduce the recombination rates of the poliovirus RdRp occur at sites both proximal and distal to the active site (47, 48). Experimental alteration of the recombination rate of RNA viruses has been used to explore the effect of Müller's ratchet, demonstrating that limited recombination increases the influence of drift on population fitness in small populations (49–51).

Recombination is common within the human enteroviruses, a diverse group of positive-sense RNA viruses in the *Picornaviridae* family, made up of hundreds of known serotypes belonging to twelve species (52). The genus includes a number of important pathogens, including polioviruses 1–3, EV-A71, EV-D68, and coxsackieviruses A and B (52, 53). Circulating enterovirus serotypes commonly replace one another in annual cycles and often recombine across species boundaries, contributing to a constant emergence of new variants with new phenotypes (54–56). Recombination between the live attenuated oral polio vaccine strains and circulating enteroviruses, such as asymptomatic coxsackieviruses, is a common source of vaccine-derived outbreaks of poliomyelitis and has presented a major challenge to eradication efforts (57, 58).

While homologous recombination can give rise to viable recombinants, nonhomologous recombination in RNA viruses commonly gives rise to subgenomic RNA species, referred to as defective interfering (DI) RNA (59). Such RNAs have been identified in dengue virus (60, 61), mumps virus (62), Sendai virus (63), vesicular stomatitis virus (64), and brome mosaic virus (65), suggesting they are a common feature of viral replication across hosts as well as across transmission and replication modes. It has been suggested that these DI RNAs, and viral particles carrying them, known as DI particles, may act as immune decoys either intracellularly or extracellularly (66, 67). Low–replicative fidelity mutants of chikungunya virus also exhibit greater rates of nonhomologous recombination resulting in DI RNA more often, suggesting mechanistic links between the mutation and recombination rates (68).

Reassortment. Reassortment occurs in RNA viruses with segmented genomes (recently reviewed in 69). When multiple genotypes infect a single cell, the progeny inherit a mix of gene segments from the parental viruses in a process analogous to independent assortment during meiosis. Reassortment creates an efficient means to explore combinations of genotypes, while preserving epistatic interactions within gene segments. For a virus with 10 gene segments, there are 1,024 potential reassortants. These reassortment events often yield new phenotypes and have been associated with host switching in numerous viruses (reviewed in 70). The most notable example of reassortment is the process of antigenic shift in influenza viruses. IAV, which encodes 10 proteins on 8 segments of negative-stranded RNA, circulates widely within avian and mammalian populations (71). Reassortment between mammalian and avian IAV strains can associate the antigenic profile, defined by hemagglutinin (H) and neuraminidase (N) gene segments, of one strain with host-specificity determinants of another, leading to antigenic shifts (72). These reassortant viruses present novel antigens to host populations, leading to pandemic spread in the unprotected population (73). All three of the most recent IAV pandemics (1918 H1N1, 1957 H2N2, and 1968 H3N2) originated from reassortment events resulting in the exchange of the hemagglutinin of avian influenza strains with mammalian influenza viruses (71). The virus responsible for the 2009 H1N1 outbreak was a triple reassortant of avian and porcine strains (74).

Maintaining Balance: Mutation, Selection, and Drift

Mutation creates diversity, which is removed through the action of selection and drift. Mutation-selection balance and mutation-drift balance describe the pressure to maintain equilibrium

between generating diversity and limiting the influence of deleterious mutations (75, 76). Mutation rates and recombination rates of RNA viruses are balanced against the influence of drift and selection and are further constrained by the biological characteristics of the virus (such as genome length, population size, and robustness to mutation). The life histories of many RNA viruses are characterized by episodes of drift and selection during transmission events and immune pressures, making the relative influence of drift and selection variable across viruses (8).

The evolutionary parameters of genetic entities are shaped by past selection to maintain mutation-selection balance, an equilibrium state at which the population is not overcome by deleterious mutations. Maintaining balance between their limited genetic capacity, high mutation rates, and population size dynamics creates intimate connections between RNA virus biology and evolutionary dynamics. Disturbing this balance, through genetic, pharmacological, and enzymatic means, is an established antiviral strategy (77–80). One pharmacological mutagen, a nucleoside (rGTP) analog called ribavirin, has been used as a drug for many RNA viruses (77). Mutations that confer resistance to mutagens, by altering the inherent RdRp mutation rate, readily emerge in response to ribavirin treatment (79, 81) and altered intracellular nucleotide pools (82). The number of mutations that can alter fidelity, and the attenuated phenotypes of recombination-deficient and fidelity mutants, suggests that the mutation rates of RdRps are highly tuned (12, 48).

In the years since the identification of high-fidelity mutants in poliovirus, similar strategies have been used to identify additional examples of viral mutants with altered evolutionary parameters. High-fidelity mutants have been identified in coxsackievirus (83) and foot-and-mouth disease virus (84). Viruses with lower-fidelity mutator strains have also been described in a number of viral species, including poliovirus (12), coxsackievirus (85), and chikungunya virus (86). Viruses with altered recombination in poliovirus (48) and reassortment in influenza have also been described (87). These have been valuable experimental tools to identify the structural determinants of fidelity in viral polymerases (88, 89). Moreover, studies in vivo have shown that alterations in the evolutionary parameters of these viruses have significant effects on viral spread and pathogenesis. Altering mutation and recombination rates of viruses through mutation confers attenuated phenotypes to poliovirus (48, 51, 90), chikungunya virus (86), and coxsackievirus (85). These approaches have also been proposed for use in live attenuated vaccines (91–95).

Robustness

Viral genomes, especially RNA viruses, are often dense with functional RNA structures and protein coding regions. The intense evolutionary constraint on RNA virus genomes is reflected in the distributions of mutation fitness effects for RNA viruses, which suggest that most mutations are lethal or deleterious (13, 37, 39). Despite this fragility, RNA viruses exhibit high mutation rates. At small population sizes, high mutation rates lead populations to evolve mechanisms of genetic robustness by evolving to genotypes with few deleterious mutational neighbors (96–99). Conversely, during passage in large population sizes, deleterious mutations can be relieved through complementation, leading to the accumulation of individuals that are less robust, or more brittle, to mutation (98, 100).

The term genetic, or mutational, robustness refers to any mechanism that reduces the impact of deleterious mutations on the virus population (101). Strategies for robustness have been previously organized into two categories: (*a*) intrinsic to the viral genome and (*b*) extrinsic to the viral genome, or environmental (102, 103). Genetic robustness is often associated with the similar concept of evolvability, or the capacity of a genotype to access adaptive mutations (99). Increased robustness tends to reduce the phenotypic effect of mutation and can therefore limit evolvability (104, 105). However, in some cases, increased robustness does not appear to occur at the cost of adaptive mutations (99).

Intrinsic robustness. Sources of intrinsic robustness are encoded within the viral genome. These include fundamental properties such as codon bias and protein structure that have been shaped by past selection to limit the detrimental effect of mutation. Viral proteomes and genomes exhibit unique structural characteristics that confer robustness to mutation, including modularity and redundancy (106–108). Surveys of viral protein structure have suggested viral proteins exhibit looser packing of residue side chains within the hydrophobic core of viral proteins (105). This is expected to buffer the energetic cost associated with mutation.

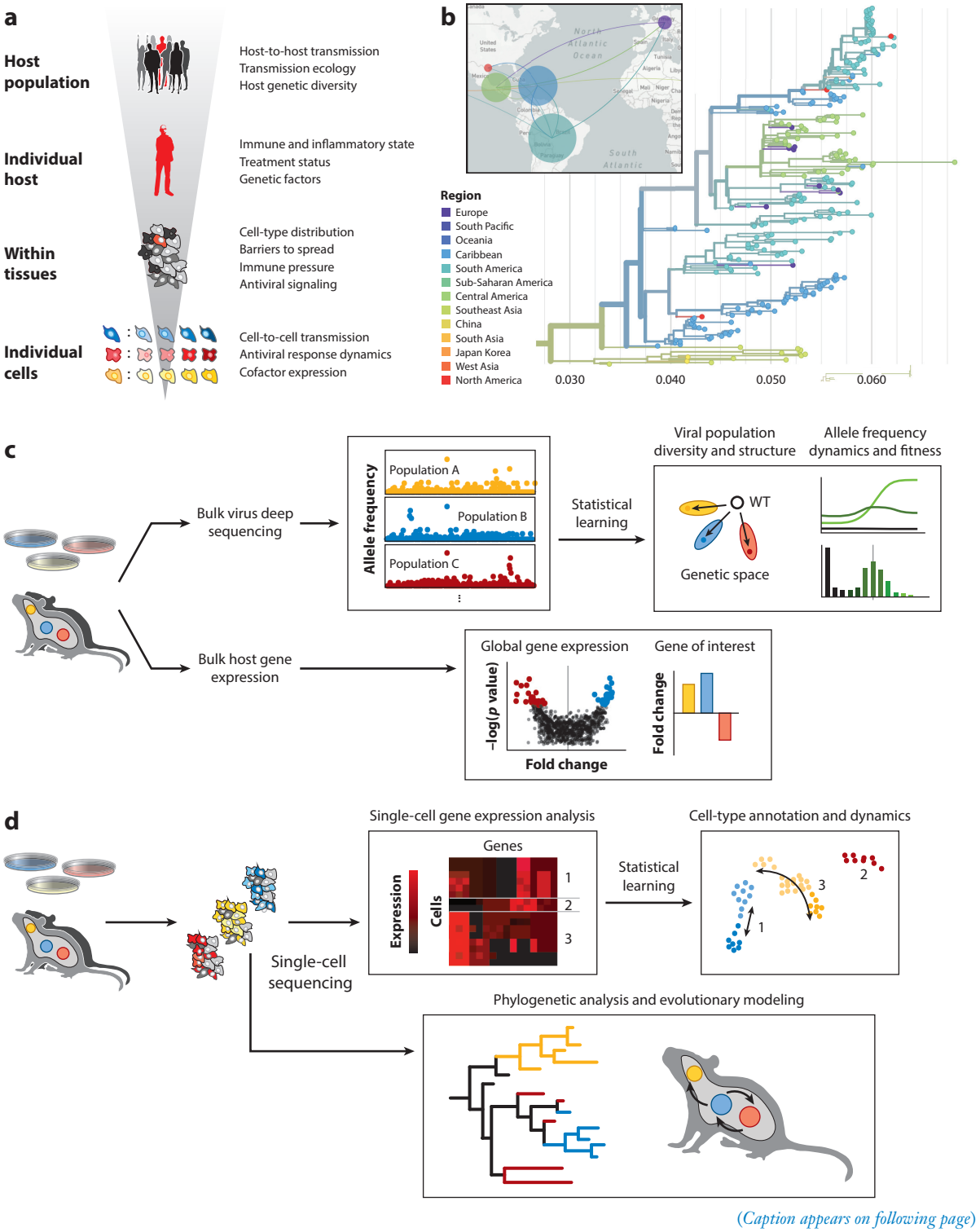
Codon bias in viral coding regions is one aspect of intrinsic robustness that has been extensively explored through the use of codon-shuffled viruses. Altering the position-specific codon usage of a virus through synonymous mutation places the virus in a new genetic neighborhood. The parental genotype is faced with a new collection of neighboring variants, each with its own fitness. Although these engineered genomes encode the same primary amino acid sequence, and retain important RNA secondary structure, they exhibit altered fitness in vitro and in vivo (109, 110). These studies suggest that codon bias is critical in maximizing viral robustness by limiting the likelihood of detrimental mutations. The propensity to access detrimental nonsynonymous or nonsense mutations at specific codons is called codon volatility. When viral genomes are engineered to maximize this volatility, by placing numerous codons one mutation away from stop codons, viral fitness was especially impaired, demonstrating the importance of the genetic neighborhood in evolution and robustness (111).

Extrinsic robustness. RNA viruses have acquired additional sources of mutational robustness by co-opting host factors. The cellular chaperone system is particularly vital to the success of viral infections (112). Three major chaperone families play a role in eukaryotic protein folding: Hsp70, Hsp90, and TRiC. While their normal host functions include stress response and folding of proteins, each chaperone family has been implicated in regulating viral infection. Hsp70 plays a role in the replication cycle of many viruses, including dengue virus (113), poliovirus (114), and rabies virus (115). Hsp90 similarly plays a role in the life cycle of poliovirus (116, 117), respiratory syncytial virus (118), and reovirus (119), among others (112). TRiC has been implicated in the replication of human immunodeficiency virus (HIV), hepatitis C virus, and rabies virus (120–122).

Given the high mutation rates of RNA viruses and the extent of translational stress imposed on cells by infection, chaperone activity may be necessary to buffer the effect of destabilizing variants (**Figure 3**). Recent work has explored the effect of perturbing host proteostasis on the evolutionary capacity of IAV (123). Virus was passaged in cells displaying enhanced heat shock factor 1 (HSF1) expression or reduced Hsp90 activity. Although the treatments did not affect viral titers, the Hsp90-inhibited population showed delayed accumulation of high-frequency mutations with respect to the HSF1 enhanced (and control) environments. Conversely, HSF1-induced populations displayed more rapid accumulation of adaptive mutations. The high- and low-proteostasis environments each selected for a unique repertoire of adaptive variants, highlighting the impact of cellular proteostasis systems on viral evolution and robustness.

Quasispecies

The high mutation rates and mutational robustness characteristic of RNA viruses lead populations to rapidly establish a diverse spectrum of genotypes. As a result, viral populations rapidly explore their mutational neighborhood, populating adjacent genotypes in the network through subsequent mutational steps (**Figure 1*c***). In sufficiently large population sizes, RNA virus populations exist as a large collection of genotypes surrounding a master sequence, often referred to as a mutant swarm or quasispecies. The term quasispecies was proposed by Manfred Eigen in 1971 (124) to



(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

Experimental approaches to studying viral evolution and host response. (*a*) Evolution of viruses proceeds across a wide range of spatial and temporal scales, each with its own evolutionary considerations. (*b*) Phylogeographic analysis of dengue virus serotype 2 evolution in the Americas and Caribbean from Nextstrain (161), a collaborative project for tracing and forecasting viral dynamics. (*c*) Bulk evolutionary experiments from cultured cells and animals are used to understand the short-term evolution of viral populations. Using deep sequencing, these studies can identify patterns of evolution between viral populations and estimate the fitness effects of thousands of mutations simultaneously. (*d*) Experimental approaches that address the heterogeneity of biological samples provide a higher-resolution view into the selective environment and the evolution of viral populations in specific cells. Bulk evolutionary experiments from cultured cells and animals are used to understand the short-term evolution of viral populations. Using deep sequencing, these studies can identify patterns of evolution between viral populations and estimate the fitness effects of thousands of mutations simultaneously. (*e*) Experimental approaches that address the heterogeneity of biological samples, such as single-cell sequencing, provide a higher-resolution look into the selective environment and the evolution of viral populations in specific cells through single-cell analyses.

refer specifically to the behavior of infinite populations with very high mutation rates; however, this concept has been extended to large, finite populations.

Quasispecies-like behavior has significant implications for viral biology and evolution. In a given environment, the deterministic effect of selection acting on the mutational fitness effects of genotypes in the population will give rise to reproducible equilibrium populations, with the most fit genotypes making up the majority of sequences. Instead of being described by a single consensus sequence, these populations are better described by these modal, master sequences, which anchor the distribution of mutants in sequence space. As environments change, variants within the quasispecies can rapidly emerge, shifting the population's genetic structure.

As stated (124) and later clarified, quasispecies are a special case of population genetics and emerge as an effect of mutation-selection balance when specific conditions are satisfied (125, 126). Although natural populations are not infinite, the evolutionary significance of the swarm-like behavior of large viral populations is that it enables rapid adaptation to altered selective conditions. These swarm dynamics of viral populations are relevant to the evolution of viruses specifically at high population sizes. An effective analogy for the difference in evolutionary dynamics might be found in the use of classical and quantum mechanics to explain and predict physical phenomena. Although the laws of physics are universal, at different scales, particular models are more useful.

Complementation and Cooperation

In large viral swarms, interactions can occur between individual genotypes. This phenomenon is known as density-dependent selection, where the relative frequency of individual genotypes shapes the fitness of other genotypes in the population (81, 127). Cooperativity has been defined as a specific form of complementation where two genotypes create a new phenotype (e.g., become more fit) when grown in combination rather than separately (128). Cooperation also entails that selection act on the cooperative allele specifically because it benefits other genotypes (and was not selected for in a purely selfish manner) (129). Such cooperation has been identified in numerous RNA viral species (130–135). Shirogane and colleagues (128, 131) identified cooperativity arising after passaging of a recombinant, nonfusogenic measles virus. Interaction between the wild-type and a mutant (destabilized) form of the fusion protein restored production of syncytia in the (previously nonfusogenic) recombinant virus. The oligomeric nature of the fusion protein complex may lend itself to cooperativity, where populations of mixed alleles can regulate the stoichiometry of mutant fusion proteins interacting in a single complex (128, 131).

Similar stoichiometric regulation is seen in other multimeric proteins. Influenza virus passaged in cell culture naturally evolved a mixed population at neuraminidase residue 151, which exhibited a higher fitness than either individual mutant in isolation (132). This mixed population consisted

of the wild-type, cleavage-competent neuraminidase allele and a mutant allele capable of binding, but not cleaving, its receptor. This cooperation is likely a response to the rising prevalence in circulating populations (circa 2005) of a hemagglutinin variant with decreased binding affinity for the same sialic acid receptors neuraminidase normally cleaves (132, 136). It is important to note that these mixed populations are only seen in IAV populations that have been passaged in cell culture and are not found in samples from active infections (137). Bordería et al. (133) adapted coxsackievirus B3 to a novel cell type and identified multiple cooperative alleles in a receptor binding domain after 40 passages. Interestingly, the fitness gains of this population could be replicated by creating an artificial quasispecies, but only when the four most prevalent haplotypes were mixed at the same frequencies they achieved in the passaging experiment (133).

Population Size

Population size is a critical parameter in the evolution of populations, and it is one that fluctuates widely during viral infection cycles. An infection elicited by an individual viral genome can produce 10^{15} progeny within an infected host. The term effective population size refers to the population size at which a model population would exhibit the same diversity. Although viral population sizes are often very large, effective population size reflects the loss of diversity associated with any recent population bottlenecks and is much lower, approximating the harmonic mean of the population size over time (138). Effective population size estimates vary widely for different viruses (139, 140) and when compared between and within hosts (140).

To overcome the negative influence of drift from transmission of small populations, and to maintain diversity in the population, viruses have evolved mechanisms to maintain larger population sizes during transmission, called en bloc transmission (**Figure 1*d***). En bloc transmission, or the transmission of multiple viral particles as a single unit, can occur through association with multivesicular bodies (MVBs), cellular or virological synapses, binding of enteric bacteria, and/or virion aggregation (141–150). Because the sample size associated with transmission is increased, the potential mutational cost associated with transmission bottlenecks is partially relieved. En bloc transmission therefore constitutes a mechanism of mutational robustness (**Figure 1*d***).

One means of en bloc transmission is via interaction with bacteria (142–144) (**Figure 1*d***). Rather than transmitting individually, multiple enteric viral particles can bind a single bacterial cell, leading to concerted infection of individual host cells and new hosts (142, 144). Multiple virus particles associate with lipopolysaccharides in the outer membrane of the bacteria, leading to increased virion stability as well as enhanced viral attachment to the poliovirus receptor (142). Such stabilization could be key in virion resistance to environmental stress and thus in transmission from host to host. Further work has shown that this bacteria-enhanced viral attachment to target cells also results in increased coinfection of cells, in part due to strains of bacteria that themselves adhere better to target cells (144). This increased rate of coinfection correlates with subsequent elevated levels of recombination between coinfecting viruses. When the number of infectious particles is limited, such as early on in an infection or after a population bottleneck, en bloc transmission can tip the balance toward more frequent coinfections, resulting in a more forgiving selective environment and thus enhancing the robustness of a viral population.

En bloc transmission is also employed to overcome the bottlenecks associated with the transmission of viruses within the host, where the movement of multiple viruses between cells is facilitated by MVBs (reviewed in 151) (**Figure 1*d***). Interestingly, at least two types of MVBs are exploited, depending on the virus under study. Infectious hepatitis A and E viral particles were found to be secreted from infected cells in exosome-like MVBs (141, 152), while poliovirus, coxsackievirus B3, and rhinoviruses make use of the autophagosome system (141, 153, 154). Virus-containing

MVBs are distinct in composition from their normal, cellular counterparts, which may account for their altered trafficking. While autophagosomes are typically destined for lysosomal fusion, poliovirus-containing autophagosomes are instead secreted out of the cell (potentially due to a lack of syntaxin 17) (141).

There are a number of important distinctions with respect to the evolutionary pressures acting on RNA viruses transmitted en bloc versus those transmitted individually. En bloc transmission of multiple viral particles increases the opportunity for complementation, cooperation, and recombination between viral genomes. This is supported by the increased infectivity of poliovirus in MVBs versus free poliovirus (141). Furthermore, certain modes of en bloc transmission (such as MVBs and viral synapses) mask their passenger viruses from neutralizing antibodies (146, 149, 155), presumably ensuring greater rates of transmission in the presence of memory immune responses. Notably, the majority of instances of en bloc transmission occur prior to cell lysis. In cases where cells do eventually succumb to infection and lyse, two distinct populations of viruses are released from cells: those transmitted en bloc and those that are free. These studies suggest that these two populations of virus face different selective pressures. After infection, the balance of transmission types (en bloc versus individual) could significantly influence the evolutionary pressures acting on viral populations.

VIRUS EVOLUTION ACROSS SCALES

Selection and drift act on viral populations across many spatial and temporal scales, and the influence of each on the evolution of virus population varies through the life history of the virus (**Figure 3*a***). The influence of specific selective pressures and evolutionary processes varies across each scale, but all contribute to the evolutionary trajectory of viral populations. In addition, selective and stochastic bottlenecks occurring during host-to-host, intraspecies, and cell-to-cell transmission all contribute to the evolution of viral populations and are unique to each virus.

Virus populations, whether considered globally or within individual hosts, exist as a collection of subpopulations, or demes. Within each host population, host, organ, or cell, viral populations evolve under distinct selective conditions that contribute to the evolution of the viral metapopulation (156) (**Figure 3*a***). One useful framework for considering the relationship between the alternative influences of drift and selection across structured populations is Wright's shifting balance theory (157). It suggests that populations can overcome selective barriers through cycles of intermittent selection, drift, and migration. The theory proceeds in three phases that operate concurrently. In the random drift phase, individual small populations are founded with specific mutations fixed by drift. Then, those viable populations undergo mass adaptation, wherein individual populations increase in fitness through the action of selection. In the third phase, interdemic selection, migration and competition between populations drives up the fitness of the metapopulation.

Using a microfluidic evolution machine, Rotem et al. (158) addressed Wright's theory experimentally. They found that allowing evolution to proceed first within millions of small, partitioned subpopulations, followed by bulk selection, leads to the emergence of higher-fitness genotypes compared to those that emerge through bulk passage. Recent studies suggest that such migration between selective environments, in a manner similar to that described by shifting balance theory, is an important parameter to consider with respect to the emergence of adaptive mutations within the host. For instance, modeling suggests that when drug penetration is incomplete in specific tissues, migration of viral populations between these different selective microenvironments can give rise to lineages resistant to multiple drugs (159). This model was later tested in a simian model of HIV using deep sequencing to monitor the emergence of drug-resistant mutations in demonstrating rapid migration between compartments (160).

Global Evolutionary Dynamics

At the global scale, viral genotypes, such as IAV strains, sweep through host populations over timescales of months to decades (**Figure 3*b***) (71, 161). Evolutionary dynamics at these spatial and temporal scales emerge from complex host demographic and ecological factors, as well as from the constraints of more basic selective pressures such as the stability and function of the viral genome and proteome (162–164).

Phylogenetic tools are used to infer the relationships between sampled viral sequences and to estimate the influence of selection and drift (165). These tools use sequence alignment and probabilistic inference to estimate the divergence time and evolutionary rates based on evolutionary models. Recent years have seen advances in computational tools such as Bayesian phylogenetic inference (166), phylogeographic tools (29, 161, 167, 168), and portable and inexpensive sequencing technologies such as nanopore sequencing (169–171). The rapid deployment of these tools in response to viral outbreaks has enabled real-time tracking and forecasting of viral dynamics (161). Accurate experimental determination of evolutionary parameters such as mutation and recombination rates, or the fitness effects of mutation along the genome, will drastically improve these models and the resulting evolutionary inferences (172, 173).

Host-to-Host Transmission

Transmission between individual hosts is one point where the influence of bottlenecks and drift leads to evolutionary changes in virus populations (174). Environmental challenges, physical dilution, and selective barriers result in transmission of small populations of founding virus with allele frequencies altered from those in the host, resulting in drift. Individual hosts exhibit variability in demographic factors such as age, immune and inflammatory state, and genetic factors that affect susceptibility to infection. Models of RNA virus evolution are beginning to incorporate the influence of environmental and immune factors on RNA virus evolution and dynamics (175–177).

Transmission between host species presents further evolutionary barriers to transmitted populations (178–182; recently reviewed in 183). During transmission of arthropod-borne RNA viruses (arboviruses), changes in selective environment between hosts are especially pronounced and must be endured through each transmission cycle. As such, the fitness landscapes encountered by the viral population are altered in alternative hosts (38, 184–187).

Arboviral hosts, such as birds, mammals, and marsupials, all differ from the insect vector in fundamental characteristics, such as temperature, life span, physiology, immune system function, and the host factors that are available to support viral replication. Recent feeding experiments have demonstrated the complex interactions of drift and selection during arboviral transmission events. After feeding on West Nile virus–infected birds, mosquitoes acquired a viral population in each bloodmeal that represented a unique subsampling of the diversity in the bird (180). Although this might be expected to result in significant drift in the viral populations over successive passages, the strong purifying selection characteristic of transmission back to the avian host quickly removed nonsynonymous variants, slowing the rate of evolution. This study is significant in light of previous studies that have suggested that arboviruses evolve more slowly than single-host RNA viruses due to strong selective bottlenecks at the point of host transmission (188, 189).

Within-Host Evolution

Mutations that sweep host populations first emerge within individual hosts (190). The probability that a variant will emerge depends on the influence of drift, selection, and population structure in the host environment. Multicellular hosts present a heterogeneous and dynamic selective

environment to the infecting viral population. Each microenvironment in the host exerts distinct selective pressures that affect the spread and pathogenesis of the viral population (51). Therefore, quantifying and modeling of virus evolution within individual hosts requires improved spatial and temporal resolution in sequencing experiments to explore the selective environment in vivo (**Figure 3*c***).

Studies to address patterns of selection in the host have focused on drug selection and immune selection. Parameswaran et al. (191) examined populations in human patients during primary and secondary dengue virus (serotype 3) infection, comparing viral populations derived from peripheral blood monocytes and those derived from plasma. Their analysis revealed differences in the diversity in prM and NS3 proteins, suggesting altered selective pressures between these populations due to B and T cell responses, respectively (191).

The ability of RNA virus populations to evolve rapidly is a critical determinant of infection and pathogenesis. During poliovirus infection in mice, viral populations establish reproducible tissue-specific patterns of diversity within individual organs (51). Populations sequenced from spleen, kidney, and liver of infected mice exhibited distinct quasispecies structures that were reproducible across individual mice. When recombination and mutation were altered through mutations in the viral polymerase, populations isolated from these tissues no longer exhibited these specific patterns. This alteration was correlated with limited systemic spread and an attenuated phenotype, suggesting that rapid evolution is required to overcome selective challenges in the host (51).

Single-Cell Evolution

The process of infection and host response is a highly heterogeneous process that takes place in a complex in vivo environment. Cellular systems organized into tissues and organs, each with its own unique immunological and biochemical properties, affect the outcome of infection and pathogenesis associated with specific viruses. New experimental paradigms, such as microfluidics and high-resolution population sequencing, allow us to approach studies of evolutionary dynamics with renewed clarity and experimental control (192–194). These technologies allow the manipulation of single cells and viral particles to control experimental parameters and quantify virus evolution and cellular response at the most granular level, stratifying individual cell types and quantifying their response to stimuli (195, 196).

Even in culture, cells exhibit remarkable heterogeneity, especially during viral infection (194, 197). Experimental approaches commonly used in the study of virus evolution and the host responses to infection have limited the study of infection and evolution to bulk measurement. However, bulk measurement and consensus sequencing do not capture the heterogeneity intrinsic to these processes and mask important evolutionary processes. During IAV infection, single-cell transcriptome sequencing of cultured MDCK cells reveals very heterogeneous responses to infection (197). Cells exhibit a wide range of infected phenotypes, and only very few exhibit robust antiviral signaling.

CONCLUSION

The high mutation rates and large population size dynamics inherent to RNA viruses set them apart from other organisms, providing a unique lens through which to understand evolution. To efficiently traverse their evolutionary landscape, RNA viruses must maintain an equilibrium between their high rates of mutation and the forces of selection and genetic drift. This balance is stabilized through mechanisms of robustness. These include those inherent to the virus itself, such as altered protein composition and structures more tolerant to mutation, or specific patterns

of codon usage bias that avoid deleterious mutations, and those co-opted from the host cell, such as the activity of cellular chaperones, or components involved in en bloc transmission.

The evolutionary strategies employed by RNA virus populations facilitate their rapid adaptation to changes in their selective environment. Even within a single host, the virus must evolve to the varied selective environments of individual tissues and cell types. Therefore, the evolutionary capacity of an RNA virus population is critical to establishing successful infections, determining tropism, and eliciting pathogenesis. Numerous lines of evidence show that the short-term evolutionary capacity of a virus is necessary to establish an infection, and that restricting (or exceeding) this capacity results in attenuated viral populations. Moreover, these constrained viral populations exhibit more limited spread, suggesting that evolutionary capacity determines the extent of RNA virus spread and pathogenesis (48, 51, 81).

Due to the importance of viral evolution across spatial and temporal scales, an emerging concept in the treatment and prevention of RNA viruses focuses on the use of genetic and chemical approaches to alter the evolutionary dynamics of RNA viruses. As mentioned above, virus-targeted approaches that alter evolutionary parameters, such as chemical mutagens or vaccine strains with altered mutation and recombination rates, can be used to treat or prevent viral infection. The identification of high-fidelity and low–recombination rate RdRps is particularly promising for vaccine design (91, 199). These variants reduce the rate at which the viral population can explore sequence space, making adaptive mutations less frequent and slowing the evolutionary rate of the virus. Incorporating these variants into live vaccine genotypes may be able to prevent vaccine-derived outbreaks that occur through the reversion of attenuating mutations through mutation and recombination (200). More recently, host-targeted approaches have also emerged that alter the evolutionary landscape of RNA viruses, such as inhibitors of proteins. Such examples of new modes of evolutionary medicine are exciting but still only emerging.

Another important developing concept in RNA virus evolution is the polyploid nature of infection. Often, more than one genome initiates infection and, even in cases where a single viral RNA initiates infection, that soon gives rise to many diverse progeny genomes. Therefore, polyploidy within the infected cell provides a source of genetic and phenotypic complementation to an extreme not seen in other organisms. En bloc transmission within MVBs serves to increase the rate of coinfection (and thus viral diversity) of targeted cells within a host, as well as to potentially mask circulating viruses from the host immune system. Work to uncover the exact makeup of virus-containing MVBs could help identify specific ways to target them and reduce spread (141, 198).

Emerging methods in the fields of single-cell biology, bioinformatics, RNA sequencing, phylogenetics, and population genetics are being combined in new ways to provide a more detailed view of RNA virus evolution across scales and complex selective landscapes. Exploiting these new technologies allows us to monitor and respond to the evolutionary dynamics of RNA virus populations. RNA viruses continue to inform our understanding of the basic mechanisms of evolution, but we are also beginning to unravel how the mechanisms of RNA virus evolution influence the pathogenesis, persistence, and transmission of RNA viruses within and between hosts.

SUMMARY POINTS

1. RNA viruses exhibit the highest mutation rates and recombination rates in nature and exhibit a remarkable capacity to evolve.
2. New technologies allow us to measure the fitness and evolutionary fate of individual mutations in vivo and in vitro.

3. Selection across spatial and temporal scales is reflected in the evolutionary path of viral populations.
4. New mechanisms of robustness have been identified that overcome the effect of drift during intrahost and interhost transmission.

DISCLOSURE STATEMENT

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