

Nidovirales: Evolving the largest RNA virus genome

Alexander E. Gorbalenya^{a,*}, Luis Enjuanes^b, John Ziebuhr^c, Eric J. Snijder^a

^a *Molecular Virology Laboratory, Department of Medical Microbiology, Leiden University Medical Center, LUMC E4-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands*

^b *Department of Molecular and Cell Biology, Centro Nacional de Biotecnología, Madrid, Spain*

^c *School of Biomedical Sciences, The Queen's University of Belfast, Belfast, United Kingdom*

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Abstract

This review focuses on the monophyletic group of animal RNA viruses united in the order *Nidovirales*. The order includes the distantly related coronaviruses, toroviruses, and roniviruses, which possess the largest known RNA genomes (from 26 to 32 kb) and will therefore be called 'large' nidoviruses in this review. They are compared with their arterivirus cousins, which also belong to the *Nidovirales* despite having a much smaller genome (13–16 kb). Common and unique features that have been identified for either large or all nidoviruses are outlined. These include the nidovirus genetic plan and genome diversity, the composition of the replicase machinery and virus particles, virus-specific accessory genes, the mechanisms of RNA and protein synthesis, and the origin and evolution of nidoviruses with small and large genomes. Nidoviruses employ single-stranded, polycistronic RNA genomes of positive polarity that direct the synthesis of the subunits of the replicative complex, including the RNA-dependent RNA polymerase and helicase. Replicase gene expression is under the principal control of a ribosomal frameshifting signal and a chymotrypsin-like protease, which is assisted by one or more papain-like proteases. A nested set of subgenomic RNAs is synthesized to express the 3'-proximal ORFs that encode most conserved structural proteins and, in some large nidoviruses, also diverse accessory proteins that may promote virus adaptation to specific hosts. The replicase machinery includes a set of RNA-processing enzymes some of which are unique for either all or large nidoviruses. The acquisition of these enzymes may have improved the low fidelity of RNA replication to allow genome expansion and give rise to the ancestors of small and, subsequently, large nidoviruses.

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1. Introduction: nidoviruses as a distinct group of ssRNA+ viruses

RNA viruses have evolved a remarkable diversity of genomes and replicative mechanisms. Although the size variation of RNA virus genomes is quite low (ranging from ~3 to 32 kb), four distinct classes have been defined, three occupied solely by RNA viruses and one shared with DNA viruses, to order the great diversity of RNA viruses (Baltimore, 1971). DNA viruses, on the other hand, form only two distinct classes, although their genome sizes can differ by as much as ~3 orders of magnitude. The small size of RNA virus genomes is fundamentally linked to the low fidelity of their RNA synthesis, which is mediated by a cognate replication complex containing an RNA-dependent RNA polymerase (RdRp) that lacks proofreading/repair mech-

anisms. RNA viruses may generate as much as one mutation per genome per replication round (Drake and Holland, 1999), a feature that is counterbalanced by the large population size of their progeny, which has been characterized as 'quasispecies' on the basis of its genome variability (Eigen and Schuster, 1977). As a result of this combination of properties, RNA viruses are "primed" to adapt to new environmental conditions, but they are limited in their ability to expand their genome as they must keep their mutation load below the so-called 'error threshold', above which the survival of the quasispecies becomes impossible (for reviews, see Domingo and Holland, 1997; Moya et al., 2004).

Among the four classes of RNA viruses, only the class containing viruses with single-stranded genomes of positive (mRNA) polarity (ssRNA+) exploit the full genome size range outlined above (Fig. 1). Members of this class are also the most numerous in terms of the number of families and distinct groups recognized by the International Committee for the Taxonomy of Viruses (ICTV) (Fauquet et al., 2005). With genome sizes of 26–32 kb, three groups of phylogenetically related viruses,

* Corresponding author. Tel.: +31 71 5261436; fax: +31 71 5266761.
E-mail address: a.e.gorbalenya@lumc.nl (A.E. Gorbalenya).

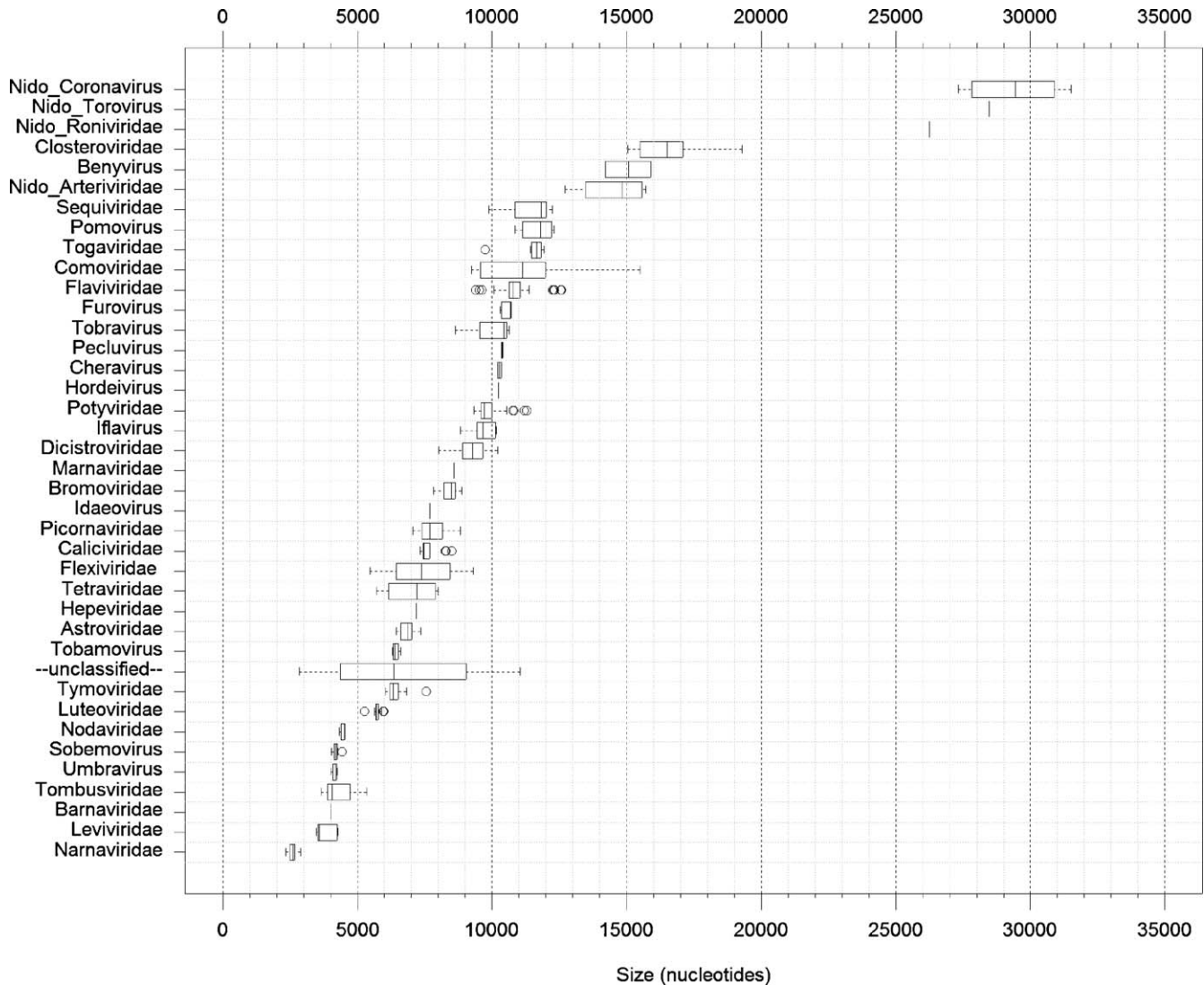


Fig. 1. Distribution of genome sizes of ssRNA+ viruses. Box-and-whisker graphs were used to plot the family/group-specific distribution of genome sizes of all ssRNA+ viruses whose genome sequences have been placed in the NCBI Viral Genome Resource (Bao et al., 2004) by December 7, 2005 (Faase and Gorbalenya, unpublished data). Four major groups of nidoviruses are highlighted with the Nido- prefix, and the *Coronaviridae* family is split into the Nido-Coronavirus and the Nido-Torovirus groups. The box spans from the first to the third quartile and includes the median, indicated by the vertical line. The whiskers extend to the extreme values that are distant from the box at most 1.5 times the interquartile range. Values beyond this distance are indicated by circles (outliers).

namely coronaviruses, toroviruses and roniviruses, are found at the upper end of this genome size scale and are clearly separated from other RNA viruses. We will discuss them in comparison with their much smaller arterivirus cousins (genome size of ‘only’ 13–16 kb), which also belong to the order *Nidovirales* (Cavanagh, 1997; de Vries et al., 1997; Snijder et al., 2005b; Spaan et al., 2005b). The ICTV recognized roniviruses (Walker et al., 2005) and arteriviruses (Snijder et al., 2005a) as distinct families, while the current taxonomic position of coronaviruses and toroviruses as two genera of the *Coronaviridae* family is being revised by elevating these virus groups to a higher taxonomic rank, that of either subfamily or family (González et al., 2003; Spaan et al., 2005a). Hereafter, we will refer to coronaviruses, toroviruses and roniviruses as ‘large’ nidoviruses and to arteriviruses as ‘small’ nidoviruses, to stress the clear genome size difference.

Nidoviruses rank among the most complex RNA viruses and their molecular genetics clearly discriminates them from other RNA virus groups. Still, our knowledge about their molecular biology, mostly derived from studies of coronaviruses, is very limited (Lai and Cavanagh, 1997; Lai and Holmes, 2001; Siddell et al., 2005; Ziebuhr, 2004). Nidoviruses attach to their host cell by binding to a receptor on the cell surface, after which fusion of the viral and cellular membrane is presumably mediated by one of the surface glycoproteins. This fusion event (either at the plasma or endosomal membrane) releases the nucleocapsid into the host cell’s cytoplasm. After genome uncoating, translation of two replicase open reading frames (ORFs) is initiated by host ribosomes, to yield large polyprotein precursors that undergo autoproteolysis to eventually produce a membrane-bound replicase/transcriptase complex. This complex mediates the synthesis of the genome RNA and a nested set of subge-

nomic RNAs that direct the synthesis of structural proteins and, optionally, some other proteins (see below). New virus particles are assembled by the association of the new genomes with the cytoplasmic nucleocapsid protein and the subsequent envelopment of the nucleocapsid structure. The viral envelope proteins are inserted into intracellular membranes and targeted to the site of virus assembly (usually membranes between the endoplasmic reticulum and Golgi complex), where they meet up with the nucleocapsid and trigger the budding of virus particles into the lumen of the membrane compartment. The newly formed virions then leave the cell by following the exocytic pathway towards the plasma membrane.

Below, we will discuss (from an evolutionary perspective) the molecular biological properties that unite and separate the large and small nidoviruses. This overview will involve an analysis of the internal and external evolutionary relationships of nidoviruses, which in some cases are very distant. These relationships have originally been delineated using bioinformatics tools and are now more and more supported by the results of functional and structural studies. This fruitful cooperation between theoretical and experimental science was especially instrumental in the dissection of the nidovirus replication apparatus, which is the central theme of this review. Due to the way in which the initial functional assignments were made, the names of many nidovirus replicative proteins were derived from those of viral or cellular homologs (Gorbalenya, 2001; Snijder et al., 2003; Spaan et al., 2005b; Ziebuhr et al., 2000). To conclude, we will broadly discuss our current understanding of the origin and evolution of small and large nidovirus genomes.

2. Nidovirus genome diversity and a common genetic plan

Nidovirus genomes have been studied for ~20 years, most extensively during the last several years. By today, more than a hundred full-length and over a thousand partial nidovirus genome sequences have been published. Four profoundly separated and unevenly populated genetic clusters have been recognized: corona-, toro-, roni- and arteriviruses. The exact relationship between these clusters is yet to be rigorously resolved (González et al., 2003). The data available from phylogenetic analysis of the most conserved replicase domain of these viruses, the RdRp, with homologs of some other RNA viruses (Gorbalenya et al., 2002) and the analysis of the domain colinearity of their replicase genes (Bredenbeek et al., 1990; Cowley et al., 2000; den Boon et al., 1991a; Draker et al., 2006; Gorbalenya et al., 1989; Gorbalenya, 2001; Snijder et al., 2003) favour clustering coronaviruses and toroviruses (Fig. 2). Arteriviruses and roniviruses form two more distant nidovirus lineages, whose relative position as shown in Fig. 2 tree remains poorly supported (Gorbalenya et al., 2002). In fact, comparison of replicase polyproteins (see below) indicates that, contrary to Fig. 2 tree topology, roniviruses may group with corona- and toroviruses to form a supercluster of large nidoviruses, and arteriviruses would then be the first to split from the nidovirus trunk (Fig. 2). Notwithstanding the fact that this revised branching of the arteri- and ronivirus lineages

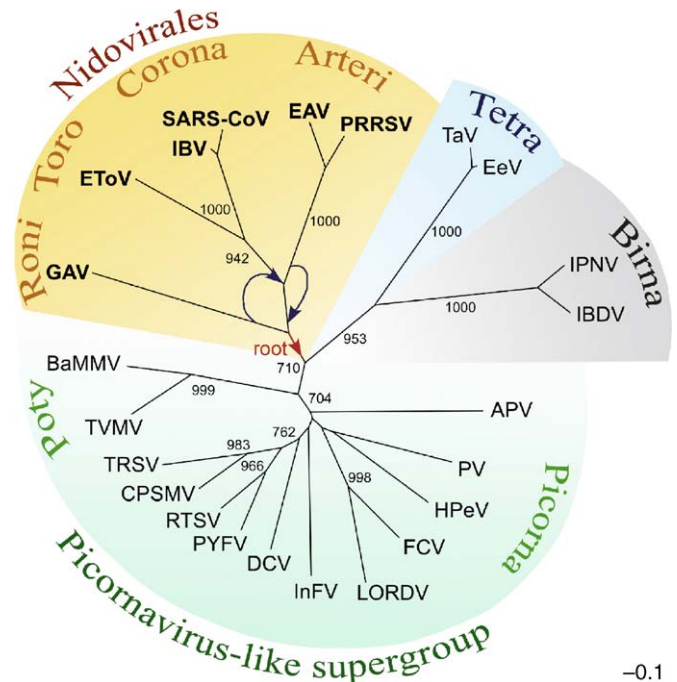


Fig. 2. RdRp-based RNA virus tree that includes nidoviruses. The most conserved part of RdRps from representative viruses in the *Picornaviridae*, *Dicistroviridae*, *Sequiviridae*, *Comoviridae*, *Caliciviridae*, *Potyviridae*, *Coronaviridae*, *Roniviridae*, *Arteriviridae*, *Birnaviridae*, *Tetraviridae* and unclassified insect viruses was aligned. An unrooted neighbour-joining tree was inferred using the ClustalX1.81 software. For details of the analysis, see Gorbalenya et al. (2002). All bifurcations with support in >700 out of 1000 bootstraps are indicated. Different groups of viruses are highlighted with different colours. The tree was modified from (Gorbalenya et al., 2002; Snijder et al., 2005b; Spaan et al., 2005b). Virus families/groups and abbreviations of viruses included in the analysis are as follows: *Coronaviridae*: avian infectious bronchitis virus (IBV), severe acute respiratory syndrome virus (SARS-CoV) and Equine torovirus (EToV); *Arteriviridae*, Equine arteritis virus (EAV) and Porcine reproductive and respiratory syndrome virus strain VR-2332 (PRRSV); *Roniviridae*: Gill-associated virus (GAV); *Picornaviridae*, human poliovirus type 3 Leon strain (PV) and parechovirus 1 (HPeV); *Iflavirus*, infectious flacherie virus (InFV); unclassified insect viruses, *Acyrtosiphon pisum* virus (APV); *Dicistroviridae*, *Drosophila C* virus (DCV); *Sequiviridae*, Rice tungro spherical virus (RTSV) and Parsnip yellow fleck virus (PYFV); *Comoviridae*, Cowpea severe mosaic virus (CPSMV) and Tobacco ringspot virus (TRSV); *Caliciviridae*, Feline calicivirus F9 (FCV) and Lordsdale virus (LORDV); *Potyviridae*, Tobacco vein mottling virus (TVMV) and Barley mild mosaic virus (BaMMV); *Tetraviridae*, *Thoesa asigna* virus (TaV) and *Euprosterina elaeasa* virus (EeV); *Birnaviridae*, infectious pancreatic necrosis virus (IPNV) and infectious bursal disease virus (IBDV). A plausible direction to the root of the nidovirus domain is indicated (red arrow). As discussed in the text, arteriviruses rather than roniviruses might have been the first to branch off from the nidovirus trunk. This revised topology of the two lineages is also indicated (blue arrows).

is yet to be independently confirmed, we will in this review consider the large and small nidoviruses as two separate phylogenetic clusters. In addition to the significant genome size variation between the four groups of nidoviruses, also other major differences were documented, involving, for example, virion morphology (see below), host range, and various other biological properties that are outside the scope of this review.

The overwhelming majority of the available nidovirus genome sequences have been reported for corona- and arteriviruses, which were the first to be fully sequenced and

analyzed (Bournsnel et al., 1987; den Boon et al., 1991a). Comparative sequence analysis and other studies of viruses from each of these families revealed three major groups in coronaviruses, called groups 1, 2 and 3, and four comparably distant genetic clusters in arteriviruses (González et al., 2003; Gorbalenya et al., 2004; Snijder et al., 2005a; Spaan et al., 2005a). So far, only very few sequences, including one full-length genome sequence for each virus group, have been reported for toroviruses (Draker et al., 2006) and roniviruses (Cowley and Walker, 2002) and, hence, information on the genetic variability of these nidovirus taxa is extremely limited. Although toroviruses and roniviruses have not been studied in great detail, the features listed below are thought to be common to nidovirus genomes and their expression (Cavanagh, 1997; Lai and Holmes, 2001; Snijder et al., 2005b; Spaan et al., 2005b).

Nidoviruses have linear, single-stranded RNA genomes of positive polarity that contain a 5' cap structure and a 3' poly (A) tail. The genomes of nidoviruses include untranslated regions (UTR) at their 5' and 3' genome termini. These flank an array of multiple genes whose number may vary in and between

the families of the nidovirus order (Fig. 3). Roniviruses have only four ORFs, toroviruses have six ORFs, arteriviruses may have between 9 and 12 ORFs and coronaviruses may encode from 9 to 14 ORFs. In all nidoviruses, the two most 5' and largest ORFs, ORF1a and ORF1b, occupy between two-thirds and three-quarters of the genome. They overlap in a small area containing a –1 ribosomal frameshift (RFS) signal that directs translation of ORF1b by a fraction of ribosomes that have started protein synthesis at the ORF1a initiator AUG. These two ORFs encode the subunits of the replicase machinery, which are produced by autoproteolytic processing of the pp1a and pp1ab polyproteins encoded by ORF1a and ORF1a/b, respectively.

The ORFs located downstream of ORF1b encode nucleocapsid and envelope protein(s), the numbers of which differ between the major nidovirus branches. Family and group-specific ORFs in this region may encode additional virion and non-structural (“accessory”) proteins (see below). These ORFs located in the 3'-part of the genome are expressed from a nested set of subgenomic RNAs (see below), a property that was reflected in the name of the virus order (nidus in Latin means nest) (Cavanagh,

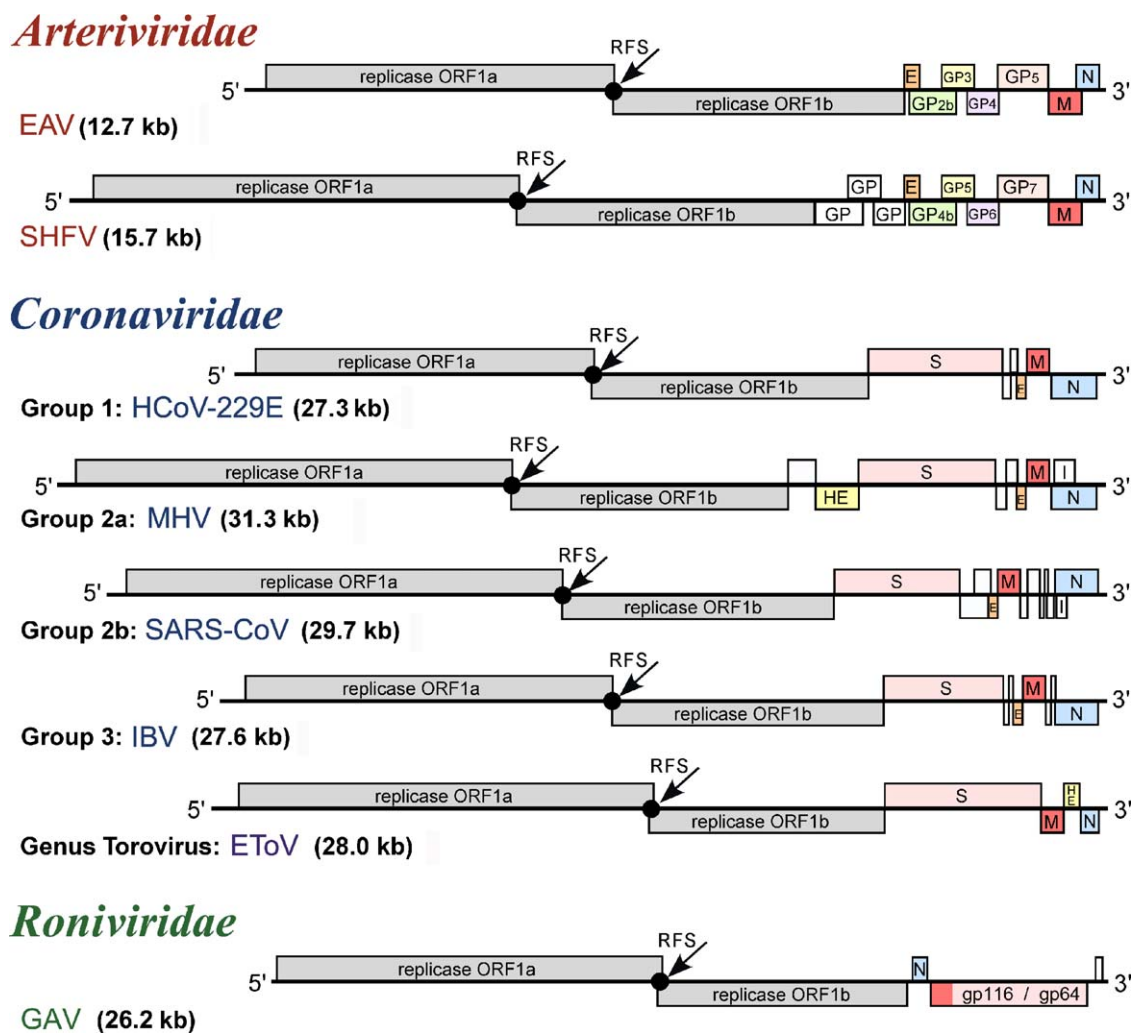


Fig. 3. Genome organization of selected nidoviruses. The genomic ORFs of viruses representing major lineages of nidoviruses are indicated and the names of the replicase and main virion genes are given. References to the nomenclature of accessory genes can be found in the text. Genomes of large and small nidoviruses are drawn to different scales. This figure was updated from Fig. 39.2 presented in Siddell et al. (2005).

1997). In large nidoviruses, compared to small nidoviruses, each of the two areas occupied by either the replicase ORFs or the 3'-proximal ORFs is expanded proportionally.

3. Replicase machinery: the conserved nidovirus backbone

What is truly unique for nidoviruses? Polycistronic genome organization may not be this feature as some other RNA viruses possessing either large genomes, e.g., the *Closteroviridae* (see review of Dolja et al., 2006), or much smaller genomes, e.g., the *Luteoviridae* (Miller et al., 1997) and *Astroviridae* (Jiang et al., 1993), employ similar genome organizations. The presence of a common 'leader' sequence at the 5'-end of all viral mRNA species (Baric et al., 1983; de Vries et al., 1990; Lai et al., 1984; Spaan et al., 1983) was initially considered to be a possible nidovirus hallmark. However, this property subsequently proved to be not universally conserved amongst nidoviruses (Cowley et al., 2002; van Vliet et al., 2002). Currently, only the organization and composition of the multidomain replicase gene can be used to discriminate nidoviruses from other RNA viruses. The power of this criterion was originally recognized upon large-scale comparisons of ssRNA+ virus genomes enabling the higher-order clustering of different virus families in several super-families/groups (Goldbach, 1986; Strauss and Strauss, 1988), including a Coronavirus-like supergroup (now known as nidoviruses) (Gorbalenya and Koonin, 1993a; Snijder et al., 1993). Thus, for example, replicase ORF1b encodes two domains that have not been identified in other RNA virus families and, therefore, qualify as genetic markers of this virus order. These are:

- I. The (putative) multinuclear zinc-binding domain (ZBD) (Cowley et al., 2000; Gorbalenya et al., 1989; Seybert et al., 2005; Snijder et al., 1990a; van Dinten et al., 2000);
- II. The uridylylate-specific endoribonuclease (NendoU) domain (Bhardwaj et al., 2004; Ivanov et al., 2004b; Posthuma et al., 2006; Snijder et al., 1990a, 2003).

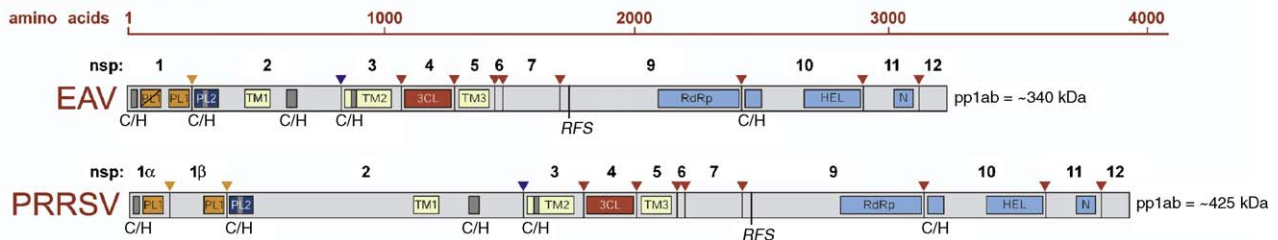
These two domains are part of a conserved array of domains whose sequential arrangement can be abbreviated as NH₂-TM1-TM2-3CL^{PRO}-TM3-RFS-RdRp-ZBD-HEL1-NendoU-COOH (Gorbalenya, 2001) (see also below). This array is specific for nidoviruses, although it includes domains that may also be common to other RNA virus families. Some of these domains, which may appear adjacent in the above domain constellation, may actually be separated by other, less conserved domains in specific nidovirus taxa. The conserved nidovirus-specific domain order was suggested to be intimately linked to essential and conserved aspects of the nidovirus replicative cycle (Gorbalenya, 2001). The domains forming the above constellation proved to be essential for nidovirus RNA synthesis and the production of progeny virions in reverse genetics experiments using the equine arterivirus (EAV) and/or various coronaviruses (see below).

The ORF1a-encoded N-terminal part of this array of domains consists of the 3C-like proteinase (3CL^{PRO}) and

flanking trans-membrane (TM) domains (TM1-TM2-3CL^{PRO}-TM3) (Gorbalenya et al., 1989) (Fig. 4). The 3CL^{PRO} has a chymotrypsin-like fold and a substrate specificity resembling that of picornavirus 3C proteinases (Anand et al., 2002; Barrette-Ng et al., 2002; Draker et al., 2006; Hegyi and Ziebuhr, 2002; Snijder et al., 1996; Ziebuhr et al., 2003; Ziebuhr and Siddell, 1999) (Smits et al., in press). The 3CL^{PRO} is responsible for the processing of the downstream, most conserved part of the replicase polyproteins and, because of this property, may also be called "main proteinase" (M^{PRO}) (reviewed by (Ziebuhr et al., 2000)). The enzyme cleaves the C-terminal half of pp1a and the ORF1b-encoded part of pp1ab at 8–11 sites having a glutamine or glutamic acid at the P1 position and a small residue (usually glycine, alanine or serine) at the P1' position (reviewed in (Ziebuhr et al., 2000; Ziebuhr, 2005)). From an evolutionary perspective, the nidovirus-wide conservation of this specificity is most remarkable. The 3CL^{PRO}s of the four main nidovirus branches are profoundly diverged and (according to conventional criteria) do not group together, although they have become significantly separated from their viral and cellular homologs. Even the catalytic center, which is uniformly conserved in cellular homologs, has evolved into quite different forms, including a Cys–His catalytic dyad in roniviruses and coronaviruses, a Ser–His–Asp catalytic triad in arteriviruses, and a Ser–His-based catalytic center in toroviruses (Anand et al., 2002, 2003; Barrette-Ng et al., 2002; Draker et al., 2006; Snijder et al., 1996; Ziebuhr et al., 1997, 2003). The crystal structures of coronavirus and arterivirus 3CL^{PRO}s revealed that both groups of enzymes have a three-domain structure, with domains I and II forming a (chymotrypsin-like) two-β-barrel fold consisting of 12 or 13 β-strands (Anand et al., 2002, 2003; Barrette-Ng et al., 2002; Yang et al., 2003). The C-terminal domains III of arterivirus and coronavirus 3CL^{PRO}s differ from each other, both in size and structure, and roniviruses and toroviruses 3CL^{PRO} may again have other versions of the C-terminal domain. Outside nidoviruses, only plant potyviruses encode a 3CL^{PRO} that has an extra C-terminal domain, a property which correlates with a special sequence resemblance between the catalytic domains of the potyvirus and corona-/ronivirus 3CL^{PRO}s (Ziebuhr et al., 2003). The three conserved hydrophobic domains encoded by ORF1a (TMs in the above formula), two of which characteristically flank the 3CL^{PRO} at either side (Gorbalenya, 2001; Snijder and Meulenberg, 1998; Ziebuhr, 2005), appear to anchor the nidovirus replication complex to intracellular membranes (Prentice et al., 2004).

The four ORF1a-encoded domains (TM1-TM2-3CL^{PRO}-TM3) are linked through an RFS to the ORF1b-encoded array of conserved nidovirus domains (Fig. 4). The RFS includes a 'slippery' heptanucleotide sequence, at which the ribosome makes the actual –1 frameshift. This 'slippery' heptanucleotide is conserved among corona-, toro- and arteriviruses, but not in roniviruses which may have evolved a different sequence to perform the same function (Brierley et al., 1987; Brierley, 1995; Cowley et al., 2000). An elaborate RNA pseudoknot, which seems to be structurally conserved among nidoviruses, is located immediately downstream of the slippery sequence (Baranov et al., 2005; Brierley et al., 1991; Cowley et al., 2000; den Boon et

Arteriviridae



Coronaviridae

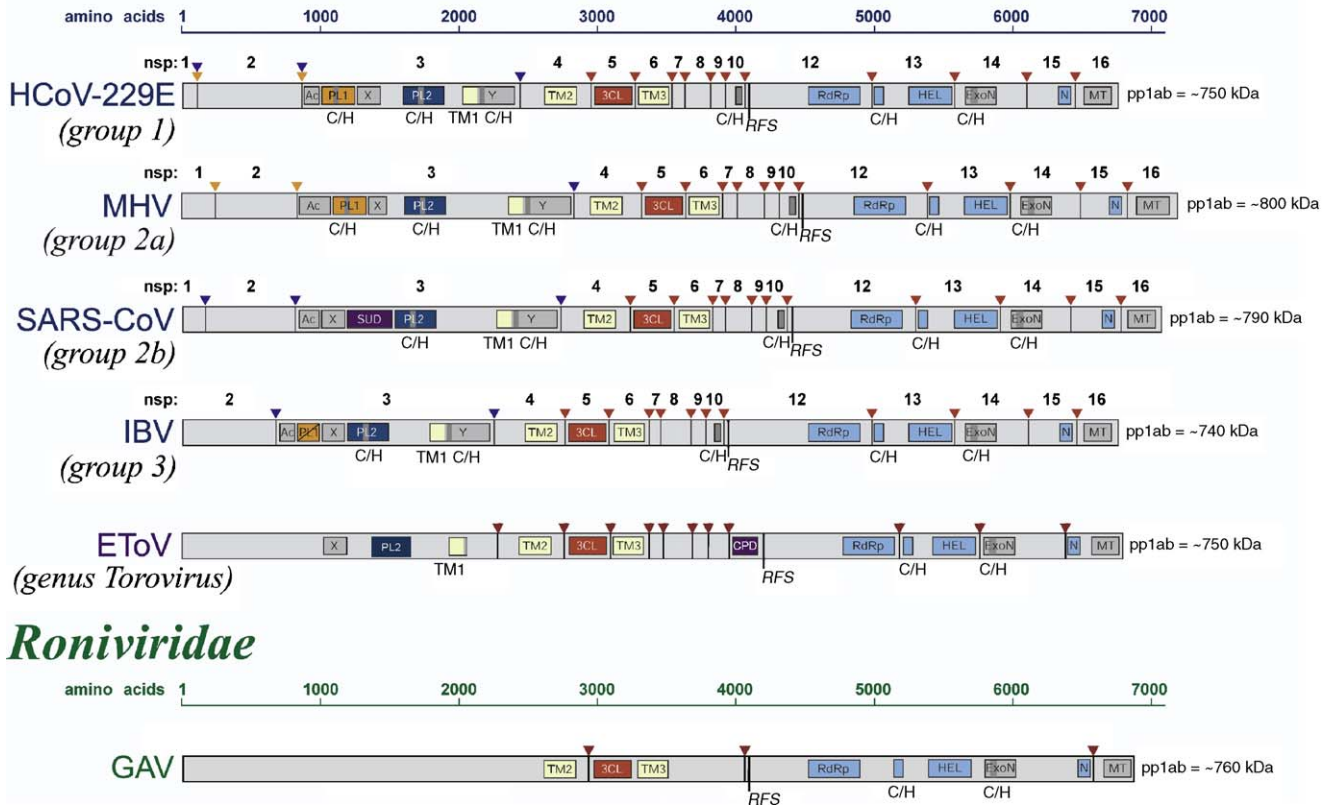


Fig. 4. Domain organization of the replicase pp1ab polyprotein for selected nidoviruses. Shown are currently mapped domains in the pp1ab replicase polyproteins of viruses representing major lineages of nidoviruses. Arrows represent sites in pp1ab that are cleaved by papain-like proteinases (orange and blue) or chymotrypsin-like (3CL^{pro}) proteinase (red). For viruses with the full cleavage site map available (arteriviruses and coronaviruses), the proteolytic cleavage products are numbered. A tentative cleavage site map for EToV is from an unpublished work by A.E. Gorbalenya. Within the cleavage products, the location and names of domains that have been identified as structurally and functionally related are highlighted. These include diverse domains with conserved Cys and His residues (C/H), putative transmembrane domains (TM), domains with conserved features (AC, X and Y), and domains that have been associated with proteolysis (PL1, PL2, and 3CL), RNA-dependent RNA synthesis (RdRp), helicase (HEL), exonuclease (ExoN), uridylate-specific endoribonuclease (N; NendoU in the main text), methyl transferase (MT) and cyclic phosphodiesterase (CPD) activities. Note that due to space limitations, the domain names in this figure may be abbreviated derivatives from those used in the main text. Polyproteins of large and small nidoviruses are drawn to different scales. This figure was updated from Fig. 39.4 presented in Siddell et al. (2005).

al., 1991a; Draker et al., 2006; Herold and Siddell, 1993; Plant et al., 2005; Snijder et al., 1990a).

In the ORF1b-encoded polyprotein, the C-terminal part of pp1ab, four conserved domains have been identified in all nidoviruses (from N- to C-terminus): RdRp, ZBD, 5'-to-3' helicase belonging to superfamily 1 (HEL1), and NendoU (Fig. 4). They comprise the most conserved sequences delineated among nidoviruses. The RdRp is the largest conserved domain, occupying the C-terminal region of a larger protein whose size varies significantly among nidoviruses (Bredenbeek

et al., 1990; Cowley et al., 2000; den Boon et al., 1991a; Gorbalenya et al., 1989). The nidovirus RdRps cluster with orthologs encoded by viruses comprising the Picornavirus-like supergroup (Gorbalenya et al., 1989, 2002; Koonin, 1991) (Fig. 2). The RdRps of nidoviruses carry a notable replacement of a Gly residue (with one exception, by a Ser residue (Gorbalenya et al., 1989; Stephensen et al., 1999)) that is otherwise conserved among ssRNA+ viruses and precedes two key catalytic Asp residues in the sequence that is known as 'the GDD motif', an RdRp hallmark (Kamer and Argos, 1984). The

RdRp domain is essential for RNA replication (van Marle et al., 1999b) and the in vitro RdRp activity of this domain was recently verified for SARS coronavirus (SARS-CoV) (Cheng et al., 2005).

Immediately downstream of the RdRp, the pp1ab polyproteins of all nidoviruses contain a protein that includes ZBD and HEL1 (Bredenbeek et al., 1990; Cowley et al., 2000; den Boon et al., 1991a; Gorbalenya et al., 1988, 1989) (Fig. 4). This relative position of RdRp and HEL domains is exceptional, as the helicase domain resides upstream of the RdRp in the replicase polyproteins of all other families of ssRNA+ viruses that have these domains in their repertoire (Gorbalenya et al., 1989). Distant homologs of the nidovirus HEL1 domain were identified in viruses of the alphavirus supergroup, and they are widespread among cellular organisms in all three kingdoms of life (Gorbalenya and Koonin, 1993b). Homologs of the ZBD, which is characterized by 12–13 conserved Cys/His residues, were not described. The ZBD appears to be required for the multiple enzymatic activities of the HEL1 domain, including NTPase, RNA 5'-triphosphatase and nucleic acid duplex unwinding (Bautista et al., 2002; Heusipp et al., 1997; Ivanov et al., 2004a; Ivanov and Ziebuhr, 2004; Seybert et al., 2000b, 2000a, 2005; Tanner et al., 2003). The arteri- and coronavirus ZBD-HEL1 proteins are capable of separating (probably in a processive manner) extended regions of up to several hundred base pairs of double-stranded RNA (and DNA) in the 5'-to-3' direction (Ivanov et al., 2004a; Ivanov and Ziebuhr, 2004; Seybert et al., 2000b, 2000a). Both domains were implicated in arterivirus genomic and subgenomic RNA synthesis (Seybert et al., 2005; van Dinten et al., 1997, 2000).

The most C-terminal domain of the conserved constellation of nidovirus replicase domains is NendoU (Bhardwaj et al., 2004; Ivanov et al., 2004b; Snijder et al., 2003). It is produced as part of a larger protein that includes another domain, which, depending on the nidovirus family, is either located upstream or downstream of NendoU in pp1ab (Fig. 4). Outside nidoviruses, distant homologs of NendoU were identified in some prokaryotes and eukaryotes that form a small protein family prototyped by the *Xenopus laevis* XendoU (Gioia et al., 2005; Laneve et al., 2003; Snijder et al., 2003). NendoU is a Mn²⁺-dependent enzyme that produces 2'-3' cyclic phosphate ends (Ivanov et al., 2004b) and it may function as homo-hexamers (Guarino et al., 2005). Both the SARS-CoV and human coronavirus 229E (HCoV-229E) NendoU domains efficiently cleave double-stranded RNA at specific uridylate-containing sequences, although they also are able to process (albeit less specifically) single-stranded RNA at each uridylate present in the sequence. This domain is essential for RNA synthesis and/or the production of virus progeny in coronavirus and arteriviruses (Ivanov et al., 2004b; Posthuma et al., 2006; Hertzog et al., unpublished data).

Apart from the domains discussed above, sequence conservation among the most distant nidovirus branches could be barely identified in ORF1a and even some parts of ORF1b. This may be due to technical reasons related to weak conservation in a heavily biased sequence set or due to different origins of the respective domains in the main nidovirus branches. Most recently, structural studies have started to contribute to the mapping of putative

functional domains in nidovirus replicase polyproteins (Bartlam et al., 2005; Egloff et al., 2004; Sutton et al., 2004; Zhai et al., 2005) and the nidovirus-specific domain constellation may be further expanded in the future.

The first candidate to be added to the constellation is another proteinase. Besides the conserved M^{PRO} (3CL^{PRO}), arteri-, coronavirus and toroviruses encode so-called accessory proteinases (one to four, depending on the virus) that autocatalytically process the large N-proximal regions of the replicase polyproteins in which they reside ((Baker et al., 1989; Snijder et al., 1992) (reviewed by Ziebuhr et al., 2000); see also Draker et al., 2006). They belong to the same superfamily of papain-like (PL^{PRO}) cysteine proteinases despite often having little sequence resemblance and very different sizes (den Boon et al., 1995; Gorbalenya et al., 1991) (Fig. 4). Three nidoviruses, EAV, simian hemorrhagic fever virus (SHFV) and avian infectious bronchitis virus (IBV), encode PL^{PRO}s that are not proteolytically active indicating that PL^{PRO}s may have other, non-proteolytic function(s) in the nidovirus replicative cycle (den Boon et al., 1995; Ziebuhr et al., 2001). Nidovirus PL^{PRO} domains may be linked with zinc finger (Zf) domains, whose type and relative position vary among nidoviruses (Herold et al., 1999; Snijder et al., 1995; Tijms et al., 2001). A similar association with a Zf was not reported for the numerous homologs encoded by ssRNA+ viruses, although nidovirus accessory proteinases do share the substrate specificity towards small aliphatic residues at the P1 and P1' positions that is prevailing among RNA virus-encoded homologs (Dong and Baker, 1994; Snijder et al., 1992) (for reviews, see Gorbalenya and Snijder, 1996; Ziebuhr et al., 2000). The Zf-PL^{PRO}-containing nsp1 protein of the arterivirus EAV was shown to be dispensable for replication but absolutely required for subgenomic RNA synthesis (Tijms et al., 2001). Interestingly, this protein provided a functional replacement for a remotely related PL^{PRO} in closterovirus RNA replication (Peng et al., 2002). The PL^{PRO}s of coronaviruses contain a Zn-ribbon domain connecting two catalytic domains (Herold et al., 1999). A similar organization was also found in a herpesvirus-associated enzyme with which some coronavirus PL^{PRO}s share deubiquitinating activity (Barretto et al., 2005; Lindner et al., 2005; Sulea et al., 2005). In roniviruses, the corresponding N-terminal pp1a/pp1ab region of about 2000 residues has not yet been functionally characterized, and no putative PL^{PRO} domain has been identified (Fig. 4).

4. Replicase machinery: group-specific domains

In addition to the domains that form the nidovirus-specific constellation, a number of other conserved (replicative) domains have been identified in some but not all nidoviruses. These domains reside in the pp1a and pp1ab polyproteins or, in one case, in a separate ORF.

Two such recently identified domains, the 3'-to-5' exoribonuclease (ExoN) and the ribose-2'-O-methyltransferase (O-MT), are of a special interest as they are only conserved in the ORF1b-encoded portion of pp1ab of large nidoviruses (Snijder et al., 2003) (Fig. 4). Like the members of the nidovirus-specific array of replicase domains, the ExoN and O-MT were shown to be

indispensable for viral RNA synthesis and/or the production of virus progeny in HCoV-229E (Hertzog et al., unpublished data; Minskaia et al., *in press*) and SARS-CoV (Almazan et al., unpublished data).

The ExoN domain occupies the N-terminal part of a protein located between the ZBD–HEL1 protein and the NendoU-containing cleavage product and has no equivalent in the arterivirus pp1ab (Cowley et al., 2000; de Vries et al., 1997) (Fig. 4) or in the genomes of any other RNA viruses (Snijder et al., 2003). This domain is very distantly related to numerous cellular proteins (Snijder et al., 2003) that form the extremely diverse DEDD superfamily of exonucleases, which are involved in many aspects of nucleic acid metabolism including proof-reading, repair, and recombination (Moser et al., 1997; Zuo and Deutscher, 2001). Unlike their cellular homologs, the nidovirus ExoNs contain either one (corona- and toroviruses) or two copies (roniviruses) of a Zf domain (Snijder et al., 2003). Using bacterially expressed forms of SARS-CoV ExoN, the predicted exoribonuclease activity was recently established and characterized *in vitro* (Minskaia et al., *in press*).

The O-MT domain (Feder et al., 2003; Snijder et al., 2003; von Grotthuss et al., 2003) is proteolytically released as a mature protein from the most C-terminal part of pp1ab. In toroviruses, this protein may additionally include the NendoU domain that is located immediately upstream of the O-MT domain (Fig. 4). In the arterivirus pp1ab, the equivalent position (downstream of NendoU) is occupied by an arterivirus-specific protein (nsp12) of uncharacterized function (Fig. 3). O-MT homologs were identified in the genera *Flavivirus* and *Alphavirus* of the ssRNA+ families *Flaviviridae* and *Togaviridae*, respectively, and in members of the ssRNA- *Mononegavirales*. All RNA virus-encoded O-MTs belong to a large protein family containing numerous DNA-encoded homologs, which is prototyped by the RmJ protein (Feder et al., 2003; Ferron et al., 2002; Koonin, 1993; Snijder et al., 2003; von Grotthuss et al., 2003). The O-MT activity has not yet been verified for nidoviruses.

Two other RNA-processing domains, ADP-ribose-1''-phosphatase (ADRP) and cyclic phosphodiesterase (CPD) (Martzen et al., 1999), are conserved in smaller subsets of nidoviruses (Fig. 4). The ORF1a-encoded ADRP (originally called X domain (Gorbalenya et al., 1991)) was identified upstream of PL2^{PRO} in the largest multidomain coronavirus protein (nsp3) (Snijder et al., 2003) and in a similar position in the torovirus pp1a/pp1ab (Draker et al., 2006). The ADRP is also part of the replicase polyprotein of all mammalian viruses of the alphavirus-like supergroup (Koonin et al., 1992). It has a peculiar, but not ubiquitous, distribution in cellular organisms of all kingdoms of life and a yet-to-be-identified function (Pehrson and Fuji, 1998; Allen et al., 2003; Martzen et al., 1999). The predicted ADRP activity was verified *in vitro* for bacterially expressed versions of this domain from SARS-CoV, HCoV-229E, and porcine transmissible gastroenteritis virus (TGEV) (Putics et al., 2005, 2006, *in press*) and the crystal structure of the SARS-CoV ADRP has been solved (Saikatendu et al., 2005). Substitutions of putative coronavirus ADRP active-site residues resulted in viruses that did not display apparent defects in RNA synthesis and grew to titers comparable to those of

the wild-type virus (Putics et al., 2005). The CPD domain is encoded in ORF1a immediately upstream of the ORF1a/ORF1b junction in toroviruses (Fig. 4), and, surprisingly, by ORF2 of a phylogenetically compact subset of subgroup 2a coronaviruses (Snijder et al., 1991) (Fig. 3), which thus express this gene from a separate subgenomic mRNA. Some dsRNA rotaviruses are the only other RNA viruses that encode a (distantly related) CPD domain, whose homologs are abundant in cellular organisms (Mazumder et al., 2002; Snijder et al., 2003). Deletion of the CPD-encoding ORF2 did not have a noticeable effect on replication of mouse hepatitis virus (MHV) in tissue culture (Schwarz et al., 1990). In contrast, a mutation in MHV ORF2 was tolerated *in vitro* but caused attenuation in the natural host (Sperry et al., 2005).

The coronavirus nsp3 contains, in addition to the ADRP and PL^{PRO}, many other domains, some of which are group- or virus-specific and have not been functionally characterized (Ziebuhr et al., 2001; Gorbalenya, unpublished observations). Among those is the SARS-CoV unique domain (SUD) (Snijder et al., 2003) (Fig. 4). Also, two upstream proteins, nsp1 and nsp2, appear to exist in a variety of forms, each specific for some coronaviruses (Snijder et al., 2003). MHV reverse genetics data demonstrated that coronaviruses tolerate specific substitutions and even deletions in this region of the replicase gene. For example, cleavage of the nsp1|nsp2 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 (Brockway and Denison, 2005; Denison et al., 2004; Graham et al., 2005). Taken together, the data suggest that coronavirus replicases have evolved to include a number of non-essential functions that may provide a selective advantage only in the host (see also Section 6).

In addition to the CPD of toroviruses, a variable number of other domains are proteolytically released from the nidovirus pp1a/pp1ab region delimited by TM3 and the RFS site (Fig. 4). Some of these domains seem to be conserved in corona- and toroviruses (Gorbalenya, unpublished observations), but no conservation that extends to arteriviruses and/or roniviruses has been reported. In coronaviruses, this region encompasses the nsp5–10. Genetic analysis implicated nsp10 in RNA synthesis (Sawicki et al., 2005) and recent crystallographic studies showed that nsp9 is an RNA-binding protein with a new version of the OB-fold (Egloff et al., 2004) that structurally resembles the domain II of coronavirus 3CL^{PRO}s (Sutton et al., 2004). Furthermore, nsp7 and nsp8 form a hexadecameric supercomplex that is capable of encircling RNA and may operate as a cofactor in viral RNA synthesis (Zhai et al., 2005).

The interaction between coronaviruses nsp7 and nsp8 is likely to be one of many other protein–protein interactions from the various subunits that are proteolytically released from pp1a/pp1ab. They may facilitate the formation of the replication complex and, probably, are critical to the functioning of this complex. For instance, the RdRp (nsp12) was proposed to interact with the main protease (nsp5) and nsp8 and nsp9 in the coronavirus MHV (Brockway et al., 2003) and a strong interaction between nsp2 and nsp3 was documented for the arterivirus EAV (Wassenaar et al., 1997). The full picture of the network of

interactions has only started to emerge and we will briefly return to this topic in the following chapters.

5. Family specific virion morphology and major virion components

Nidoviruses have evolved enveloped virions with different morphology to mediate entry into the cell and many extracellular functions essential for virus reproduction and spread (reviewed by Lai and Holmes, 2001; Snijder and Meulenberg, 1998). Both the number and composition of structural proteins vary between viruses of the four major branches and may even vary among viruses of the same branch. Weak sequence similarities between functionally equivalent proteins have been reported only for relatively closely related toroviruses and coronaviruses (den Boon et al., 1991b; Snijder et al., 1990b). The structural proteins of arteriviruses and roniviruses may have originated from unrelated ancestors or they have diverged beyond the point where ancestral relationships with other nidovirus orthologs could be recognized from sequence comparisons.

Given these differences, it is no wonder that virions of the four major nidovirus branches have (strikingly) different architectures, although they all have an external lipid bilayer with associated proteins (envelope) that encloses the internal nucleocapsid structure (Enjuanes et al., 2000; Spaan et al., 2005b) (Fig. 5). Coronaviruses, toroviruses and the significantly smaller arteriviruses have spherical virions. In addition, elongated rod-shaped virions are observed inside the torovirus-infected cells, and ronivirus particles were shown to be rod-shaped. Virions of the *Coronaviridae* and the *Roniviridae* families carry large projections that protrude from the envelope (peplomers). These oligomeric structures provide them with the characteristic “crown” observed by electron microscopy, which inspired the name of the coronavirus family. Ronivirus envelopes are also studded with prominent peplomers (Walker et al., 2005), but only relatively small and indistinct projections have been observed on the arterivirus particle surface (Snijder et al., 2005a).

The major envelope proteins are spike (S) and membrane (M) in coronaviruses and toroviruses, GP5 and M in arteriviruses, and gp116 and gp64 in roniviruses. Only the S and M protein species of corona- and toroviruses share very limited sequence similarities, indicating possible common origins. S proteins differ in size, but all have a highly exposed globular domain and a stem portion containing heptad repeats organized in a coiled-coil structure (Bosch et al., 2004; de Groot et al., 1987; Ingallinella et al., 2004; Liu et al., 2004; Snijder et al., 1990b; Supekar et al., 2004; Xu et al., 2004). At least in coronaviruses, the S protein forms trimers (Delmas and Laude, 1990). The four glycoproteins of arteriviruses are organized into two structures: one formed by a disulfide-linked heterodimer of GP5 with the M protein and another by a heterotrimer of minor structural proteins GP2, GP3, and GP4 (reviewed by Siddell et al., 2005). The M proteins of coronaviruses, toroviruses and arteriviruses have a triple-spanning membrane topology with the amino-terminus being located on the outside of the virus and the carboxy-terminus residing on the inside. In virions of the coronavirus TGEV, the M protein may have a quadruple-

spanning membrane topology leading to the exposure of both ends of the protein on the surface of the virion (Escors et al., 2001a). The two envelope proteins of the *Roniviridae* seem to be generated by post-translational processing of a precursor glycoprotein whose N-terminal region has the predicted triple-spanning membrane topology typical of the M proteins of all other nidoviruses (Cowley and Walker, 2002) (Fig. 5).

Coronaviruses, including SARS-CoV, have an internal core shell that seems to be formed by the helical nucleocapsid and the M protein (Escors et al., 2001b). The torovirus nucleocapsid resembles a toroid, which inspired the name of this nidovirus (reviewed by Snijder and Horzinek, 1993), while the nucleocapsid of roniviruses has an extended, helical organization. Unlike the three other groups, arteriviruses probably have an icosahedral core shell (reviewed by Snijder and Meulenberg, 1998). In all nidoviruses, the nucleocapsid includes only a single protein species that is uniformly named nucleocapsid protein (N), even though the N proteins of viruses from the four major branches are probably not evolutionarily related. Unlike its arterivirus counterpart (Molenkamp et al., 2000), the N protein of coronaviruses was reported to be essential for efficient sg RNA synthesis and genome replication (Almazan et al., 2004; Schelle et al., 2005). The crystal structures of the capsid-forming core domain of the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) N protein (Doan and Dokland, 2003) and the N-terminal RNA-binding domain of the SARS-CoV N protein (Huang et al., 2004) have been solved.

Nidovirus particles can include other proteins that may be dispensable in some nidoviruses or are non-essential at all. One protein, called E, is ubiquitous in viruses of the corona- and arterivirus branches (Fig. 3). This protein is essential for arteriviruses, but not for all coronaviruses. Relative to the major structural proteins, the E protein is present in a reduced number of copies (around 20) in coronavirions. Deletion of the E gene may lead either to a block of virus maturation, preventing virus release and spread (group 1 coronavirus TGEV) (Ortego et al., 2002), or to a ten to one hundred thousand-fold reduction in virus titers (group 2 coronaviruses MHV and SARS-CoV) (Fischer et al., 1998; Kuo and Masters, 2003; DeDiego et al., unpublished observations). The coronavirus E protein belongs to a family of proteins known as viroporins, which modify membrane permeability (Gonzalez and Carrasco, 2003) by forming ion channels in the virion envelope. The E protein may also modify the plasma membrane of infected cells, which could facilitate the release of virus progeny (Wilson et al., 2004). Additional proteins may be present in virions of some coronaviruses and/or toroviruses (Enjuanes et al., 2000; Spaan et al., 2005b; Tan et al., 2005) and they will be discussed in the following chapter.

6. Virus-specific accessory genes: fast adaptability to a new niche?

In addition to the family specific structural protein genes, the 3'-proximal region of the genome may include ORFs encoding proteins that we will call “accessory” (Fig. 3). They are specific for either a single species or a few viruses that form a compact phylogenetic cluster. Based on both studies with naturally

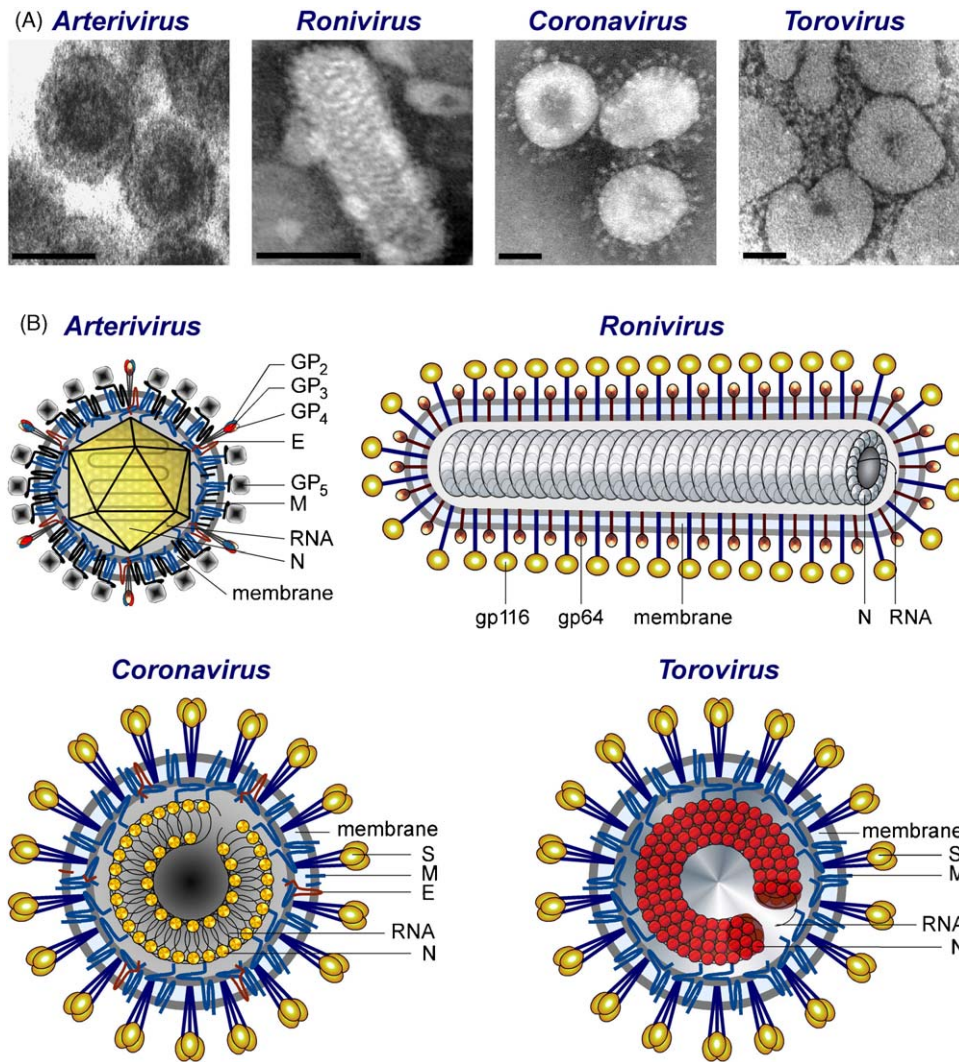


Fig. 5. Architecture of particles of members of the order *Nidovirales*: electron micrographs (A) and schematic representations (B). N, nucleocapsid protein; S, spike protein; M, membrane protein; E, envelope protein; HE, hemagglutinin-esterase. Coronavirus M protein interacts with the N protein. In arterivirus, GP5 and M are major envelope proteins, while GP2, GP3, GP4, and E are minor envelope proteins. Toro- and roniviruses lack the E protein present in corona- and arteriviruses. An equivalent of the M protein has not yet been identified in roniviruses (although see also Fig. 3). This figure was reproduced from Fig. 20.1 presented in Snijder et al. (2005b). Images were reproduced (with permission) from (Snijder and Muelenberg, 1998) (arterivirus), (Spann et al., 1995) (ronivirus), Dr. Fred Murphy, Centers for Disease Control and Prevention (CDC), Atlanta, USA (coronavirus) and (Weiss et al., 1983) (torovirus).

occurring (deletion) mutants and targeted mutagenesis studies, many of these genes are now known to be dispensable for virus growth in cell culture, and, as a result, may be called “non-essential” (Spaan et al., 2005a). The proteins encoded by these genes may be used to build virions or, alternatively, may function in infected cells or the infected host as a whole. Some functionally dispensable ORF1a-encoded replicase domains may also qualify as “accessory” (see Section 4). The CPD may be the most compelling example of accessory proteins encoded in different genome regions but belonging to the same class. This domain is part of the replicase polyproteins in toroviruses, but is encoded by ORF2 in specific group 2 coronaviruses (but not in other coronaviruses) (see Section 4). Below we will restrict our description to the accessory genes located in the 3′-proximal region of the genome.

Only the two nidovirus branches with the largest genomes (coronaviruses and toroviruses) seem to encode accessory

genes (Fig. 3). They have been acquired relatively late during nidovirus evolution, as suggested by their distribution within these branches. The absence of accessory genes in “small” arteriviruses cannot be currently rationalized. One might argue that it is related to the tight gene organization (with extensive overlaps between individual ORFs) in the arterivirus 3′-proximal genome regions (reviewed by Snijder and Muelenberg, 1998). Although this organization complicates the insertion of new genes without disrupting the functionality of the existing gene set, it seems to be compatible with either the insertion of an accessory gene downstream of the most 3′-proximal ORF or evolution of an accessory gene that fully overlaps with an existing gene in an alternative reading frame. Both these possibilities have been used to evolve accessory genes in coronaviruses (see below) but not in arteriviruses.

In coronaviruses, accessory genes are typically found in variable numbers (2–8), while in the torovirus genome only one

accessory gene, encoding the hemagglutinin-esterase (HE), has been identified (Spaan et al., 2005a) (Fig. 3). Coronavirus accessory genes may occupy any intergenic position in the conserved array of the four genes encoding the major structural proteins (5'-S-E-M-N-3'), or they may flank this gene array or overlap with a gene in this array (Enjuanes et al., 2000; Spaan et al., 2005a). Historically, some of the accessory genes were considered to be group-specific markers in coronaviruses (Lai and Cavanagh, 1997). With the recent expansion of our knowledge about the coronavirus diversity (Fouchier et al., 2004; Lau et al., 2005; Li et al., 2005; Marra et al., 2003; Poon et al., 2005; Rota et al., 2003; van der Hoek et al., 2004; Woo et al., 2005), they have been found not in every new virus of a respective group.

Group 1 CoVs may have two to three accessory genes located between the S and E genes and up to two others downstream of the N gene. Viruses of group 2 form the most diverse coronavirus cluster and they may have between three and eight accessory genes. In this cluster, MHV, human coronavirus OC43 (HCoV-OC43), and bovine coronavirus (BCoV) form a phylogenetically compact subset that is characterized by two accessory genes inserted between ORF1b and the S gene, encoding the CPD and HE functions, respectively (original group 2-specific markers), and other two genes located between, and partially overlapping with, the S and E genes, and yet another gene I that is internal to the N gene. From this set, homologs of only three proteins are encoded by the recently identified human coronavirus HKU1 (HCoV-HKU1) (Woo et al., 2005), which is the closest known relative of the above virus cluster. In contrast, the most distant group 2 member, SARS-CoV (Lau et al., 2005; Li et al., 2005; Marra et al., 2003; Rota et al., 2003), has seven or eight unique accessory genes, two between the S and E genes, four to five between the M and N genes, plus ORF 9b, which entirely overlaps with the N gene in an alternative reading frame. In the group 3 avian coronaviruses, prototyped by IBV, bipartite accessory genes were identified between the S and E genes (gene 3), and between the M and N genes (gene 5), respectively (Bournsnell et al., 1987; Cavanagh, 2005). Except for CPD and HE, no homologs of nidovirus accessory genes have been identified in other RNA viruses. The CPD homologs were discussed in Section 4, while the coronavirus/torovirus HE proteins are homologs of the HE protein of the ssRNA- influenza C virus (Luytjes et al., 1988; Snijder et al., 1991). The recently solved tertiary structure of an 81-residue luminal domain of the SARS-CoV 7a protein revealed a compact Ig-like fold (Nelson et al., 2005).

Many, possibly all, accessory proteins are non-essential for nidovirus replication in cell culture, but they may play an important role for replication in vivo and/or virulence. For the group 1 coronavirus TGEV, deletion of gene 7 had little effect on virus replication in cell culture but, in vivo, reduced the virus titers in target organs (lung and gut) by more than 100-fold, thus considerably decreasing virus virulence (Ortego et al., 2003). Also, the deletion of either of the two accessory gene clusters (3abc or 7ab) in feline infectious peritonitis virus (FIPV) produced mutants with an attenuated phenotype in the cat. These mutants did not cause any clinical symptoms upon infection with a dose considered to be fatal in the case of a wt virus infection (Haijema et al., 2004). For the group 2 MHV, four accessory genes proved to

be non-essential in vitro and in vivo, but their deletion produced mutants with an attenuated phenotype upon infection of the natural host (de Haan et al., 2002). The I gene was shown to be not essential for viral replication in mice (Fischer et al., 1997). Characterization of field isolates and reverse genetics experiments with SARS-CoV mutants lacking one or more accessory genes (3a, 3b, 6, 7a, 7b, 8a, 8b, and 9b) showed that these genes are not essential for virus replication in cell culture (e.g., Vero cells) and in the lung of infected animal model hosts (e.g., mouse) (Song et al., 2005; Yount et al., 2005). However, deletion of some of these accessory genes may have an attenuating effect in humans. This may be true for gene 3a, which encodes a SARS-CoV-specific structural protein (Ito et al., 2005). Other deletions, as those observed in SARS-CoV ORF 8a/b, leading to the loss of 29, 82, or 415 nts, may have been the result of the adaptation of SARS-CoV to civet cats and humans, after the virus was transferred from the natural reservoir, possibly bats. The deletion of 29 nt present in most of the human isolates has also been observed in civet cats, while the full size ortholog of ORF8a/b was identified in the SARS-like coronavirus isolated from bats (He et al., 2004; Lau et al., 2005; Li et al., 2005). Mutants of the group 3 IBV, in which gene 3ab was deleted, replicate equally well as the wild-type virus in vitro. Using isogenic IBV generated by reverse genetics, in which the expression of genes 3 and 5 was prevented, it was shown that these genes are not required for replication in tissue culture or chicken tracheal organ cultures (Casais et al., 2005; Hodgson et al., 2006). Inactivation of gene 5 also has no effect on the yield of IBV progeny in embryonic eggs, although this experiment, due to the use of a non-pathogenic virus strain, did not rule out a possible role for this gene in vivo (Casais et al., 2005).

When present on the virus surface, another accessory structural protein found in coronaviruses, HE, may increase viral infectivity by binding sialic acid moieties on the cell surface (Kazi et al., 2005). In fact, it has been shown that the coronavirus HE presumably promotes virus spread and entry in vivo by facilitating the reversible attachment of the virus to O-acetylated sialic acids (Lissenberg et al., 2005). While HE may provide a strong selective advantage during natural infection, many laboratory strains of mouse hepatitis virus (MHV) no longer produce this protein. Apparently, their HE genes were inactivated during cell culture adaptation. Additional experiments suggested that wild-type HE reduces the in vitro propagation efficiency, indicating that the expression of "luxury" proteins may come at a fitness penalty. Probably, under natural conditions the costs of maintaining HE are outweighed by the benefits.

The variability of the HE gene within toroviruses is also remarkable. The HE gene is present in porcine, bovine, equine, and putative human toroviruses (Cornelissen et al., 1997; Duckmanton et al., 1999; Kroneman et al., 1998). Most newly characterized bovine torovirus variants seem to have emerged from a genetic exchange, during which the 3' end of the HE gene, the N gene, and the 3' nontranslated region of a bovine virus (Breda type strain) have been swapped for those of a porcine torovirus. Moreover, some porcine and bovine torovirus variants carried chimeric HE genes, which apparently resulted from recombination events, presumably through a double template

switch, involving hitherto unknown toroviruses. From these observations it has been inferred that toroviruses may be even more promiscuous than other mammalian nidoviruses, the coronaviruses and arteriviruses (Smits et al., 2003). The extensive evolution of the torovirus HE gene may have been a host-adaptation strategy or a way to evade the host immune response.

It is apparent from the above overview, that coronaviruses are capable of expanding their repertoire of essential genes with many new and non-essential ones specific for a few closely related viruses. Although the analysis of accessory protein functions is technically challenging, and still in its infancy, it seems likely that this genome plasticity might provide the respective nidoviruses with selective advantages and fast adaptability in their natural hosts. As these accessory genes represent only minor extensions to the set of essential genes, this adaptability may be a direct benefit from the coronavirus ability to evolve and replicate a giant-size genome.

7. RNA synthesis in *Nidovirales*: what makes it special?

The synthesis of the replicase polyproteins is directed by the genome RNA, while the structural and accessory proteins are produced from sg mRNAs whose number may vary between 2 and 9 in different nidoviruses. For years, the detailed analysis of the RNA species involved in the synthesis of sg mRNAs (transcription) and genomic RNA (replication) has been one of the busiest areas of nidovirus research and the data produced often divided the field (for reviews see Brian and Spaan, 1997; Lai and Cavanagh, 1997; Lai and Holmes, 2001; Sawicki and Sawicki, 1995; Snijder and Meulenberg, 1998; van der Most and Spaan, 1995).

In the previous sections, we used the results from a comparative analysis of the replicative apparatus of nidoviruses to discuss the conserved features that distinguish nidoviruses from other viruses and the features that separate large and small nidoviruses. Since the replicase machinery has evolved to mediate RNA synthesis, the above-mentioned specifics must be linked to the unique aspects of nidovirus replication/transcription. Initially, it appeared that copying (and joining) of non-contiguous genome sequences during the synthesis of sg mRNAs (Baric et al., 1983; de Vries et al., 1990; Lai et al., 1984; Spaan et al., 1983) could be one of the major unifying aspects common and specific only to nidoviruses. More recent studies on the relatively well characterized corona- (Zuniga et al., 2004) and arteriviruses (Pasternak et al., 2001; van Marle et al., 1999a) and the poorly characterized toro- (van Vliet et al., 2002) and roniviruses (Cowley et al., 2002) revealed another common theme in nidovirus RNA synthesis, which, however, does not distinguish either all or the large nidoviruses from (all) other RNA viruses.

Nidovirus sg mRNAs are generated as an extensive 3'-coterminal nested set from which ORFs in the 3'-proximal region of the polycistronic genome are expressed. With a few notable exceptions, only the 5'-proximal ORF is expressed from each sg mRNA species and, consequently, the number of sg mRNA species synthesized is roughly proportional to the number of ORFs located downstream of ORF1b. In the genomes of all nidoviruses studied, a copy of a short characteristic AU-

rich sequence, at present mostly referred to as transcription-regulating sequence (TRS), is located upstream of ORF1a and immediately upstream of each of the downstream ORFs that can also occupy the 5'-proximal position in one of the sg mRNAs. As their name implies, the TRSs are thought to regulate the transcription of the genome template. Most probably, they act as signals for either attenuation or termination of minus strand RNA synthesis in all nidoviruses, thus producing subgenome-size minus strands that are later utilized as templates in sg mRNA synthesis (Sawicki et al., 2001). Similar mechanisms for attenuation of minus strand RNA synthesis were described for a number of polycistronic plant RNA virus genomes of different families and sizes (Gowda et al., 2001; Miller and Koev, 2000).

In arteriviruses and coronaviruses, the TRSs associated with the ORFs located in the 3'-proximal domain of the genome (known as body TRSs) are believed to direct attenuation of minus strand RNA synthesis which, in a process guided by a base-pairing interaction between complementary sequences, is resumed at the TRS upstream of ORF1a (known as leader TRS) to complete the synthesis of the subgenome-size minus strands. This process has been dubbed 'discontinuous extension of minus strand RNA' (for a recent review see Sawicki and Sawicki, 2005). The negative-sense RNAs are subsequently used as templates for the synthesis of complementary sg mRNAs, which have two adjacent parts (known as leader and body) that correspond to two noncontiguous parts of the genome RNA. The sg- and genome-length RNAs share a 5' leader sequence of 55–92 nt (coronaviruses) or 170–210 nt (arteriviruses) (Lai and Cavanagh, 1997; Snijder and Meulenberg, 1998; Zuniga et al., 2004). A sg mRNA of similar composition, carrying a leader sequence that matches the 5'-terminal 18 nt of the genome, is synthesized to express ORF2 of equine torovirus (Snijder et al., 1990c; van Vliet et al., 2002), whereas the three remaining, smaller torovirus sg mRNAs (Snijder et al., 1990c; van Vliet et al., 2002) and both sg mRNAs of roniviruses (Cowley et al., 2002) lack a common 5'-leader sequence, and are probably produced in a process that does not involve discontinuous RNA synthesis.

The use of continuous versus discontinuous subgenomic RNA synthesis among nidoviruses separates arteri- and coronaviruses from roniviruses, with toroviruses being an intermediate. This grouping is different from the one based on genome size, indicating that the evolution of transcription was a complex process that was not directly linked to genome expansion. Irrespective of the use of discontinuous subgenomic RNA synthesis, nothing in the available data on RNA synthesis explains why the nidovirus replicase machinery must include the large number of (unique) enzymatic and other subunits that has been described in Section 3. Indeed, the relatively complex mechanism for discontinuous RNA synthesis could be considered (in full agreement with the available data!) to be a variant of the replicative similarity-assisted template switching (Pasternak et al., 2001) that operates during RNA recombination in viruses with relatively small genomes and "simple" replicase complexes (Lai, 1992). A much more detailed understanding of the molecular processes involved in nidovirus RNA synthesis will probably be required to find a plausible explanation for this paradox.

8. Mechanisms of evolution: mutations and recombination

Two common evolutionary mechanisms – mutation and recombination – have been implicated in the generation of nidovirus genome diversity. In contemporary coronaviruses, like SARS-CoV and HCoV-OC43, the mutation rate was estimated (Salemi et al., 2004; Vijgen et al., 2005) to be in the range that is typical for other RNA viruses, with much smaller genomes, whose replicase fidelity is known to be low (Castro et al., 2005; Domingo and Holland, 1997; Drake and Holland, 1999). Mutations are accepted at an uneven rate across the nidovirus genome, with the structural protein genes, especially those encoding envelope glycoproteins, and the 5'-proximal half of ORF1a evolving relatively fast and ORF1b evolving relatively slowly (Chouljenko et al., 2001; Song et al., 2005). The observed differences must be linked to the roles played by the respective products of these genome regions in the nidovirus life cycle (see above). In the most conserved parts of the genome, nonsynonymous replacements start to accumulate significantly only at relatively large evolutionary distances separating the major nidovirus groups. In these cases, replacements have sometimes been accepted at places of extraordinary conservation, e.g., in the active site of the 3CL^{pro}, which are not known to have been mutated in their DNA-encoded homologs. Although little is known about the absolute time scale of nidovirus evolution, it was argued that a high mutation rate may have been at work for a very long time to generate the contemporary diversity of the most conserved enzymes in RNA viruses (Koonin and Gorbalenya, 1989).

There is plenty of evidence, especially in corona- (Makino et al., 1986) and toroviruses (Herrewegh et al., 1998), for frequent recombination between contemporary nidoviruses (or their immediate ancestors), either in the field or in experimental settings (for reviews see Brian and Spaan, 1997; Lai, 1996). This recombination commonly involves closely related viruses, e.g., belonging to the same group of coronaviruses, and is believed to occur during replication and to be mediated by replicase-driven template switching in a homologous genome region. As a result, chimeric progeny genomes are produced with a mosaic relationship to their parental templates. This type of recombination, which is common in ssRNA+ viruses (Lai, 1992), could be effective in removing deleterious (point) mutations (Chao, 1988; Worobey and Holmes, 1999) that might otherwise accumulate in the big nidovirus genome and could drive it into extinction. To counteract the adverse consequences of a high mutation rate for their big genomes, nidoviruses may employ yet other, unique repair mechanism(s), possibly involving the diverse RNA-processing enzymes described in Section 3 (see also below).

An aberrant variant of homologous recombination (Lai, 1992) may have also been involved in the expansion and shrinking of the nidovirus genome. Arteri- and coronaviruses have multiple (possibly up to four) copies of PL^{pro} (den Boon et al., 1995; Gorbalenya et al., 1991; Ziebuhr et al., 2001), which are likely to have arisen from duplication of a locus. PL^{pro} duplications may have occurred independently in arteri-

coronaviruses, since the PL^{pro}s of these two nidovirus branches do not seem to interleave with each other (Ziebuhr et al., 2000; Ziebuhr et al., 2001). The pattern of distribution of viruses with one and two PL^{pro}s in the coronavirus tree suggests that their evolution may have been accompanied by either independent duplication or loss of PL^{pro} domains (Ziebuhr et al., 2001). Duplications were also documented in a region immediately upstream of PL1^{pro} in HCoV-HKU (Woo et al., 2005) and in the 3'-proximal region of the arterivirus SHFV (Godeny et al., 1998). Also, the coronavirus nsp9 was proposed to have originated from a duplication of a 3CL^{pro} domain (Sutton et al., 2004).

Heterologous recombination, directed by template switching in nonhomologous regions, might have promoted the relocation of the CPD and HE genes in an ancestor of either toroviruses or group 2a coronaviruses. Under the alternative scenario, the CPD and HE genes must have been acquired independently by these ancestors (Snijder et al., 1991). It is heterologous RNA recombination that may have generated the diverse gross changes in the nidovirus genome that were discussed in previous chapters. Almost nothing is known about the parameters of heterologous recombination in nidoviruses, including the origins of parental (donor) sequences of which (a part of) the replicase domains must have been acquired. This complete lack of information on the origin of specific sequences extends from the accessory genes in the 3'-part of the coronavirus genome to the non-essential, lineage-specific replicative domains and also includes the essential ExoN and O-MT domains that are specific for large nidoviruses.

9. Origins of the *Nidovirales* and nidovirus families

The complex genetic plan and the replicase gene of nidoviruses must have evolved from simpler ones. The reconstruction of such ancestral events is in no way a straightforward exercise for a group of viruses in which major internal (i.e., nidovirus-specific) and external relationships (with other viruses and cellular systems) are extremely remote and the direction of evolution may not be readily recovered (Zanotto et al., 1996). Even though tentative, such an effort may have a definitive merit as it puts to a critical test the maturity of our knowledge base and involves across-virus comparisons to produce a large scale picture that otherwise would have been obscured. We offer below a speculative scenario which is based on the assumption that, generally, RNA viruses expand rather than shrink their genomes, which may not be true at any given time point.

It is safe to assume that the last common ancestor (LCA) of the *Nidovirales* may have had a genome size close to that of the contemporary arteriviruses. In this scenario, the transition from this arterivirus-like nidovirus LCA to the LCA of the large nidoviruses may have been accompanied by the acquisition of the otherwise unique ExoN domain (Snijder et al., 2003) and a (gradual?) genome size enlargement of about 14 kb (Fig. 6). The correlation between these two events fits perfectly in the relationship between the quality of genome copying and genome size observed in various biological systems. In this context, the ExoN domain may have been acquired to improve the low

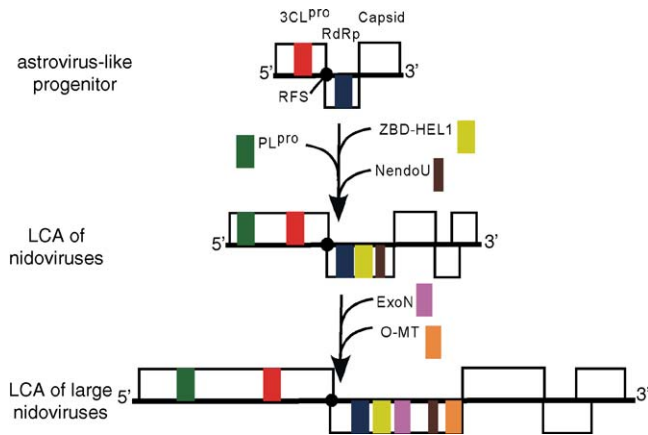


Fig. 6. Origin and evolution of the nidovirus genome plan. A tentative evolutionary scenario leading to the origin of the LCAs of nidoviruses and large nidoviruses from a progenitor with an astrovirus-like genome organization is illustrated. The three shown genomes are fictitious, although they are drawn to a relative common size scale and include major replicative domains found in genomes of respective contemporary viruses, as discussed in the text.

fidelity of RdRp-mediated RNA replication through its 3' → 5' exonuclease activity (Minskaia et al., *in press*), which might operate in proof-reading mechanisms similar to those employed by DNA-based life forms (reviewed by Kunkel and Bebenek, 2000; Kunkel and Erie, 2005). This parallel does not imply that the ExoN-containing replicase complex of large nidoviruses may work like a DNA polymerase complex. To perform its vital function for copying large genomes, ExoN may work in concert with other nidovirus RNA processing enzymes, like their distant homologs do in two cellular intron-processing pathways (Fig. 7). Based on this parallel, it was suggested that the genetically segregated and down-regulated HEL1, ExoN, NendoU, and O-MT domains (Fig. 3) may provide RNA specificity, and that the relatively abundant CPD and ADRP, when available (Figs. 3 and 4), may modulate the pace of a reaction in a common pathway (Snijder et al., 2003), which, as we propose, could be part of an oligonucleotide-directed repair mechanism.

The ExoN function likely was acquired by an ancestral virus that already had a multi-subunit replicase complex whose fidelity was good enough to copy a larger than average-size genome. Based on the genetic diversity among contemporary viruses,

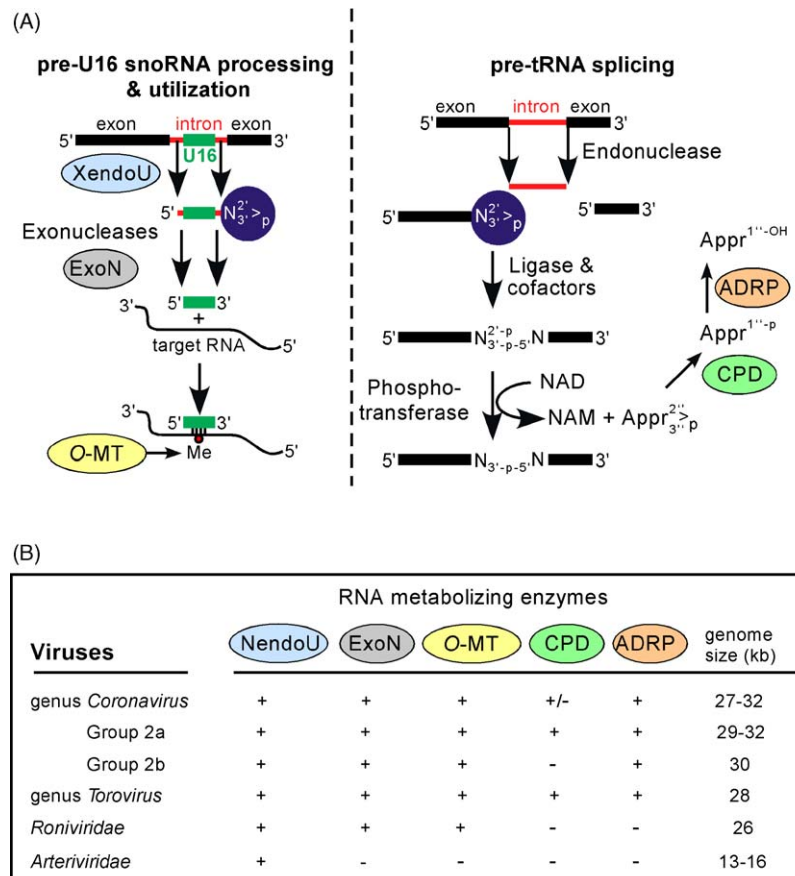


Fig. 7. RNA-processing enzymes of nidoviruses: possible cooperation and virus distribution. (A) Cellular pathways for processing of pre-U16 small nucleolar RNA (snoRNA) and pre-tRNA splicing in which homologs of five nidovirus RNA-processing enzymes are involved. Note that both pathways produce intermediates with 2'-3'-cyclic phosphate termini (blue circles), indicating the structural basis for a possible cooperation of the nidovirus homologs in a single pathway (Snijder et al., 2003). (B) Table summarizing the conservation of five (putative) RNA-processing enzymes among representatives of large and small nidoviruses. This figure was updated from Fig. 5 presented in Snijder et al. (2003).

we can assume that the domain composition of the replicase polyproteins of the arterivirus-like nidovirus LCA was more complex than that of other known RNA virus families. At the stage at which (presumably) the nidovirus LCA emerged, the two genetic marker domains, ZBD and NendoU, may have been acquired (Fig. 6). They may have been used to build a prototype repair mechanism sufficient to copy a ~14 kb genome that later, following the acquisition of ExoN, evolved into a more advanced system that was subsequently used in the *Coronaviridae/Roniviridae* branch. It is likely that extensive evolution producing a number of major virus lineages, which may either have become extinct or eluded identification thus far, preceded the origin of the Nidovirus LCA.

Among contemporary viruses, the *Astroviridae* (Jiang et al., 1993) may most strongly resemble the reconstructed nidovirus LCA in two major respects (Fig. 6). First, they have a nidovirus-like genetic plan with overlapping ORFs 1a/1b encoding replicase polyproteins and a downstream located ORF for the capsid protein that is expressed from an sg mRNA. Second, the backbone of the astrovirus replicase gene, which is composed of three domains, 3CL^{pro}-RFS-RdRp, matches the central part of the nidovirus-specific domain arrangement. These three domains are key elements in the control of genome expression and replication and therefore the ancestral relationship of *Astroviridae* and *Nidovirales* would also be in agreement with functional considerations.

It is also interesting to note that astroviruses are at the upper border of the genome size of RNA virus families that lack a HEL gene (Fig. 1). A remarkably strong positive correlation was noted between an ssRNA+ virus genome size of more than ~6 kb and the presence of a HEL domain in the replicative polyproteins of such viruses (Gorbalenya and Koonin, 1989). This correlation implies that the acquisition of the HEL domain may have been a turning point in RNA virus evolution that allowed enlargement of RNA virus genomes to sizes of more than 6–7 kb. Mechanistically, the acquisition of a HEL domain may have liberated RNA viruses from constraints related to the unwinding of large double-stranded RNA regions. Efficient control over this reaction may be essential for regulating replication, expression and transport of RNA virus genomes (Kadare and Haenni, 1997). The acquisition of the HEL domain apparently coincided with a major diversification of ssRNA+ viruses to produce more than 30 families and groups that include viruses with different genetic layouts and genomes with sizes ranging from ~6 to 32 kb.

In conclusion, we may then speculate that an ancestral virus with the genome and replicase gene organization of astroviruses, which already feature the largest genomes among their classmates (Fig. 1), may have acquired a HEL1 domain from a virus of the alphavirus-like supergroup or another source. The HEL1 evolved its 5' → 3' polarity and was, in the association with ZBD, inserted downstream of the RdRp domain, all novelties among RNA viruses that may have been essential for the genome expansion. The acquisition of other domains might have followed. To ensure control over the expression of gradually growing replicase polyproteins, the PL^{pro} was acquired, eventually resulting in the LCA of nidoviruses (Fig. 6).

10. Concluding remarks

In stunning contrast to the three-log size variation among the genomes of the contemporary DNA viruses, the entire diversity of the much more numerous RNA viruses is “squeezed” into a bandwidth of just one order of magnitude. Despite this huge scale difference, RNA viruses must have undergone extensive evolution to expand their genomes, as is illustrated by the *Nidovirales* order (this review) and the *Closteroviridae* family (see the review by Dolja et al., 2006). The three groups of nidoviruses with large genomes stand alone at the upper edge of the RNA virus genome size scale. The large size gap that separates these viruses from others, which densely populate the bottom half of the genome size scale (Fig. 1), suggests that expansion of viral genomes above ~15 kb is a formidable challenge that has been rarely met in RNA virus evolution. As speculated in this review, the large nidoviruses may have cleared this barrier with the largest margin because of prior evolution resulting in unprecedented genome expansions in several of their ancestors (Figs. 1 and 6). The comparison of the large corona-, toro- and roniviruses with their much smaller *Arteriviridae* cousins provides an intellectual framework for rationalizing the genome expansion in the *Nidovirales*. In this review, the common and unique features that have been recognized in either the large or even all nidoviruses were briefly discussed. Obviously, the progression of our understanding of nidovirus evolution depends on advancements in many research areas, including studies on nidovirus-specific RNA and protein synthesis, interactions of these viruses with their hosts, virus sampling in the field, and diverse evolutionary analyses. These advancements may help to dissect the molecular mechanisms that nidoviruses have evolved to synthesize and express their giant genomes and maintain the necessary balance between fast adaptation to changing environmental conditions on the one hand and genome stability on the other. To better understand this aspect, information on how nidoviruses make use of the unprecedented complexity and special features of their replicase machinery will be of prime interest. Furthermore, a comprehensive understanding of the parameters of nidovirus adaptability may result in the identification of the driving forces that have shaped nidovirus evolution. In this context, it will be essential to continue the sampling of the natural diversity of nidoviruses, also to see whether the genome size gap between the small and large nidoviruses will be filled with viruses with intermediate-size genomes, and whether nidoviruses infecting other phyla, e.g., plants and insects, can be identified. Ultimately, we may hope to learn whether the large nidoviruses (particularly, coronaviruses) have reached the theoretical genome size limit that may have been set for RNA viruses by nature. Finally, and more practically, our progress in understanding the fundamental aspects of RNA virus genome expansion may help to monitor and control infections caused by (known and potentially emerging) nidoviruses.

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