Thinking Outside the Triangle: Replication Fidelity of the Largest RNA Viruses

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Abstract

When judged by ubiquity, adaptation, and emergence of new diseases, RNA viruses are arguably the most successful biological organisms. This success has been attributed to a defect of sorts: high mutation rates (low fidelity) resulting in mutant swarms that allow rapid selection for fitness in new environments. Studies of viruses with small RNA genomes have identified fidelity determinants in viral RNA-dependent RNA polymerases and have shown that RNA viruses likely replicate within a limited fidelity range to maintain fitness. In this review we compare the fidelity of small RNA viruses with that of the largest RNA viruses, the coronaviruses. Coronaviruses encode the first known viral RNA proofreading exoribonuclease, a function that likely allowed expansion of the coronavirus genome and that dramatically increases replication fidelity and the range of tolerated variation. We propose models for regulation of coronavirus fidelity and discuss the implications of altered fidelity for RNA virus replication, pathogenesis, and evolution.

INTRODUCTION

RNA viruses constitute some of the most ubiquitous and lethal human pathogens. The emergence of Ebola virus, new human influenza viruses, severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome coronavirus (MERS-CoV) underscores the capacity of RNA viruses to cause new human diseases. RNA viruses—notably HIV and influenza—also demonstrate emergence of resistance to antivirals and vaccines. Such adaptive potential results from several characteristics of RNA viruses including rapid replication cycles, enormous population size, and extensive genetic diversity. However, high adaptive potential is not without cost; low-fidelity replication imposes genome size constraints such that most RNA virus genomes do not exceed ~15 kb (1–3). In this review we highlight significant advances in understanding the determinants of RNA virus replication fidelity and the implications of altered fidelity for virus replication, fitness, and pathogenesis. We devote particular attention to the coronaviruses (CoVs), which encode genomes up to 32 kb long and thus are an exception to the constraint on RNA genome size and complexity. Additionally, we summarize recent experimental data and comparative genomics analyses demonstrating that CoVs have evolved a network of replicase proteins that increase the efficiency of RNA replication and regulate replication fidelity through a novel RNA proofreading activity. Finally, we discuss the possibility that CoV RNA replication involves a large multisubunit polymerase similar in organization to replicative DNA polymerase complexes.

SARS-CoV: severe acute respiratory syndrome coronavirus

MERS-CoV: Middle East respiratory syndrome coronavirus

Replication fidelity: the accuracy with which genetic information, either DNA or RNA, is copied

CoV: coronavirus

Proofreading: an error-correcting process involving the removal of a mismatched nucleotide during RNA or DNA synthesis

Zoonotic: describes an infectious agent capable of transmission between animals and humans

HUMAN CORONAVIRUSES: EMERGENCE, GENOME ORGANIZATION, AND REPLICATION

Emergence and Human Disease

CoVs cause significant morbidity and mortality in humans (4), from mild common colds to lethal respiratory and systemic diseases. Five CoVs are known to circulate in humans (HCoVs): HCoV-NL63, HCoV-229E, HCoV-OC43, HCoV-HKU1, and MERS-CoV. Although SARS-CoV is not known to be present in human populations, a SARS-like CoV recently was discovered in Chinese horseshoe bats (Rhinolophidae) (5). This virus can use human ACE2 (angiotensin-converting enzyme 2) as a receptor and is >99% identical to SARS-CoV, indicating that the immediate SARS-CoV zoonotic precursor is likely present in bat populations. Molecular clock analyses suggest that all endemic HCoVs originated as zoonotic infections and emerged into humans over several hundred years: HCoV-NL63 between 1190 and 1449 CE (6), HCoV-229E between 1501 and 1883 CE (7, 8), and HCoV-OC43 between 1866 and 1918 CE (9). The timing of HCoV-HKU1 emergence remains unknown (10). SARS-CoV was identified in 2003 (11) and MERS-CoV in 2012 (12), both within a year of entering the human population, emphasizing the capacity of CoVs to cross species barriers and thrive in new host environments.

Though CoVs infect a large number of mammalian and avian species (13), the majority of the identified diversity within alpha- and betacoronaviruses exists in bats (14). CoVs are predicted to have coevolved with bats for millions of years, possibly since the evolutionary split between bats and birds (15, 16). All known HCoVs have proposed bat origins with the exception of HCoV-OC43, which emerged from a bovine CoV (6). However, movement of zoonotic CoVs into human populations may require passage or coemergence with other mammalian species, as exemplified by SARS-CoV emergence (17), which involved cyclical passage between civet cats and humans. The closest known relative of HCoV-229E recently was discovered in alpacas, suggesting interspecies transmission to humans might have been through alpacas (8) instead of directly from bats (7).

MERS-CoV is the most recently emerged HCoV, and its transmission history is still being defined. MERS-CoV originally was isolated in June 2012, and new cases continue to be identified, with more than 243 laboratory-confirmed cases as of April 2014 and more than 93 deaths. Emerging data suggest the involvement of an additional species in maintenance of MERS-CoV transmission to humans, and at present dromedary camels are the prime suspects (18, 19). There is clear evidence for limited human-human transmission (20, 21), and the continued evidence for new infections suggests the potential for further adaptation.

(+)ssRNA: positive-sense single-stranded RNA

Nsp: nonstructural protein

RdRp: RNA-dependent RNA polymerase

Genome Organization and Replication Strategy

The order Nidovirales contains four families: the Arteriviridae, Coronaviridae, Mesoniviridae, and Roniviridae. CoVs comprise the Coronavirinae subfamily within the Coronaviridae family and demonstrate a remarkable diversity across many animal species. All CoVs have positive-sense single-stranded RNA [(+)ssRNA] genomes, which possess a 5′ cap structure and a 3′ poly(A) tail and are contained within a pleomorphic host membrane–derived envelope (reviewed in 22). The genome organization is similar for all CoVs, with the viral genome divided roughly into two major regions: the nonstructural protein genes and the structural and accessory protein genes (**Figure 1*a***). Compared with other positive-strand RNA viruses, CoVs encode the most extensive ensemble of replicase and transcriptase proteins, which can include up to 16 nonstructural proteins (nsp1–16) (22). Proteins involved in viral replication and transcription compose the first two-thirds of the viral genome and are contained in two open reading frames, ORF1a and ORF1b. ORF1a is translated from genome RNA with every translational initiation, resulting in the expression of polyprotein 1a (pp1a), which is composed of nsp1–11. Translation of ORF1b requires a −1 ribosomal frameshift immediately following the nsp10 coding region, which occurs less than 40% of the time and results in the fusion polyprotein 1ab (pp1ab), which contains nsp1–16 (23, 24). Consequently, nsp1–11 are produced more abundantly than nsp12–16. Processing of both polyproteins by two or three viral proteases yields 16 mature nsps and several intermediate precursors, with the vast majority of these mature nsps known or predicted to function in virus replication complex formation and RNA synthesis (22, 25, 26).

CoV RNA synthesis can be divided into two general stages: genome replication and subgenomic mRNA synthesis (22). Genome replication is initiated following translation of the viral genome by host machinery and processing of replicase nsps by viral proteases. The (+)ssRNA genome is transcribed into a full-length (−)ssRNA intermediate, which is then used as the template for amplification of the (+)ssRNA genome (**Figure 1*b***). Expression of the 3′ structural and accessory proteins occurs from subgenomic mRNAs (sgmRNAs) initiated by a discontinuous transcription mechanism. Negative-strand subgenomic RNA [(−)sgRNA] templates are generated from the 3′ end of the (+)ssRNA genome and are regulated by short transcriptional regulatory sequences (TRSs) located in the 5′ untranslated region (UTR) of the (+)ssRNA genome, termed the leader TRS, and by those present immediately upstream of each 3′ ORF (**Figure 1*b***). Recognition of each 3′ TRS by the viral RNA-dependent RNA polymerase (RdRp) can result in either read-through or dissociation of the (−)sgRNA template from the (+)ssRNA genome. The (−)sgRNA-RdRp complex then reassociates with the 5′ leader TRS, thus generating a set of (−)sgRNAs that contain one or more ORFs as well as 3′ and 5′ termini identical to the negative-strand genome template. These (−)sgRNA templates are then used as the primary templates to generate sgmRNAs that possess 5′ and 3′ sequences identical to each other and to the genome. This nested set of mRNAs is the basis for the name of the order Nidovirales, of which CoVs are members. The sgmRNAs also are used as amplification templates for subsequent rounds of (−)sgRNA synthesis (27).

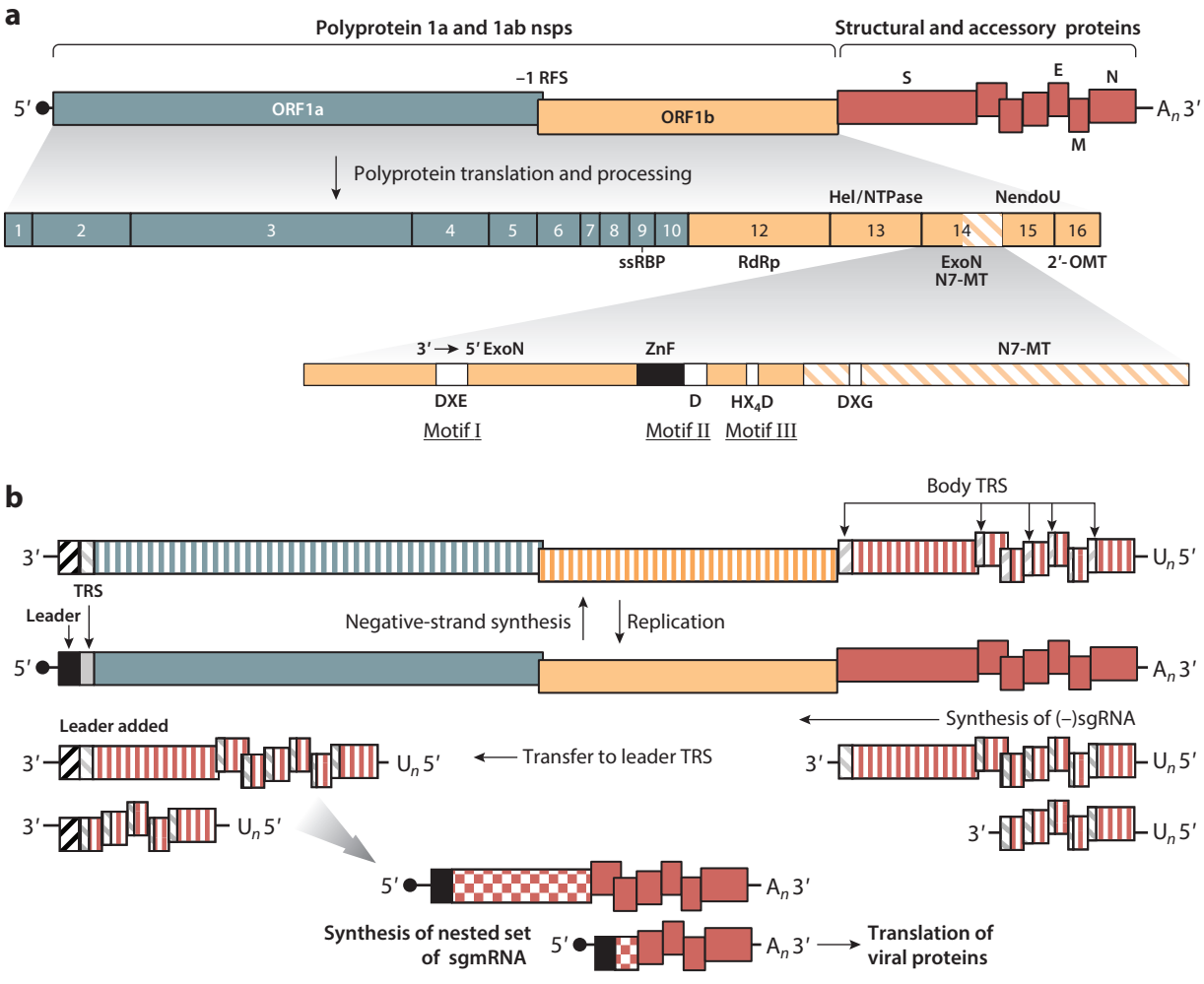

Figure 1

Coronavirus genome organization and replication strategy. (*a*) Open reading frame (ORF) 1ab encompasses roughly two-thirds of the genome and encodes the replicase nonstructural proteins (nsp1–16). Nsp11 is ~15 amino acids long with no known function and thus is not shown. The other one-third of the genome encodes structural and accessory proteins including the spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins. The structural and accessory proteins are shown for a generic CoV, as the accessory proteins vary both in number and in position between CoVs. The nonstructural proteins nsp1–11 are translated from ORF1a (*blue*), whereas translation of the ORF1b proteins (nsp12–16) only occurs following a −1 ribosomal frameshift (RFS). Polypeptides containing nsp1–11 (pp1a) or nsp1–16 (pp1ab) are cleaved by up to three viral proteases to generate individual nsps. The functions of several nsps are noted: nsp9, single-stranded RNA–binding protein (ssRBP); nsp12, RNA-dependent RNA polymerase (RdRp); nsp13, helicase (Hel) and NTPase; nsp14, 3′→5′ exoribonuclease (ExoN) and *N*7-methyltransferase (N7-MT); nsp15, uridylate-specific endonuclease (NendoU); nsp16, 2′-*O*-methyltransferase (2′-OMT). The active site residues of nsp14 involved in proofreading are distributed across three motifs (I–III). Additionally, a zinc finger domain (ZnF) is encoded, which is unique among the DEDD superfamily of exonucleases to which nsp14 ExoN belongs. (*b*) CoV replication (*top*) involves the production of full-length negative-strand intermediates (*striped*) that are used to generate new positive-strand genomes (*solid*). Transcription and translation (*bottom*) of the 3′ ORFs requires the generation of negative-strand subgenomic RNAs [(−)sgRNAs] (*striped*) that are then used to make viral subgenomic messenger RNA (sgmRNA). Only the 5′-most ORF (*checkered*) is translated for each sgmRNA. Abbreviation: TRS, transcriptional regulatory sequence.

Replicase Proteins and Novel RNA-Modifying Functions

Studies of the CoV RNA synthesis machinery have focused on the identification and functions of nsp1–16 (**Figure 1*a***). Several CoV nsps have been shown to play prominent roles in RNA synthesis and modification by harboring one or more enzymatic activities: the nsp8 primase (28, 29), nsp12 RdRp (30–32), nsp13 helicase (Hel) and NTPase (33, 34), nsp14 *N*7-methyltransferase (N7-MT) (35) and exoribonuclease (ExoN) (36), nsp15 uridylate-specific endoribonuclease (NendoU) (37), and nsp16 RNA 2′-*O*-methyltransferase (2′-OMT) (38). Other viral proteins without predicted or confirmed enzymatic activities also contribute to viral RNA synthesis and modification, including nsp7, which interacts with nsp8 to form a large hexadecameric ring-like structure (39), and nsp10, which is critical for viral RNA synthesis (40) and interacts with both nsp14 and nsp16 (41–44). The nsp12 RdRp is the catalytic core of the CoV RNA synthesis machinery and consists of at least two domains: an N-terminal domain unique to CoVs and a C-terminal catalytic domain that is predicted to adopt a structure similar to other viral RdRps (45). CoV nsp12 contains an SDD active site motif that is conserved in all members of the Nidovirales order (46). Work by te Velthuis et al. (30), using full-length nsp12 containing a native N terminus, demonstrated that nsp12 contains weak primer-dependent RdRp activity; another group (32) has reported that nsp12 RdRp can initiate de novo. Primase activity is likely provided by nsp8, which contains low-fidelity RdRp activity similar to known DNA-dependent DNA primases (28, 29). Even less is known about the unique N-terminal region, except that portions or all of the domain is required for in vitro RdRp activity (31) and that it appears to lack homology to any protein identified to date. The encoding of such a diverse repertoire of RNA-modifying functions suggests that CoVs have evolved a network of proteins that might be more analogous to cellular RNA and/or DNA synthesis machinery. This possibility, particularly relating to the mechanisms by which CoVs maintain the integrity of their large RNA genomes, is discussed further below.

ExoN: exoribonuclease

Mismatch repair: a postreplicative process distinct from proofreading that detects and repairs misincorporated or damaged bases

RNA VIRUS REPLICATION FIDELITY: DETERMINANTS AND THE EFFECT ON VIRAL POPULATIONS

A Comparison with Cellular Organisms

Both cellular life forms and all viruses must faithfully replicate their genomes to ensure the transmission of genetic information. For cellular organisms, faithful reproduction of genetic material is mediated by a network of proteins tasked with detecting and repairing errors during or subsequent to DNA synthesis. In its entirety, this process is referred to as replication fidelity, and it encompasses at least three critical steps: (*a*) selection and extension of the correct nucleotide onto the replicating DNA strand by a DNA polymerase, (*b*) removal of mismatched nucleotides by intrinsic 3′→5′ ExoN activity within the DNA polymerase or by a closely associated 3′→5′ ExoN–containing subunit, and (*c*) correction by the cellular mismatch repair system of errors that have escaped proofreading (**Figure 2**) (47–49). Each of these processes contributes to cellular DNA replication fidelity, but the relative contributions of these steps toward the estimated error rate of 10^{-9} to 10^{-11}, or one error per 10^{9} to 10^{11} nucleotides, vary. Correct nucleotide selection, recognition of a properly formed base pair, and extension by the DNA polymerase together provide a greater contribution to fidelity (10^{-5}) than either proofreading (10^{-2}) or the mismatch repair system (10^{-3}), though the relative contributions are likely error type specific (47, 50, 51). The fidelity of DNA and RNA polymerases likely results more from polymerase dynamics—such as recognition of the shape of correct versus incorrect base pairs and conformational changes within the polymerase active site—than from the capacity of the polymerase to selectively discriminate between nucleotides (52–58). Beyond these mechanisms, DNA-based cellular organisms also have

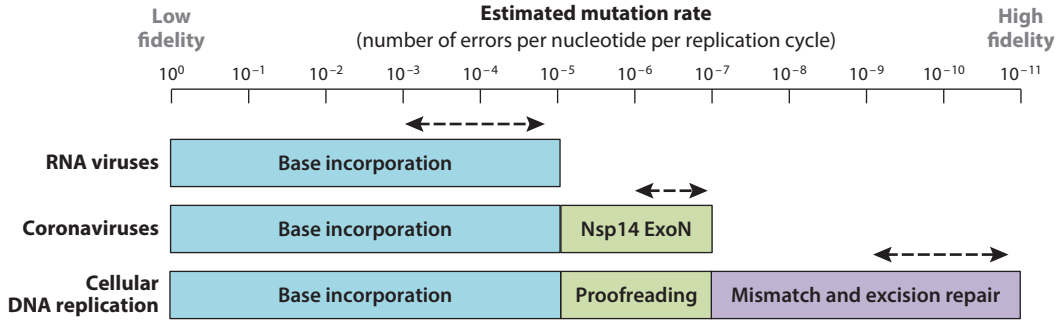

Figure 2

Replication fidelity in RNA viruses and DNA-based organisms. Shown is the estimated range of mutation rates (*dashed arrows*) for RNA viruses, coronaviruses, and cellular DNA replication. The relative contributions of polymerase base incorporation (*cyan*), proofreading (*green*), and mismatch and excision repair (*purple*) toward the estimated mutation rates are shown. See the text for specific references regarding the estimated mutation rates. Abbreviation: nsp14 ExoN, nonstructural protein 14 exoribonuclease.

machinery dedicated to repairing damaged bases, removing UV-induced pyrimidine dimers, and rejoining double-strand breaks. Overall, cellular DNA replication is orchestrated by numerous accessory proteins and by an ensemble of DNA polymerases: 5 for *Escherichia coli*, 8 for *Saccharomyces cerevisiae*, and at least 16 for humans (recently reviewed in 47).

Compared with cellular organisms, RNA viruses are minimalists in terms of replication fidelity. The primary determinant of RNA virus genome replication, with the exception of the retroviruses, is the RdRp. Positive-strand RNA viruses with genomes <20 kb lack proofreading and other postreplicative repair mechanisms (59) and therefore rely on the viral RdRp for maintenance of genome sequence integrity. This reliance on the viral RdRp theoretically limits the fidelity of RNA viruses to an upper limit in the range of $\sim 10^{-5}$. In fact, most RNA viruses replicate with estimated error rates between 10^{-3} and 10^{-5}, which results in approximately one mutation per genome per round of replication for a typical ~10-kb genome (2, 60). This fidelity range is similar to that observed with exonuclease-deficient DNA polymerases, and in fact, error-prone Y family DNA polymerases polymerize with fidelities much lower than 10^{-5} (49, 61–63). Thus, the low fidelity of RNA virus genome replication is likely not because RdRps polymerize RNA with significantly lower fidelity than their DNA polymerase counterparts; rather, low fidelity is primarily a result of the lack of proofreading activity (59, 64). This hypothesis is supported by the recent discovery that CoVs and other members of the Nidovirales order with genomes >20 kb encode a 3′→5′ ExoN (36, 65) involved in maintaining CoV replication fidelity (66–69). The estimated mutation rates of murine hepatitis virus (MHV) and SARS-CoV are approximately 2.5×10^{-6} and 9×10^{-7} mutations per nucleotide per replication cycle, respectively (**Figure 2**). Genetic inactivation of CoV ExoN activity reduces these estimated mutation rates to the range observed in other RNA viruses and comparable to that of DNA polymerases lacking exonuclease activity (67, 68).

Diversity of RNA Virus Populations

MHV: murine hepatitis virus

RNA viruses are among the most diverse replicative units in existence (70), and they demonstrate a remarkable capacity for adaptation due in part to high mutation rates during replication. RNA viruses replicate exponentially, resulting in populations that can theoretically include a substitution at every site in the genome (71). As a result of this replicative capacity, RNA viruses exist as populations of heterogeneous yet genetically related viruses, often referred to as mutant swarms,

mutant clouds, or quasispecies. The defining principle of viral quasispecies evolution is that viral populations are composed of variants capable of interacting in a cooperative or antagonistic manner (see 71, 72 for recent reviews on viral quasispecies evolution). As a result, quasispecies evolution posits that it is then the viral population, and not an individual variant, that is the subject of natural selection. Mutant swarms have been shown to act as a collective during infection (73); one of the most definitive examples of this phenomenon occurs during poliovirus infection. Poliovirus is neurotropic in humans and mouse models. Poliovirus populations that have been genetically bottlenecked with reduced diversity lose their neurotropism, whereas expanding diversity of the poliovirus population by passage in the presence of RNA mutagen reestablishes neurotropism (74). This cooperativity might allow initially deleterious mutations or less fit variants to be maintained within a population, potentially facilitating more rapid adaptation under selective pressure or in new host environments (73, 75).

Altered-Fidelity Variants

The replication fidelity of RNA viruses is likely evolutionarily constrained within a range that balances genome stability with the generation of sufficient genetic diversity. Therefore, the mechanisms by which RNA virus replication fidelity is maintained must be evolutionarily finely tuned to achieve these contrasting but important goals. A frequent misconception regarding viral mutation rates, particularly in response to a perceived danger of mutator variants (69, 76), is that increasing the mutation rate proportionally increases the rate at which viral populations adapt (77, 78). In contrast to this supposition, although the normally high mutation rates of RNA virus replication contribute to the adaptive capacity of RNA viruses, increasing the mutation rate beyond that of the wild-type virus often results in a decrease in fitness (69, 76, 79). This is primarily due to the probability that the majority of random mutations are deleterious (60, 71, 79). RNA viruses are thought to replicate at an error threshold; thus, even marginal increases in mutation rates could result in an excess of deleterious mutations within the population (see 72 for an excellent review). On the other hand, increasing fidelity also results in decreased pathogenesis and spread, either through a decrease in replication speed, an impaired ability to adapt, or both (71, 80–82).

Though many factors likely contribute to the high adaptive potential of RNA viruses, one of the key contributors is the viral RdRp. Much like their DNA polymerase counterparts, RdRps catalyze nucleotide polymerization and are the core machinery by which RNA viruses replicate their genomes. The structural and dynamic aspects of RdRp fidelity remain an active area of research, but early studies with the HIV reverse transcriptase and with *E. coli* and bacteriophage T4 DNA polymerases demonstrated that point mutations can increase or decrease polymerase fidelity, suggesting that changes to RdRp fidelity might be possible (83–85). The first example of an RdRp with altered fidelity was the poliovirus RdRp G64S mutation (74, 86). This mutation was identified independently by two groups following passage of poliovirus (PV) in the presence of the antiviral nucleoside analog ribavirin, and it resulted in a ~3-fold increase in replication fidelity. Later studies by both groups demonstrated that this single mutation resulted in viral attenuation in vivo, and that attenuation was likely the result of restricting the genetic diversity of the virus population (80, 81). These studies provided the first evidence that increased fidelity was attainable for an RNA virus. Subsequently, fidelity variants with mutations in viral RdRps have been isolated for other picornaviruses and arboviruses (**Figure 3** and **Table 1**) (67, 68, 74, 76, 80, 81, 86–99). Most reported altered-fidelity mutants deviate from the fidelity of the wild-type virus by a maximum of 4- to 5-fold and often are attenuated in vivo (**Figure 3**). A recent study using coxsackievirus B3 (CVB3) reported that mutations within the CVB3 RdRp that reduced fidelity by more than ~3-fold resulted in virus nonrecovery and suggested that this might represent the

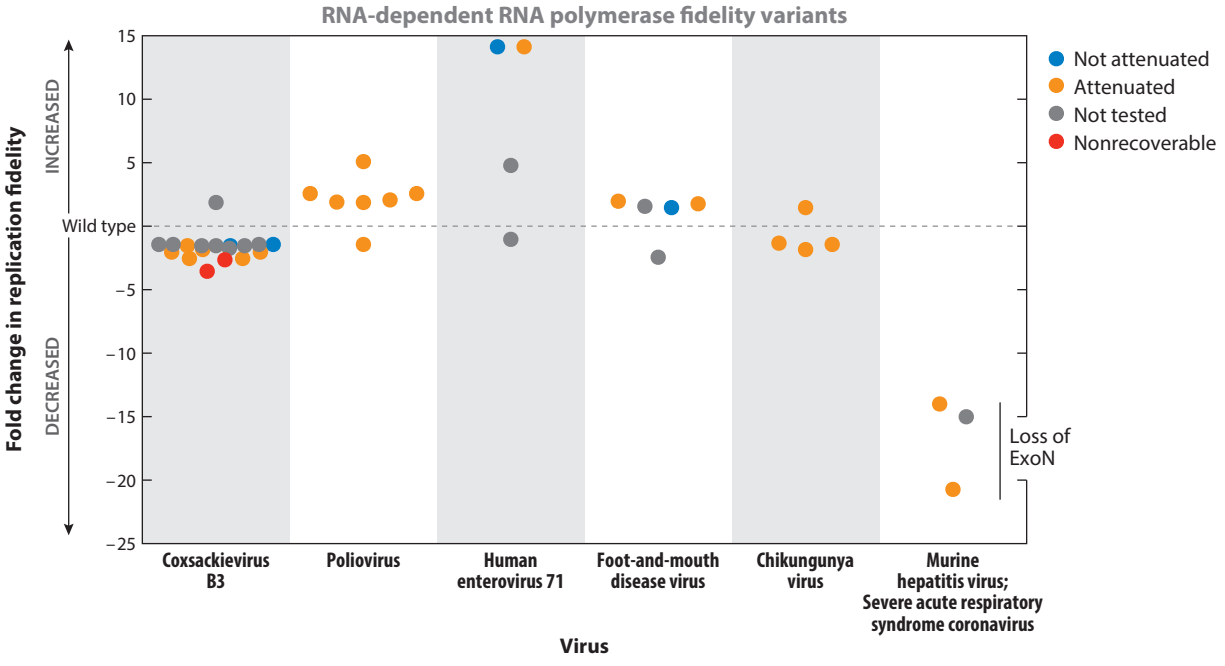

Figure 3

Fidelity variants of RNA viruses. Published RNA virus fidelity variants (excluding retroviruses) are shown. For each variant, the fold change in replication fidelity was calculated using previously published data and compared with the wild type (*dotted horizontal line*). See the text and **Table 1** for specific references for each point. All points are colored according to whether or not the fidelity-altering mutation resulted in attenuation in vivo: blue, not attenuated; orange, attenuated; gray, attenuation not tested. The two red points denote mutations resulting in nonrecoverable viruses.

lower limit of RdRp fidelity for the picornaviruses (76). To our knowledge, no other group has reported RdRp mutants with lower fidelity. An upper limit for alterations in RdRp fidelity has yet to be described. These studies support the hypothesis that RNA viruses replicate within an evolutionarily selected range of fidelity, and that deviation outside of this range profoundly impacts virus fitness in vitro and in vivo.

The CoV Exoribonuclease in Replication Fidelity and Pathogenesis

In contrast to the picornaviruses and arboviruses, CoVs encode a 3′→5′ ExoN activity in nsp14 that is critical for replication fidelity. The larger members of the Nidovirales order (i.e., Coronaviridae and Roniviridae) with genomes of 26 to 32 kb encode ExoN, whereas it is absent in the smaller Arteriviridae family members with genomes <16 kb (25, 65). The recent identification of the Nam Dinh virus (NDiV), an insect nidovirus that encodes ExoN in its 20-kb genome, provides an important link in the transition from small to large nidoviruses (100, 101). CoV nsp14 is approximately 530 amino acids long and contains two enzymatic activities (**Figure 1*a***): N-terminal ExoN activity and C-terminal N7-MT activity (35). The ExoN domain contains four acidic residues, DEDD, in three motifs, which are a defining characteristic of the DEDD superfamily of RNA and DNA exonucleases (102). In addition to these four invariant residues, CoV nsp14 ExoN contains a highly conserved histidine residue within motif III resulting in an HX_4D arrangement (25, 36). Members of this DEDDh subgroup include proofreading enzymes such

Table 1 Fidelity variants of RNA viruses

Virus	Reference(s)	Mutation(s)	Protein	System[a]	Method[b]	Fold change[c]	Attenuated[d]
CVB3	76	L241I	3Dpol	Virus	Fragment sequencing	−1.5	No
		I230F, M145L, S299T	3Dpol	Virus	Fragment sequencing	−1.5	–
		S299T, A372V	3Dpol	Virus	Fragment sequencing	−1.5	–
		S164P	3Dpol	Virus	Fragment sequencing	−1.6	No
		P48K	3Dpol	Virus	Fragment sequencing	−1.6	Yes
		I230F, S299T	3Dpol	Virus	Fragment sequencing	−1.6	–
		F232Y, S299T	3Dpol	Virus	Fragment sequencing	−1.6	–
		F232Y, S299T, A372V	3Dpol	Virus	Fragment sequencing	−1.6	–
		I230F, M145L	3Dpol	Virus	Fragment sequencing	−1.8	–
		A239G	3Dpol	Virus	Fragment sequencing	−1.9	Yes
		Y268W	3Dpol	Virus	Fragment sequencing	−2.1	Yes
		Y268H	3Dpol	Virus	Fragment sequencing	−2.1	Yes
		F232Y	3Dpol	Virus	Fragment sequencing	−2.6	Yes
		I230F	3Dpol	Virus	Fragment sequencing	−2.6	Yes
		F232V	3Dpol	Protein	Kinetic parameters	−2.7	–
		F232L	3Dpol	Protein	Kinetic parameters	−3.6	–
	88, 98	S299T	3Dpol	Virus/protein	Multiple methods	−1.5	–
		A372V	3Dpol	Virus/protein	Multiple methods	1.8	–
PV	74, 80, 81, 86, 89	G64V	3Dpol	Virus	Fragment sequencing	1.8	Yes
		G64L	3Dpol	Virus	Fragment sequencing	1.8	Yes
		G64A	3Dpol	Virus	Fragment sequencing	2.0	Yes
		G64S	3Dpol	Virus/protein	Multiple methods	2.0–4.0	Yes
		G64T	3Dpol	Virus	Fragment sequencing	2.5	Yes
	93	T362I	3Dpol	Virus/protein	Kinetic parameters	−1.5	Yes
	96	K359R	3Dpol	Virus/protein	Kinetic parameters	5.0	Yes
HEV71	94, 95	G64N	3Dpol	Virus	Fragment sequencing	−1.1	–
		G64T	3Dpol	Virus	Fragment sequencing	4.7	–
		G64R	3Dpol	Virus	Fragment sequencing	14.0	–
		S264L	3Dpol	Virus	Fragment sequencing	14.0	Yes
FMDV	97	M296I	3Dpol	Virus/protein	Multiple methods	−2.5	–
	91	R84H	3Dpol	Virus	Fragment sequencing	1.4	No
	92	D5N	3Dpol	Virus	Fragment sequencing	1.5	No
		A38V	3Dpol	Virus	Fragment sequencing	1.7	Yes
		D5N, A38V, M194I, M296V	3Dpol	Virus	Fragment sequencing	1.9	Yes
CHIKV	87	C483Y	Nsp4	Virus	Fragment sequencing	1.4	Yes
	90	C483A	Nsp4	Virus	Fragment sequencing	−1.4	Yes
		C483W	Nsp4	Virus	Fragment sequencing	−1.5	Yes
		C483G	Nsp4	Virus	Fragment sequencing	−1.9	Yes

(*Continued*)

Table 1 (*Continued*)

Virus	Reference(s)	Mutation(s)	Protein	System[a]	Method[b]	Fold change[c]	Attenuated[d]
MHV	67	D89A, E91A	Nsp14	Virus	Full-genome sequencing	−15.0	–
SARS-CoV	68	D90A, E92A	Nsp14	Virus	Full-genome sequencing	−20.7	–
	69	D90A, E92A	Nsp14	Virus	Full-genome sequencing	−14.0	Yes

Abbreviations: CHIKV, chikungunya virus; CVB3, coxsackievirus B3; FMDV, foot-and-mouth disease virus; HEV71, human enterovirus 71; MHV, murine hepatitis virus; PV, poliovirus; SARS-CoV, severe acute respiratory syndrome coronavirus.

[a]"Virus" denotes the recovery of virus, whereas "protein" denotes recombinantly expressed protein.

[b]Sequencing was performed on either viral supernatants, viable plaques, or total intracellular viral RNA.

[c]Fold change was calculated using values reported in each reference. A range is reported if values were independently reported by more than one group.

[d]Dashes indicate that the attenuation phenotype is unknown.

as the ε subunit of *E. coli* DNA polymerase III (Pol III). This similarity early on suggested a role for nsp14 ExoN in proofreading and/or other aspects of RNA processing (25). In contrast with its cellular counterparts, CoV ExoN also contains a unique zinc finger domain of unknown function between motifs I and II. Biochemical confirmation of ExoN activity using bacterially expressed nsp14 from HCoV-229E demonstrated that ExoN is capable of cleaving both ssRNA and dsRNA in a 3′→5′ direction (36). Recombinant HCoV-229E genomes containing mutations that inactivated ExoN activity did not allow recovery of replication-competent virus and exhibited profound defects in viral RNA synthesis. In contrast, MHV and SARS-CoV containing mutations in motifs I and III were replication competent, albeit with reductions in viral RNA synthesis (67, 68). Alanine substitution of the DEDDh residues does not impair N7-MT activity of purified nsp14 in vitro (35, 103); however, other mutations within nsp14 ExoN have been demonstrated to affect N7-MT activity in vitro, indicating that ExoN and N7-MT functions are evolutionarily linked or potentially serve a novel function in CoV RNA synthesis (103).

Recombinant MHV lacking ExoN activity (ExoN$^-$) accumulated 15-fold more mutations compared with wild-type MHV with intact ExoN (ExoN$^+$) (67). Recombinant SARS-CoV ExoN$^-$ also demonstrated an almost identical mutator phenotype in culture and during mouse infection using ExoN$^-$ variants of virulent mouse-adapted SARS-CoV (MA-SARS) (68, 69). In both cases, between 14- and 20-fold more mutations were present within the ExoN$^-$ viruses as compared with ExoN$^+$ viruses (**Table 1** and **Figure 3**). ExoN inactivation profoundly attenuated the pathogenesis of MA-SARS in young, aged, and immunocompromised mice (67, 69). Both the genotype and phenotype of ExoN$^-$ MHV and SARS-CoV were stable over extended passage in culture and in mice. Furthermore, MA-SARS ExoN$^-$ did not revert to virulence even during persistent infection of SCID (severe combined immunodeficient) mice. Although the precise mechanism of fidelity regulation by ExoN remains to be defined, all available biochemical and virological evidence supports the conclusion that nsp14 ExoN provides a critical proofreading function during CoV replication (36, 42, 66–69, 104). Both MHV ExoN$^-$ and SARS-CoV ExoN$^-$ viruses demonstrated increased sensitivity to the RNA mutagen 5-fluorouracil (5-FU) as compared with ExoN$^+$ viruses (66). Next-generation sequencing of SARS-CoV RNA following treatment with 5-FU indicated that the ExoN$^-$ virus populations accumulated 40-fold more mutations compared with untreated ExoN$^-$ population, and 24-fold more mutations compared with 5-FU-treated wild-type ExoN$^+$ virus (66). These results together demonstrate a role for ExoN in maintaining

CoV replication fidelity, establish a link between CoV fidelity and pathogenesis, and provide the most direct evidence that ExoN is the first known proofreading enzyme encoded by an RNA virus.

It remains to be determined whether nsp14 ExoN mediates other functions in virus replication or host interactions. Arenaviruses such as Lassa fever virus (LASV) are the only other mammalian RNA viruses known to encode a 3′→5′ ExoN (105). LASV nucleoprotein (NP) is a DEDDh ExoN that specifically degrades double-stranded RNA (dsRNA) and thus is a critical component of immune evasion (105). SARS-CoV nsp1 has been shown to induce endonucleolytic cleavage of host mRNAs (106); however, there is as yet no direct evidence that nsp14 ExoN degrades host mRNAs. CoV ExoN was shown to cleave both dsRNA and ssRNA in vitro (36), and ExoN-mediated cleavage of ssRNA resulted in larger cleavage products as compared with cleavage of dsRNA. These data suggest that nsp14 ExoN could potentially exert differential activity on diverse RNA species (36). More recent work demonstrated that the small nonenzymatic CoV protein nsp10 can stimulate ExoN activity by almost 35-fold and renders ExoN capable of cleaving 3′ mismatched residues (42). Thus, the specificity and activity of ExoN on various viral or host RNA substrates conceivably could be determined through interactions with other viral proteins. Aside from nsp10, CoV nsp14 ExoN could also function in tandem with the viral endonuclease nsp15 NendoU to degrade RNA targets. In fact, CoV nsp15 NendoU has an uncharacterized interferon antagonist activity, and the arterivirus homolog of nsp15 was recently shown to inhibit interferon β induction (107, 108). The capacity of ExoN to cleave a variety of RNA substrates and the uncharacterized interferon antagonist activity identified for nsp15 NendoU suggest that these two proteins could be important for suppression of anti-CoV immune responses (108). If so, such a function for ExoN could contribute to the attenuation of MA-SARS ExoN$^-$ observed in vivo.

CORONAVIRUS GENOME SIZE: SOLVING THE EIGEN PARADOX

RNA Virus Genome Size and the Eigen Paradox

Unlike in DNA viruses and DNA-based organisms, the genome size distribution of ssRNA genomes from different virus families is quite narrow; the largest ssRNA virus genomes are ~32 kb in length (65). Excluding the nidoviruses, (+)ssRNA viruses range in size from ~2,300 to ~20,000 bases, with the majority of ssRNA virus genomes measuring ~10 kb (**Figure 4*a***) (65). Assuming near-equivalent error rates, RNA viruses with larger genomes would be predicted to accumulate more mutations per genome during replication, and would in turn be predicted to accumulate more deleterious mutations, leading to virus extinction. This has led to a theoretical limitation to RNA virus genome size termed the Eigen paradox or Eigen trap, after Manfred Eigen's work describing self-replicating molecules (1, 109, 110). In an analysis of CoV evolution, Nga et al. (100) depicted the Eigen trap as a triangle on which genome size, replication fidelity, and genome complexity are located at the apices (**Figure 4*b***). In the center of this triangle are the RNA viruses, which due to their low-fidelity replication are confined within the Eigen trap. Because they are unable to increase replication fidelity, RNA viruses are evolutionarily constrained to have genomes of relatively low complexity and small size.

However, a growing body of work suggests that CoVs have found a way to escape this trap. Recent comparative genomics studies of complete nidovirus genomes proposed that acquisition of ExoN allowed expansion of the smaller ancestor nidovirus genome (65, 100, 111). Once CoVs acquired mechanisms to increase their fidelity and limit the accumulation of deleterious mutations, increases in both genome size and complexity were possible. The acquisition of additional replicase proteins likely allowed both the continued expansion and the divergence of nidoviruses into present-day CoVs (**Figure 4*c***) (111). Conceptually, expansion of ORF1a could have allowed

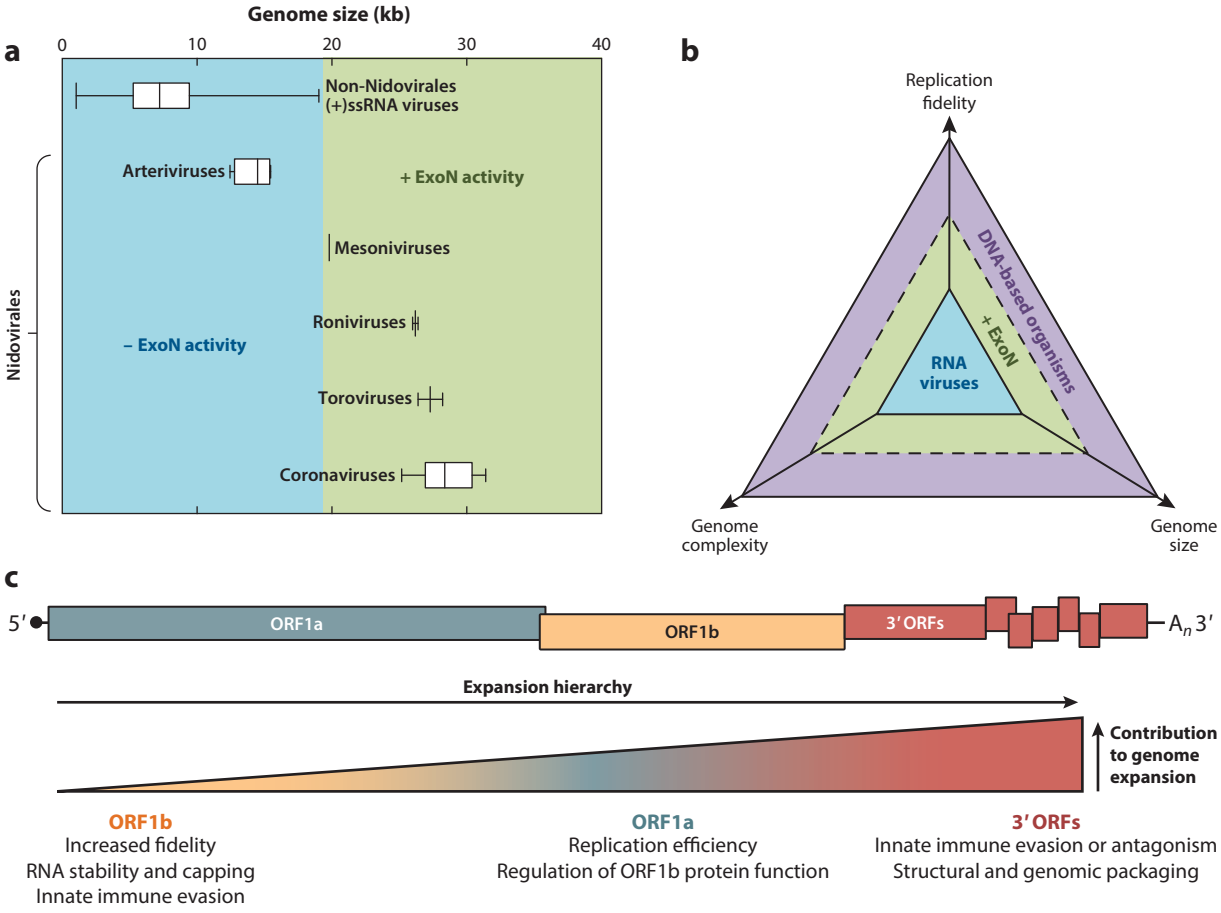

Figure 4

Size of positive-sense single-stranded RNA [(+)ssRNA] virus genomes and expansion of the coronavirus (CoV) genome. (*a*) Median genome size for (+)ssRNA viruses, excluding the nidoviruses, in comparison with each family within the order Nidovirales. Full-length genomes were obtained from the National Center for Biotechnology Information (NCBI) Viral Genomes Resource. Each bar depicts the median genome size and extends from the 25th to 75th percentile; the whiskers show the minimum and maximum values. Genomes containing (*green*) and lacking (*cyan*) 3′→5′ exoribonuclease (ExoN) activity are shown. (*b*) The relationship between replication fidelity, genome complexity, and genome size. Fidelity, complexity, and size increase as the arrows move from the center to the edges of the triangle. RNA viruses have low replication fidelity, low genomic complexity, and small genomes and thus are constrained within the small Eigen triangle (or trap). Acquisition of ExoN likely helped CoVs (*green*) escape this trap by increasing replication fidelity. Panel *b* adapted with permission from Nga et al. (100). (*c*) A schematic of the CoV genome (*top*) depicts open reading frame (ORF) 1a in blue, ORF1b in orange, and the 3′ ORFs in red. The order in which these regions are thought to have expanded—ORF1b→ORF1a→3′ ORFs—is shown (*bottom*). The relative contribution to overall CoV genome size, as defined by the total number of bases added, is denoted by the height of the triangle. See Lauber et al. (111) for additional details. Some predicted activities and functions likely acquired as a result of genomic expansion are also shown.

for increased replication efficiency, possibly through the regulation of ORF1b proteins (111). This hypothesis is supported by studies showing the association of ORF1a products such as nsp7–10 with the ORF1b proteins (38, 41–44, 112–114). Furthermore, the lack of homologous nsp7–10 sequences outside of the coronaviruses and toroviruses suggests that acquisition of these proteins might have also facilitated genomic expansion of the "large" nidoviruses (65). Acquisition of

several 3′ accessory ORFs, excluding essential structural proteins, was shown to have contributed the most to CoV genomic expansion, as defined by the total number of nucleotides added. Both the number and the function of these 3′ ORFs vary tremendously among CoVs.

Processivity: the average number of consecutive nucleotides a polymerase is capable of adding during a single template-binding event

A Model for a Putative Multisubunit Polymerase Complex

Whereas the simplest of RNA viruses encode only an RdRp, other RNA viruses encode a variety of RNA-modifying enzymes, such as RNA capping (or cap snatching) machinery and helicase activity. Due to their large genomes (65, 111), unique RNA-modifying functions, and putative proofreading capability (25, 65–69, 111), CoVs may have more complex RNA replication systems than those described for many other RNA viruses. Additionally, the limited polymerase activity observed for nsp12 RdRp (30–32) alone likely reflects the need for additional viral proteins in order to faithfully and rapidly replicate the CoV genome during infection. These observations suggest the possibility that CoVs employ a multisubunit polymerase complex for viral RNA synthesis. Such a complex has yet to be described experimentally, likely due to challenges of recapitulating in vitro a complex containing nsp12 RdRp and six or more additional replicase proteins. Although detailed biochemical and structural studies will be essential in understanding how the CoV replicase is assembled, a model can be proposed (104, 115) on the basis of known and predicted activities and by analogy to DNA polymerase complexes (**Figure 5**), specifically DNA Pol III.

One of the most extensively studied multisubunit polymerases is *E. coli* DNA Pol III, which is the major polymerase during chromosomal replication (recently reviewed in 116). *E. coli* DNA Pol III is a holoenzyme (DNA Pol III HE) that contains a catalytic core ($\alpha\varepsilon\theta$), a processivity factor (β_2 sliding clamp), and a multisubunit clamp loader that loads β_2 onto the DNA template (116). The catalytic core contains three subunits: the polymerase (α); the $3'\rightarrow5'$ exonuclease (ε); and the small nonenzymatic θ subunit, which stabilizes and stimulates ε (116–118). Much like the Pol III core, CoV nsp12 RdRp likely interacts with nsp14 ExoN, as both proteins would need to be in close proximity for error removal and repair (**Figure 5**). The viral helicase nsp13 is likely upstream but closely associated with nsp12 RdRp (33) to ensure the availability of a single-stranded template. This model is consistent with previous studies describing that a large majority of CoV RNA is present as partially double-stranded RNA, suggesting that multiple RNA templates are being synthesized for each negative-strand template (119). Nsp15 and nsp16 could also be associated with this complex; however, given the undefined role of nsp15 during replication and the role of nsp16 in RNA capping, these proteins might form distinct complexes. Other proteins, particularly those from ORF1a, likely interact with the CoV polymerase core (112–114), which would be consistent with the hypothesis that some ORF1a proteins were acquired to regulate ORF1b proteins and/or to increase the efficiency of replicating an increasingly large CoV genome (111).

Though the functions of several of the ORF1a proteins are just beginning to be defined, several studies support the hypothesis that nsp7–10 are associated with this polymerase core. CoV nsp8 was shown to harbor RdRp and primase activity (28) and to interact with nsp7 to form a large toroidal hexadecamer structure (39). The ~30-Å central pore of the SARS-CoV nsp7-nsp8 hexadecamer is lined with positively charged amino acids, which allow the supercomplex to bind dsRNA. These data suggest that the nsp7-nsp8 hexadecamer could function as a processivity factor for the CoV polymerase during RNA replication, much like the β_2 clamp within the *E. coli* Pol III HE (reviewed in 120) and other processivity factors. The small nonenzymatic nsp10 associates with both CoV methyltransferases: nsp14 N7-MT and nsp16 2′-OMT (41–44). Binding of nsp10 to nsp16 is required for 2′-OMT activity, an enzymatic activity that is critical in mitigating detection of CoV RNA by the innate immune system (121). Nsp10 also stimulates nsp14 ExoN activity in vitro (42), though the significance of an nsp10-nsp14 interaction during virus replication

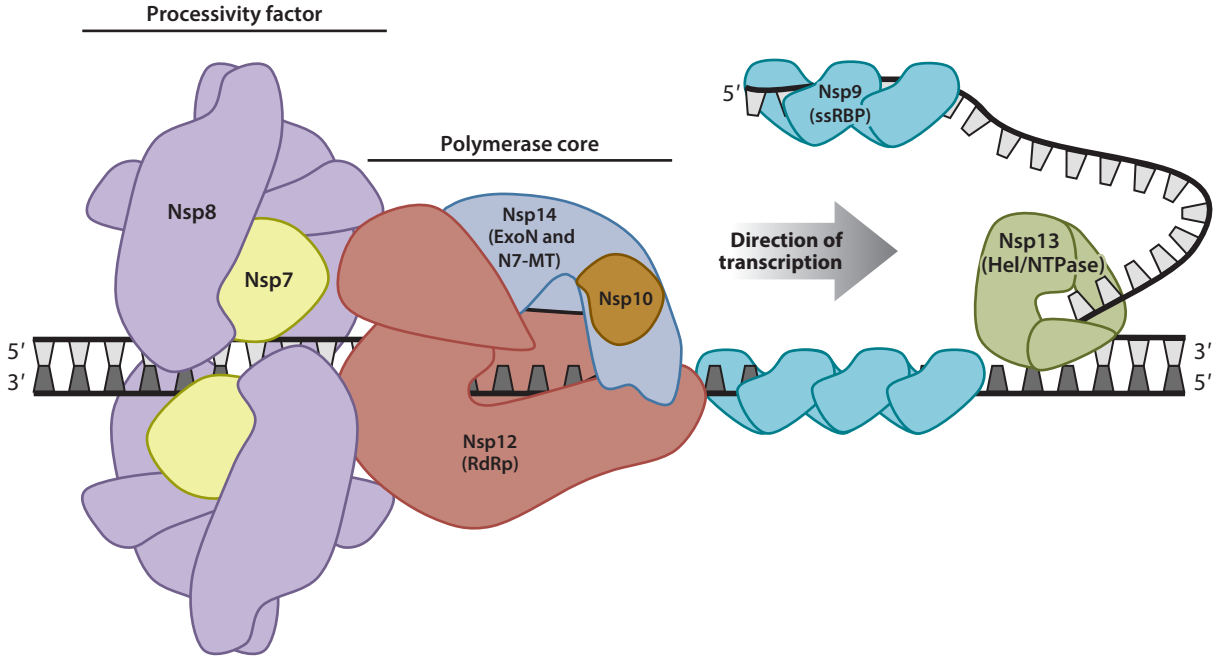

Figure 5

Model of the putative coronavirus multisubunit polymerase. The viral genome is shown in light gray and negative-sense template RNA in dark gray. The viral helicase and NTPase (nsp13 Hel/NTPase; *green*) is shown removing a newly synthesized viral genome, and the viral single-stranded RNA–binding protein (nsp9 ssRBP; *cyan*) is shown protecting the single-stranded RNA. The remaining portions of the model are based on known structures (nsp7 and nsp8) and/or reported protein-protein interactions. The polymerase core is predicted to include the viral RNA-dependent RNA polymerase (nsp12 RdRp; *red*), the 3′→5′ exoribonuclease and *N*7-methyltransferase (nsp14 ExoN and N7-MT; *blue*), and the nonenzymatic protein nsp10 (*orange*). This core is shown tightly associated with the putative processivity factor (nsp7 and nsp8; *yellow* and *purple*, respectively). Figure adapted with permission from Smith et al. (104).

has yet to be described. Nsp9 appears to function as an ssRNA-binding protein (122), suggesting that its function is to protect the single-stranded template as well as single-stranded newly synthesized RNA, a function analogous that of the SSB protein during DNA replication. Indeed, interactions between nsp9 and other replicase proteins have been observed (122). The continued elucidation of the functions of ORF1a proteins will be essential in establishing their importance during replication and their potential evolutionary linkage to specific ORF1b proteins.

CONCLUDING REMARKS

RNA virus replication fidelity is emerging as a new field of study that incorporates polymerase biochemistry, in vitro evolution experiments, virus fitness studies, and bioinformatic analyses. The studies are yielding surprising insights that have important implications for how we think about virus replication, pathogenesis, and evolution, as well as translational approaches to virus inhibition and attenuation.

Impact of Decreased Fidelity on Virus Fitness

Consider the following statement: An increased mutation rate favors the virus. This widely held view is based on two clear observations: (*a*) RNA viruses replicate with much lower fidelity than

DNA-based organisms, and (*b*) RNA viruses demonstrate rapid adaptation and emergence of resistance to antivirals and vaccines. The assumption built on these observations is that if decreased fidelity is beneficial for the virus, further decreases in fidelity are even better. Yet the data presented here for several virus families suggest that decreasing fidelity results in impaired replication, decreased competitive fitness, and attenuated virulence. Although only a few virus families have been investigated for the effect of decreased fidelity, it is certainly possible that examples will be identified where decreased fidelity favors the virus under specific circumstances, such as selection of resistance mutants to antivirals or revertants of conditional mutants.

New Models for RNA Synthesis and Regulation of Fidelity

Data presented in this review support the hypothesis that CoVs use a multiprotein replication complex that incorporates a processivity factor, a proofreading exoribonuclease, an RNA-dependent RNA polymerase, and a helicase, as well as predicted stimulatory cofactors and capping activities. These data and models argue that CoV RNA synthesis, modification, and fidelity might be more akin to those of DNA-based organisms with multiprotein DNA replication complexes. This has significant implications for understanding CoV replication but also for investigating the interface between DNA- and RNA-based life. The fact that CoVs are the largest known replicating RNA-based organisms suggests the possibility that they may exist at the boundary of what is possible in an RNA virus that has to balance genome stability with the population diversity required for adaptation. Alternatively, the demonstrated capacity of CoVs for zoonotic infections and host-species movement might argue that CoVs use a much larger range of fidelity and genome complexity to explore a greatly expanded sequence and phenotype space.

Goldilocks? Maybe

The Goldilocks metaphor is often used in cosmology and evolution to describe the fortuitous conditions that allowed the emergence and evolution of life. One is tempted to apply a similar metaphor to virus replication fidelity. Certainly the available data as presented in this review indicate that increasing or decreasing the fidelity of a number of divergent RNA viruses impairs fitness and is attenuating. However, the fact that fidelity can be moved off center—notably by at least 20-fold for CoVs—suggests that a range of fidelity is available to RNA viruses, potentially allowing for selection of more or less diverse populations. If Goldilocks is applicable, it would be to a range rather than a single optimal fidelity set point. CoVs represent the most obvious opportunity to explore this possibility, because they at minimum require the interaction of RdRp with a proofreading ExoN. This would represent at least two possible fidelity states, one high-fidelity state during RdRp-ExoN interaction and one low-fidelity state where RdRp interaction with ExoN is altered.

Fidelity as a Target for Inhibition and Attenuation

Could altered-fidelity viruses be used as live attenuated vaccine candidates? The best data for the effect of altered fidelity are presented here and show that either increased or decreased fidelity is attenuating in a range of RNA virus families. Thus it is possible that fidelity regulation is so central to all aspects of virus replication and pathogenesis that altering fidelity may be a broadly applicable approach to stable attenuation. For CoVs it appears that genetic inactivation of ExoN results in both a genotype and a phenotype that are attenuated and are resistant to reversion in vitro and in vivo during passage or persistent infection. Further, inactivation of ExoN results in a profound increase in sensitivity to RNA mutagens for SARS-CoV. Thus, the process of replication

fidelity may be a new target for virus family–wide attenuation and inhibition of virus replication and pathogenesis.

SUMMARY POINTS

1. RNA viruses replicate with lower fidelity than DNA-based organisms due to the lack of mechanisms for error recognition and repair.
2. RNA viruses exist as populations of genetically related variants, also known as mutant swarms or quasispecies, that are the units of selection.
3. To date, small RNA viruses are thought to regulate fidelity principally through the RNA-dependent RNA polymerase.
4. The tolerated range of increased or decreased fidelity for RNA viruses lacking proofreading may be very narrow.
5. Incorporation of a proofreading exoribonuclease allowed expansion of nidovirus genome size and complexity, as observed in coronaviruses.
6. Coronaviruses tolerate a 20-fold decrease in fidelity; the limits of increased or decreased replication fidelity for CoVs have not been defined.
7. Coronaviruses may assemble a multiprotein complex containing RNA-dependent RNA polymerase and proofreading activities.
8. Fidelity determinants may represent highly conserved and nonredundant targets for viral inhibition and live-virus attenuation.

FUTURE ISSUES

1. For coronaviruses, identifying all of the components of a multiprotein replication complex by in vitro reconstitution and structural studies will allow prediction and testing of our model for assembly and function of the replication complex in RNA synthesis and fidelity.
2. It will be exciting to test whether RNA viruses explore the range of fidelity under different selective pressures, and even during the course of a single infectious cycle.
3. The contributions of host cell proteins to RNA virus replication fidelity should be explored to define whether different environments stimulate or impair virus replication fidelity.
4. It will be important to define the multiple contributing factors to replication fidelity for CoVs and other RNA viruses, particularly robustness to mutations and RNA-dependent RNA polymerase selectivity and speed.
5. Studies of other families of RNA viruses, including negative-strand segmented genomes such as that of influenza virus, are needed to better understand common and divergent mechanisms and ranges of tolerated replication fidelity.
6. The availability of increased-fidelity and decreased-fidelity strains of multiple RNA viruses will allow testing of the impact of fidelity on host-range expansion, adaption, and experimental evolution.

7. Increasing availability and affordability of next-generation sequencing will allow in-depth analysis of the effects of fidelity and virus population diversity on virus replication, pathogenesis, and fitness.

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