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Current Opinion in Virology

Implications of altered replication fidelity on the evolution and pathogenesis of coronaviruses

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RNA virus evolution results from viral replication fidelity and mutational robustness in combination with selection. Recent studies have confirmed the impact of increased fidelity on RNA virus replication and pathogenesis; however, the impact of decreased fidelity is less defined. Coronaviruses have the largest RNA genomes, and encode an exoribonuclease activity that is required for high-fidelity replication. Genetically stable exoribonuclease mutants will allow direct testing of viral mutational tolerance to RNA mutagens and other selective pressures. Recent studies support the hypothesis that coronavirus replication fidelity may result from a multi-protein complex, suggesting multiple pathways to disrupt or alter virus fidelity and diversity, and attenuate pathogenesis.

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Introduction

RNA viruses cause many existing and recently emerged human diseases, and contain diverse replication machinery capable of generating enormous numbers of viral progeny. Despite this diversity, the size of RNA virus genomes spans only an order of magnitude (10^3 to 10^4), while DNA virus genomes vary in size over three orders of magnitude (10^4 to 10^7) [1,2]. Such differences in genome size between RNA and DNA viruses have been theoretically coupled to the inherent low fidelity of the RNA-dependent RNA polymerases (RdRps) used in viral RNA synthesis (10^{-3} to 10^{-4} mutations/nucleotide/round of replication; subsequently referred to as μ), which has long been proposed to result from the lack of proofreading within replicase complexes during viral RNA synthesis [3]. While this concept of constitutively low replication fidelity has been useful for predicting RNA virus evolution, viral systems have recently begun to be developed to directly test the impact of altered replication fidelity on viral evolution, genome size, viral replication, and/or viral pathogenesis. Studies using poliovirus and chikungunya virus have demonstrated that even modest increases in RNA replication fidelity (2–4-fold) result in decreased viral fitness *in vitro* and pathogenicity *in vivo* [4,5,6•]. Similarly, only very small decreases in replication fidelity (<4-fold) were tolerated during poliovirus replication [5,7]. Thus, work with these viruses demonstrates rather stringent limits on the variation in fidelity tolerated during viral replication, suggesting a finely tuned balance between genome stability and the diversity required for survival. Mutational robustness, or the capacity of a virus population to tolerate mutations, has also been shown to influence sensitivity to environmental conditions or mutagens. Coxsackie virus demonstrates increased sensitivity to RNA mutagens compared to poliovirus in a population size-dependent manner, suggesting that population tolerance for mutational diversity is likely a distinct feature of virus families [8•].

These exciting developments, in combination with advances in next generation sequencing, will allow direct experimental testing of the roles of fidelity and diversity in RNA virus genome size and complexity, host range expansion and adaptation, tropism, sensitivity to RNA mutagens or environmental stressor such as temperature, virulence or attenuation, and antigenicity and immune escape. The impact of decreased replication fidelity on attenuation or pathogenesis *in vivo* is less well studied, however, recent studies with the coronaviruses (CoVs) suggest that they may be excellent models to examine these important questions.

Coronavirus genome size: is bigger smarter?

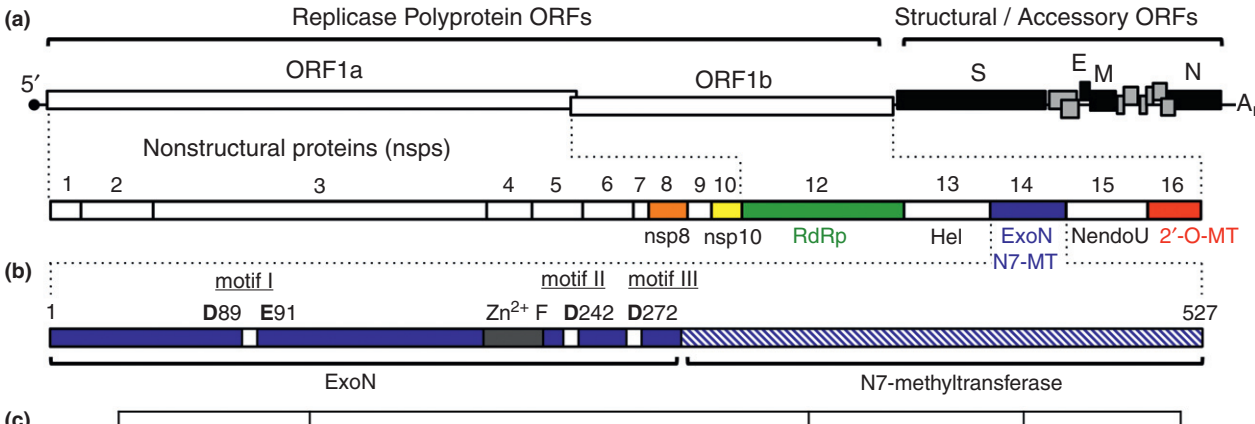
Bioinformatic models suggest that RNA genome size is constrained to an upper threshold of approximately 30 kb for an RNA based organism; beyond which, both the stability and the faithful replication of the viral genome cannot be maintained [1,9]. Coronaviruses fail to abide by these theoretical rules, and encode the largest known RNA genomes (27–32 kb) at almost twice the length of the next-largest non-segmented RNA viruses [1]. Assuming a fixed genomic mutation rate, coronaviruses and other large nidoviruses (e.g. roniviruses, toroviruses) must possess mechanisms to limit the accumulation of deleterious mutations while concomitantly maintaining the genetic diversity required for adaptation. Without such mechanisms, larger RNA viruses would accumulate

excessive numbers of deleterious mutations leading to a dramatic loss of fitness [10]. The demonstrated capacity of coronaviruses for host switching, [11] and the emergence of SARS-CoV into the human population [12,13] demonstrate that coronaviruses have strong adaptive capacity all while encoding such large genomes. Finally, the lack of larger or more complex RNA genomes exceeding those of the nidoviruses suggests that they may represent the upper limit of replicating RNA molecules, continually negotiating genome size and/or complexity with replicative stability. How then do the coronaviruses tolerate such a large and complex genome, and how did it arise?

Coronaviruses encode multiple RNA modifying enzymes, including a 3′-to-5′ exoribonuclease

Coronaviruses encode the most complex array of viral replicase proteins of any positive-strand RNA virus family [14]. The coronavirus replicase polyproteins contain up to 16 nonstructural protein domains (nsp1–16), many of which have known or predicted functions in viral RNA synthesis or modification (Figure 1) [9,14,15], including: RNA primase [16], RNA-dependent RNA polymerase (RdRp) [17], helicase/ATPase [18,19], N-methyltransferase [20], endoribonuclease [21], and 2′-O-methyltransferase [22] activities. Additionally, the coronaviruses encode a 3′-to-5′ exoribonuclease (ExoN) domain within nsp14 [23]. ExoN is encoded in all larger members of the *Nidovirales* order (i.e. *Coronaviridae* and *Roniviridae*), but is absent within the smaller *Arteriviridae* family members ('small nidoviruses') (Figure 2) [9]. Furthermore, ExoN activity has not been predicted or demonstrated to be present within any RNA viruses containing genomes smaller than the arteriviruses. Coronavirus nsp14-ExoN is a predicted member of the 'DEDD' superfamily that includes both RNA and DNA exonucleases from a diverse group of eukaryotic and prokaryotic organisms [9,24], and which derives its name from the four invariant acidic amino acids distributed across three

Figure 1



(c)

Proteins	Interaction	Method	Ref.
nsp8-primase nsp12-RdRp	Nsp8 is required for nsp12 polymerase activity	purified proteins *in vitro*	[16,37,38]
nsp10 nsp14-ExoN	Nsp10 causes 35-fold enhancement of nsp14-ExoN exonuclease activity	purified proteins *in vitro*	[35]
Nsp10 nsp16-2′-O-MT	Nsp10 is required for nsp16 2′-O-MT activity of SARS-CoV	purified proteins *in vitro*	[34]
nsp10 nsp5 (3CLpro)	Nsp10 ts mutant alters nsp5 protease activity	recombinant virus	[49]

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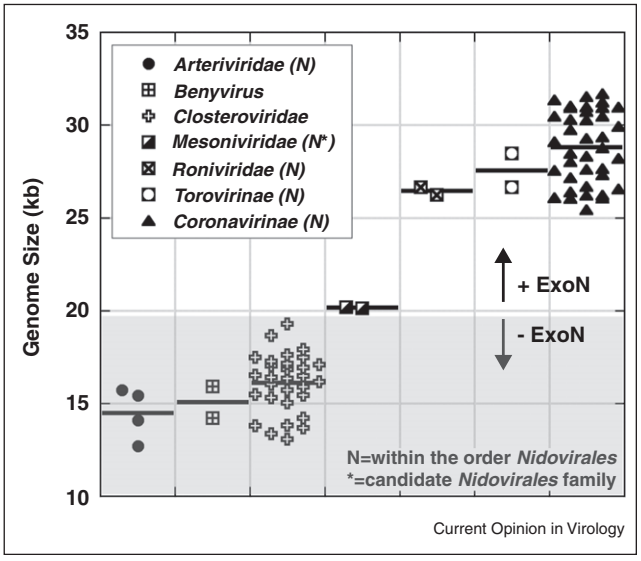
Coronavirus genome organization and identified viral nsp interactions. **(a)** Coronaviruses express 16 nonstructural proteins (nsps1–16) from open reading frames ORF1a and ORF1b, along with several structural and accessory proteins. Several nsps have been shown to have roles in RNA synthesis or modification: nsp12 (RdRp, green), nsp13 [helicase (Hel)], nsp14 [3′-to-5′ exoribonuclease (ExoN) and N7-methyltransferase (N7-MT); blue], nsp15 [endoribonuclease (NEndoU)], and nsp16 [2′-O-methyltransferase (2′-O-MT), red]. **(b)** ExoN shares conserved motifs (I, II and III) with other identified 3′-to-5′ exonucleases, and requires the amino acids D-E-D-D (white boxes) for activity. Nsp14 also contains a zinc finger (Zn^{2+} F, grey box) and a functional N7-methyltransferase domain. **(c)** Multiple nsps have been demonstrated to form higher-order complexes, and/or functionally regulate one another *in vitro* and in recombinant viruses.

Figure 2



Relationship between ssRNA virus genome size and the presence or absence of ExoN. The sizes of full viral genomes identified using the NCBI Viral Genome Resource [48] (with the exception of Yellow head virus strain YHV1999, GenBank ID: FJ848675.1) are plotted, and the average genome size per virus family or subfamily is shown as a horizontal black bar. Members of the order *Nidovirales* are denoted with an N, while the proposed new *Nidovirales* candidate family, *Mesoniviridae* [31], is denoted with an asterisk.

conserved sequence motifs: motifs I (DE), II (D) and III (D). Nsp14-ExoN is distinguished from other cellular ExoN homologs by the presence of a highly conserved putative zinc-finger domain positioned between motifs I and II [9] that could potentially confer specificity for RNA over DNA, and thus be important for proper targeting and function. What then is the known or predicted relationship of nsp14-ExoN to CoV genome size, stability and replication fidelity?

ExoN and the expansion of the RNA genome

The original prediction of the CoV ExoN domain within nsp14 suggested that it might serve a role as a possible proofreading exonuclease, an activity without precedent in RNA viruses [9]. Though proofreading and repair mechanisms were long thought to occur only during DNA replication, it is now clear that both DNA-dependent RNA polymerases (DdRps) and RNA-dependent RNA polymerases (RdRps) are capable of proofreading (reviewed in [25]). Cellular DdRps, such as human RNA pol II, have been shown to excise misincorporated nucleotides via 3′-to-5′ exonuclease activity that is stimulated upon binding of specific cleavage-stimulatory factors [26,27]. While nuclease activity has been reported for the influenza virus RdRp [28], evidence supporting either an intrinsic 3′-to-5′ exonuclease activity of viral RdRps, or the occurrence of proofreading-repair mechanisms during replication of other RNA viruses has yet to be found [3]. The identification of the CoV ExoN within the large nidoviruses suggested that acquisition of ExoN allowed for genome expansion to double that of the arteriviruses [1,9]. Closteroviruses (RNA viruses outside of the order *Nidovirales*) have non-segmented genomes larger than the arteriviruses, but smaller than the coronaviruses, and do not encode any known exonucleases [1], leaving a theoretical genome size gap of >10 kb between genomes lacking and encoding ExoN (Figure 2). However, important support for the ExoN hypothesis has been provided by the recent discovery of two invertebrate nidoviruses (Nam Dinh virus, NDiV; and Cavally virus, CAVV) containing genomes of approximately 20 kb that encode an nsp14-ExoN homolog [29•,30,31], further narrowing the boundaries of the genome size gap to <4 kb for genomes lacking and containing ExoN. Is ExoN-mediated genome expansion due to alterations in RNA genome replication fidelity?

ExoN is required for replication fidelity and is a probable RNA-dependent RNA proofreading enzyme

The predicted enzymatic activity [9] of nsp14-ExoN was confirmed *in vitro* for bacterially expressed SARS-CoV nsp14, demonstrating that expression of nsp14 alone was sufficient for 3′-to-5′ exoribonuclease activity [23]. Recombinant mutant MHV-A59 and SARS-CoV viruses containing motif 1 (DE to AA) substitutions (named S-ExoN and M-ExoN), which were shown to significantly diminished or abolished ExoN activity [23], are viable and have less than 1 log reduction in peak titers [32••,33] compared to WT. Single cycle replication of M-ExoN and S-ExoN resulted in 15–20-fold increases in mutation accumulation and similar calculated increased mutation rates (μ) as compared to their respective WT counterparts. The estimated mutation rate of the ExoN mutants ($\mu \sim 10^{-4}$ to 10^{-5}) more closely aligns with RNA viruses encoding smaller genomes, while the WT viruses containing intact ExoN activity appear to have a profound increase in replication fidelity ($\mu \sim 10^{-6}$ to 10^{-7}). Both the *in vitro* exoribonuclease activity and the demonstrated requirement for ExoN for high fidelity replication are consistent with the hypothesis that nsp14-ExoN is involved in RNA-dependent RNA proofreading. Given that ExoN activity is distinct from RdRp activity, could ExoN be a component of a larger multi-protein polymerase proofreading complex?

ExoN is likely a proofreading component of a larger multi-subunit error recognition and repair complex

Identification of ExoN activity distinct from the viral RdRp [23] suggests that nsp14-ExoN is a component of a larger multi-protein complex that includes nsps8, 10, 12 and 16 and possibly others. As other DEDD superfamily exonucleases are subunits of larger proteins

with polymerase activity, and serve to recognize and repair mismatched nucleotides, ExoN would be predicted to associate with other viral and/or cellular proteins. Several lines of evidence support this hypothesis ([Figure 1]). Nsp14 also contains N7-methyltransferase activity (N7-MTase) [20,34], demonstrating a requirement for ExoN to function in that context, and potentially interact with other virus-encoded proteins in the capping pathway, specifically nsp16, a 2′-O-methyltransferase (2′-O-MTase) [22]. While, nsp14 possesses independent ExoN activity, very recent work demonstrates that ExoN activity *in vitro* is enhanced up to 35-fold by the binding of nsp10 [35••], a protein with no known independent enzymatic activity. Nsp10 is also required for activation of SARS-CoV nsp16 2′-O-MTase activity [34], and surprisingly, nsp10–16 and nsp10–14 interactions appear to bind at overlapping sites on the surface of nsp10 [35••,36]. Given the distinct functions of nsp10–16 and nsp10–14 binding events [35••], nsp14 could possibly form at least two higher-order complexes during viral replication, one involved in putative viral RNA proofreading and composed of nsps10, 12 and 14 (at minimum), and a second viral RNA capping complex composed of nsps10, 14 and 16. While there is currently no experimental evidence for cooperation of nsp14 with the nsp15 endonuclease, such an interaction has been proposed for viral RNA modification [9]. Finally, nsp8 (primase) interacts with nsp7 and is required for nsp12 RdRp activity *in vitro* [37,38], further supporting the possibility of a multi-protein complex. Such multi-protein complexes would allow for testing if multiple proteins regulate virus replication fidelity, and if natural or induced variability in fidelity exists under differing replication conditions. If so, could the modular nature of the coronavirus replicase represent a genetically encoded system for fidelity regulation?

Is replication fidelity of RNA viruses fixed or responsive to selective pressure?

Mutation rates of RNA viruses have been the subject of extensive research [39••], with the general paradigm being that the inherent low fidelity of viral RdRps is a major contributor to mutation rates. While such biochemical restraints on RdRp function help in understanding replication fidelity determinants, the existence of high fidelity RdRp variants [6,40,41], as well as the high fidelity replication of coronaviruses, demonstrates that increased RdRp fidelity is both feasible and attainable for RNA viruses. Consistent with the need to balance adaptability with genome maintenance, viral replication in the presence of RNA mutagens demonstrates that RNA viruses can only accommodate limited changes in mutation rates without incurring significant fitness costs [8•,42,43]. Such sensitivity not only suggests that RNA viruses replicate close to a maximum error threshold [44], but also suggests that high mutation rates are a product of selection. Additionally, selection for further reductions or enhancements in replication fidelity has been described for RNA viruses [6•,40,41,45–47], suggesting that replication fidelity may represent an evolutionarily defined range rather than a fixed value, to maintain optimal population fitness. Given the likely modular nature of a coronavirus proofreading complex and the tolerance for fidelity variation (up to 20-fold at least), they may represent a unique model by which to measure viral mutation rates under variable selective pressures.

Conclusions

The multiple viral systems becoming available for study of increased and decreased fidelity create important new platforms and research applications, particularly the opportunity to study the impact of altered fidelity as a universal approach for attenuation of entire taxonomic groups of RNA viruses. Increased replication fidelity has already been shown to be attenuating for several RNA viruses. In contrast, the concept of decreased fidelity as an attenuation strategy for live viruses has been subject to concerns, when in fact there is no experimental evidence that decreasing virus replication fidelity accelerates emergence of virulence, or expansion of host range. If fidelity is a long-term evolved phenotype, then fidelity alterations could tip the balance toward continual emergence of attenuating mutations that would trump the emergence of virulence while possibly maintaining and expanding the repertoire of immune response. Stable coronavirus mutants that tolerate profoundly decreased replication fidelity may represent an excellent model to study the implications of decreased replication fidelity on attenuation and vaccine design in human viruses with strong animal models.

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