

# THE CORONAVIRUS REPLICASE: INSIGHTS INTO A SOPHISTICATED ENZYME MACHINERY

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## 1. INTRODUCTION

The order *Nidovirales* currently comprises three families of plus-strand RNA viruses, the *Coronaviridae*, *Arteriviridae*, and *Roniviridae*, which are thought to have a common phylogenetic origin.<sup>1-3</sup> The proposed close phylogenetic relationship between the three virus families is mainly based upon common features that discriminate nidoviruses from other RNA plus-strand RNA viruses, including (i) the production of a nested set of 3'-coterminally subgenome-length RNAs,<sup>4</sup> (ii) the use of ribosomal frameshifting into the -1 reading frame to express the key replicative functions,<sup>5</sup> and (iii) the conservation of a set of functional domains that are arranged in the nonstructural polyproteins in the following order (from N- to C-terminus): two- $\beta$ -barrel-fold main protease, RNA-dependent RNA polymerase of superfamily 1 (RdRp), zinc finger-containing helicase of superfamily 1, and endoribonuclease.<sup>6-9</sup> Despite their common ancestry, the nidovirus families differ considerably with regard to (i) their genome sizes (ranging from 12.7 to 31.3 kilobases), (ii) their structural proteins, and (iii) the conservation of several RNA-processing enzymes.<sup>3</sup>

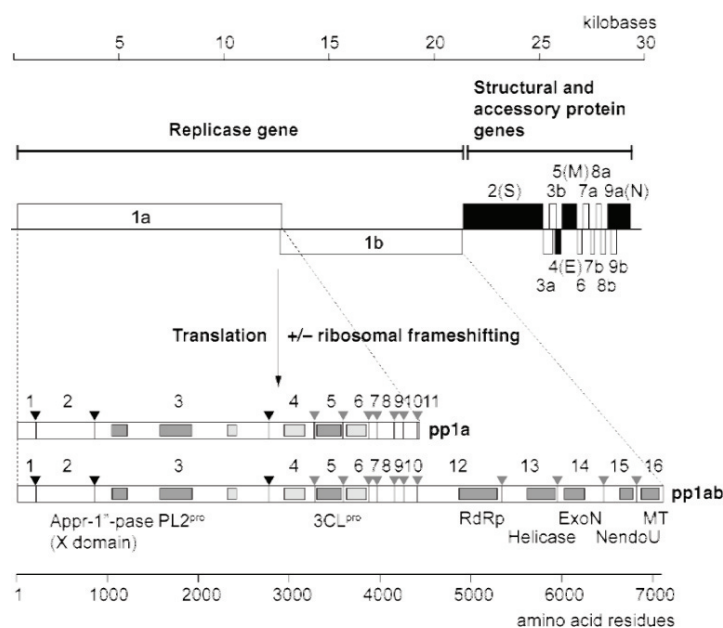
## 2. ORGANIZATION AND EXPRESSION OF THE CORONAVIRUS REPLICASE GENE

With genome sizes of between 27.3 and 31.3 kilobases, coronaviruses represent the largest nonsegmented RNA viruses currently known. About two-thirds of their genomes (>20,000 nucleotides) are devoted to encoding the viral replicase, which mediates viral RNA synthesis but probably has many more functions. The replicase is encoded by the replicase gene. This gene is composed of two large open reading frames (ORFs), designated ORF1a and ORF1b, that are located at the 5'-end of the genome. The upstream ORF1a encodes a polyprotein of 450–500 kDa, termed polyprotein (pp) 1a, whereas ORF1a and ORF1b together encode pp1ab (750–800 kDa) (Fig. 1). Expression of the C-terminal ORF1b-encoded portion of pp1ab depends on a (-1) ribosomal frameshift. As a consequence, the key replicative enzymes (RdRp, helicase, and others) are expressed at a significantly lower level than are ORF1a-encoded protein functions.<sup>5,10</sup> The coronavirus replication complex is bound to intracellular membranes through several ORF1a-encoded hydrophobic domains (reviewed in Refs. 3 and 9) and, besides replicase

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gene-encoded proteins, includes several cellular proteins and the viral nucleocapsid protein.<sup>11–13</sup>



**Figure 1.** SARS-CoV genome organization. The putative functional open reading frames (ORFs) in the 29.7-kb genome of SARS-CoV are indicated (reviewed in Ref. 14). Translation of the genomic RNA gives rise to two replicative polyproteins, pp1a and pp1ab, that mediate the key functions required for SARS-CoV genome replication and synthesis of 8 major subgenomic RNAs encoding the structural proteins (shown in black) and several SARS-CoV-specific accessory proteins. Expression of ORF1b sequences requires a programmed ribosomal frameshift into the  $-1$  reading frame during translation of the genome RNA, which occurs just upstream of the ORF1a translation stop codon. The replicative polyproteins are extensively processed by viral proteases. The processing end-products of pp1a are designated nonstructural proteins (nsp) 1 to nsp11 and those of pp1ab are designated nsp1 to nsp10 and nsp12 to nsp16. Cleavage sites that are processed by the 3C-like protease (3CL<sup>pro</sup>), which is also called main protease (M<sup>pro</sup>), are indicated by gray arrowheads, and sites that are processed by the papain-like protease, PL2<sup>pro</sup>, are indicated by black arrowheads. The major replicative domains are shown in dark gray and transmembrane domains predicted to anchor the viral replicase to intracellular membranes are indicated in light gray. Appr-1<sup>ase</sup>, ADP-ribose 1<sup>st</sup>-phosphatase; PL2<sup>pro</sup>, papain-like cysteine protease 2; 3CL<sup>pro</sup>, 3C-like main protease; RdRp, RNA-dependent RNA polymerase; ExoN, 3'-to-5' exoribonuclease; NendoU, nidoviral uridylylate-specific endoribonuclease; MT, putative ribose-2'-O methyltransferase.

### 3. PROTEASES AND PROTEOLYTIC PROCESSING

Activation of the coronavirus replication complex involves extensive proteolytic processing of the replicase polyproteins to produce 16 (in IBV: 15) mature products called nonstructural proteins (nsp) 1 to 16 (reviewed in Refs. 3 and 15). The processing involves two different types of proteases. The key enzyme is the so-called 3C-like protease (3CL<sup>pro</sup>), also called main protease (M<sup>pro</sup>), which cleaves the central and

C-terminal regions of pp1a and pp1ab at 11 conserved sites.<sup>15</sup> 3CL<sup>pro</sup> is a cysteine protease featuring a Cys-His catalytic dyad and a three-domain structure.<sup>16,17</sup> The N-terminal domains I and II adopt a two- $\beta$ -barrel-fold structure that resembles the structures of serine proteases of the chymotrypsin superfamily. The C-terminal entirely  $\alpha$ -helical domain III is critically involved in proteolytic activity and dimerization. It is now generally accepted that the 3CL<sup>pro</sup> dimer represents the active form of the protease.<sup>18,19</sup> Intermolecular interactions within the dimer have been suggested to keep the active center (in particular, the S1 subsite) in a proteolytically competent conformation.<sup>17,20</sup> Coronavirus main proteases have conserved substrate specificities [(L,I)-Q↓(S,A,G)] and, accordingly, similar substrate-binding pocket structures, as has been shown by crystal structure analyses of main proteases from different coronavirus genetic groups.<sup>17,21–23</sup> Based on this information, protease inhibitors have been developed that effectively inhibit a broad range of coronavirus main proteases.<sup>23</sup> Potentially, these compounds may be useful for treatment of known or newly emerging coronavirus infections.

The N-terminal regions of the coronavirus polyproteins, which are poorly conserved among the coronavirus groups I, II, and III, are cleaved at two (in IBV) or three sites (in all other coronaviruses) by one (IBV and SARS-CoV) or two zinc-finger-containing papain-like cysteine proteases called PL1<sup>pro</sup> and PL2<sup>pro</sup>.<sup>24–32</sup> PL<sup>pro</sup> cleavage sites are usually flanked by small residues (mainly Gly and Ala).<sup>15</sup> PL1<sup>pro</sup> and PL2<sup>pro</sup> probably evolved by gene duplication in one of the ancestors of the present-day coronaviruses.<sup>29</sup> For MHV, it was recently demonstrated that cleavage of the nsp1|nsp2 site and, even more surprisingly, the C-terminal nsp1 and the entire nsp2 sequence are not required for MHV replication *in vitro*.<sup>33–35</sup>

#### 4. POLYMERASE AND HELICASE ACTIVITIES

The coronavirus RdRp has been classified as an outgroup of the RdRp superfamily 1.<sup>36</sup> It resides in the ORF1b-encoded nonstructural proteins 12, as was predicted about 15 years ago<sup>6</sup> and now demonstrated for a bacterially expressed form of the SARS-CoV nsp12.<sup>37</sup> The RdRp catalytic domain occupies the C-terminal two-thirds of nsp12,<sup>6</sup> but also the N-terminal part of nsp12 seems to contribute to activity.<sup>37</sup> Based on MHV co-immunoprecipitation data, the N-terminal part of nsp12 has been suggested to interact with other replicase subunits, specifically with nsp5 (3CL<sup>pro</sup>), nsp8, and nsp9,<sup>38</sup> which is in line with the recently published crystal structure of an nsp7–nsp8 complex from SARS-CoV.<sup>39</sup> The X-ray crystallography study by Zhai et al.<sup>39</sup> provides first insights into the sophisticated architecture of the coronavirus replicase. It shows that eight molecules of each nsp7 and nsp8 assemble into a hexadecameric supercomplex that forms a hollow, cylinder-like structure, which, because of its internal dimensions and electrostatic properties, seems to be capable of encircling RNA. This nsp7–nsp8 supercomplex, in turn, interacts with nsp9, a small single-stranded RNA-binding protein,<sup>40,41</sup> and interactions of nsp7–nsp8(–nsp9) with yet other replicase subunits (including nsp12) are likely. On the basis of structural and functional data, it has been speculated that the nsp7–nsp8 complex might act as an important RdRp cofactor, for example, to increase the RdRp processivity.<sup>39</sup> Evidence for a critical involvement of the C-terminal pp1a processing products (nsp7–10) in viral RNA synthesis also comes from work on MHV

temperature-sensitive mutants, which has shown that nsp10, a Cys/His-rich protein, has a distinct role in minus-strand RNA synthesis.<sup>42</sup>

After the RdRp, helicases are the second best conserved enzymes of plus-strand RNA viruses. The coronavirus helicase resides in nsp13 and has been classified as belonging to the helicase superfamily 1<sup>6,43</sup> (Fig. 1). Coronavirus helicases and all their nidovirus homologs are linked to an N-terminal zinc-binding domain (ZBD) involving 12–13 conserved Cys/His residues.<sup>6</sup> The conservation of a ZBD-associated superfamily 1 helicase is considered one of the major genetic markers of nidoviruses. Over the past years, the helicase activities of HCoV-229E and, to a lesser extent, of SARS-CoV have been characterized.<sup>44–48</sup> Mutagenesis and biochemical data revealed that the ZBD is essential for the enzymatic activities of both the coronavirus and arterivirus helicases.<sup>49</sup> Coronavirus helicases proved to have multiple enzymatic activities, including nucleic acid-stimulated NTPase, dNTPase, and RNA (plus DNA) duplex-unwinding activities.<sup>44,47,48</sup> Coronavirus (and arterivirus) helicases were demonstrated to unwind double-stranded RNA substrates with 5'-to-3' polarity, that is, they move in a 5'-to-3' direction along the strand to which they initially bind.<sup>44,50</sup> The polarity of coronavirus helicase activities contrasts with that of the helicases of flavi-, pesti-, and hepaciviruses, which all operate in the opposite direction,<sup>51</sup> indicating that the biological functions of helicases from the *Nidovirales* and *Flaviviridae* might differ fundamentally from each other. Coronavirus (nidovirus) helicases belong to the few helicases that act on both RNA or DNA substrates with nearly equal efficacy. Given that the helicase is part of the (cytoplasmic) coronavirus replicase complex, it seems unlikely that the observed DNA duplex-unwinding activity is of biological significance. Coronavirus helicases are able to unwind up to several hundred base pairs of double-stranded nucleic acid in a processive manner,<sup>47,48</sup> supporting a role as a “replicative” helicase that separates regions of double-stranded RNA that the polymerase might encounter during RNA synthesis.

Coronavirus helicases are able to hydrolyze essentially all natural nucleotides to fuel their translocation along nucleic acids and concomitant unwinding of duplex RNA (and DNA) structures.<sup>44–48</sup> Besides their NTPase and dNTPase activities, coronavirus helicases were shown to possess yet another phosphohydrolyase activity, namely an RNA 5'-triphosphatase activity that employs the active site of the (d)NTPase activity. It is reasonable to believe that the nsp13-associated RNA 5'-triphosphatase catalyzes the first reaction in the synthesis of coronavirus 5' RNA cap structures.<sup>47,48</sup> Given the multiple enzymatic activities of coronavirus helicases and the previously observed diverse roles of the arterivirus helicase in viral replication, transcription and virion biogenesis,<sup>52</sup> it is reasonable to believe that coronavirus helicases are involved in more than one metabolic pathway in the viral life cycle.

## 5. RNA-PROCESSING ENZYMES

A detailed sequence analysis by Gorbalenya, Snijder and colleagues of the SARS-CoV genome revealed that the enzymology of coronavirus RNA synthesis may be significantly more complex than previously thought.<sup>8</sup> In this seminal study published in 2003, as many as five novel enzymatic activities were identified in the genomes of coronaviruses and, to a varying extent, other nidoviruses (Fig. 1). The putative activities

included (i) a 3'-to-5' exonuclease (ExoN) associated with nsp14, (ii) an endoribonuclease (NendoU) associated with nsp15, (iii) an S-adenosyl methionine-dependent ribose 2'-O-methyltransferase (MT) associated with nsp16, (iv) an ADP-ribose 1"-phosphatase (Appr-1"-pase) associated with the so-called "X domain" of nsp3, and (v) a cyclic phosphodiesterase (CPDase), which is only conserved in group II coronaviruses (excluding SARS-CoV). Four of the activities were found to be conserved in all coronaviruses, suggesting that they have an essential role in the coronaviral life cycle. Recently, the predicted exo- and endoribonuclease activities were established and characterized using bacterially expressed forms of the ExoN from SARS-CoV (Minskaia, Hertzog, Gorbalenya, Campanacci, Cambillau, Canard, and Ziebuhr, unpublished data), and the NendoU from HCoV-229E, SARS-CoV, and IBV.<sup>53,54</sup> We have recently demonstrated by reverse genetics using the HCoV-229E infectious clone that the ExoN, NendoU, and MT domains are all essential for the production of virus progeny. Substitutions of predicted active-site residues resulted in diverse defects in viral RNA synthesis and virus production<sup>54</sup> (Hertzog, Ulferts, Schelle, and Ziebuhr, unpublished data). The underlying mechanisms for the observed defects are currently being studied in detail.

The observed pattern of conservation in different nidovirus families suggests a functional hierarchy for the newly identified RNA-processing activities, with the manganese ion-dependent uridylyate-specific endoribonuclease, NendoU, playing a central role. This enzyme is universally conserved in nidoviruses and, besides the ZBD-associated helicase, represents a genetic marker of nidoviruses that is also reflected by its previous designation as "nidovirus-specific conserved domain"<sup>55,56</sup>. As mentioned above, NendoU has been demonstrated by site-directed mutagenesis of the full-length HCoV-229E clone to be essential for coronaviral RNA synthesis. However, we recently obtained mutagenesis data that argue for a more complex scenario regarding the activities of NendoU. Thus, for example, we found that several single-residue substitutions that abolished the NendoU activity in an *in vitro* RNase assay gave rise to viable (albeit low-titer) virus when transferred to the HCoV-229E infectious clone (Hertzog, Ulferts and Ziebuhr, unpublished data). These data indicate that NendoU might have more than one activity and/or cleave more than one substrate that likely differs from that used in our *in vitro* assays. Clearly, more work will be required to elucidate the function(s) of NendoU in the nidoviral life cycle.

In view of the very similar gene expression and RNA synthesis strategies employed by the various nidovirus families and genera, it is intriguing that only one (namely NendoU) of the newly identified nidovirus RNA-processing activities is conserved in arteriviruses. The basis for this differential conservation pattern of RNA-processing enzymes is currently unclear but might indicate that the enzymology involved in viral genome-length and subgenome-length RNA synthesis varies to some extent among the nidovirus families. Alternatively, members of the various nidovirus families/genera might interact differentially with yet-to-be-defined RNA-processing pathways of the host cell.

It is also worth remembering that arterivirus genomes are about 2 times smaller than other nidovirus genomes and it is possible that (some of) the extra domains conserved in corona- and toroviruses (and, to a lesser extent, in roniviruses) are required to replicate genomes of this unique size. The coronavirus ExoN activity was recently shown by sequence analysis and site-directed mutagenesis to be related to cellular enzymes of the DEDD exonuclease superfamily (Minskaia, Hertzog, Gorbalenya, Campanacci,

Cambillau, Canard, and Ziebuhr, unpublished data). This exonuclease family includes cellular 3'-to-5' exonucleases involved in proofreading, repair, and/or recombination, and it is tempting to speculate that nidoviruses use their ExoN domains in similar processes, for example, to keep the error frequency of their low-fidelity RdRp below a critical threshold.

In contrast to the essential RNA-processing activities (ExoN, NendoU, and MT), the nsp3 X domain-associated Appr-1'-pase activity was found to be dispensable for viral replication *in vitro*.<sup>57</sup> Substitutions of putative active-site residues of the Appr-1'-pase domain, which have been confirmed to abolish enzymatic activity *in vitro*, gave rise to viable virus. The virus had no apparent defects in viral RNA synthesis and grew to the same titers as the wild-type virus.<sup>57</sup> Similarly, MHV reverse genetics data demonstrated that coronaviruses tolerate specific substitutions and even deletions in the replicase gene. For example, cleavage of the nsp1|nsp2 cleavage site was shown to be dispensable for viral replication, and even deletion of the C-terminal part of nsp1 and the entire nsp2, respectively, had only minor effects on viral replication.<sup>33-35</sup> By contrast, several other mutations/deletions were tolerated *in vitro* but caused attenuation in the natural host.<sup>58</sup> Taken together, the data suggest that coronavirus replicases have evolved to include a number of nonessential functions. The fact that some of the "nonessential" protease cleavages and enzymatic activities are conserved in coronaviruses or in specific coronavirus genetic groups suggests that they provide a selective advantage only in the host.

## 6. CONCLUDING REMARKS

Although much has been learned about coronavirus replicase organization, localization, proteolytic processing, and viral replicative enzymes, there are still major gaps in our knowledge. Inspired by the SARS epidemic in 2003, numerous studies aiming at the development of coronavirus vaccines and antivirals have recently been published, and there is no doubt that coronavirus research has gained momentum over the past few years. Given the rapidly accumulating biochemical, structural, and genetic information on coronaviruses, a more detailed understanding of the molecular mechanisms involved in coronaviral RNA synthesis can be expected to emerge in the near future. It will be of particular interest to identify those proteins that are involved in the specific mechanisms of coronavirus RNA synthesis, such as the production of a nested set of subgenome-length RNAs and the replication of RNA genomes of unprecedented size. Furthermore, studies on coronavirus-encoded RNA-processing activities and their cellular homologs might reveal interesting insights into the relationship (or interplay) of coronaviral and cellular RNA metabolism pathways. In the long run, the unique structural properties of several conserved coronavirus replicative enzymes may lead to the development of selective enzyme inhibitors and possibly even drugs suitable to treat coronavirus infections of humans and animals.

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