THE CORONAVIRUSLIKE SUPERFAMILY

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If superior creatures from space ever visit earth,
the first question they will ask, in order to
assess the level of our civilization, is:
'Have they discovered evolution yet?'

Richard Dawkins
The Selfish Gene

INTRODUCTION

In the past three years, the increasing knowledge of viral genome organizations, replication strategies, and nucleotide sequences has had its impact on coronavirus taxonomy. The 'superfamily' concept[1,2], which is based on evolution and phylogeny, and which had already closed the gaps between other virus groups (e.g. the alphaviruslike and picornaviruslike superfamilies), has now been found to apply to a group of coronaviruslike viruses as well.

The sequence analysis of the genomes of the 'classic' coronaviruses infectious bronchitis virus (IBV) and mouse hepatitis virus (MHV), the Berne torovirus (BEV), and the 'unclassified togavirus' equine arteritis virus (EAV) revealed unexpected evolutionary links. The common features of these viruses are centered around the 'coronaviruslike' replicase gene and the replication and expression strategy which is associated with it. In this short comparative review, toroviruses and arteriviruses will be introduced to the coronavirologist, and similarities and differences with coronaviruses will be discussed, using MHV as the standard coronavirus for comparison throughout this paper.

Coronaviruses, Edited by H. Laude and J.F. Vautherot
Plenum Press, New York, 1994

TOROVIRUSES

In 1972, a virus was isolated from a diarrheic horse during routine diagnostic work at the University of Berne, Switzerland. The isolate, designated P138/72, displayed an unusual morphology but was not studied in more detail until a similar virus was isolated from diarrheic calves in Breda, Iowa, U.S.A., in 1979[3,4]. Berne virus (BEV) and Breda virus (BRV) were found to be antigenically related[3]. In later years, a second BRV serotype was identified[4] and similar pleiomorphic viruses were found in the stools of children and adults with gastroenteritis in Birmingham, U.K., and Bordeaux, France[5]. Immune EM experiments indicated that these human viruses were serologically related to BRV and BEV[6].

The unique morphology of BEV and BRV (see below) and the physicochemical properties of BEV[7] initially led to the proposal of a new family of enveloped animal viruses, the *Toroviridae*[8]. BEV, the only torovirus so far which can replicate in cultured cells, was chosen as the torovirus prototype.

ARTERIVIRUSES

Equine arteritis virus (EAV) was first isolated from a fetus aborted during an endemic disease outbreak in pregnant mares[9]. Serological evidence suggests that the virus is widespread in the horse population and only rarely causes disease. However, in pregnant mares abortion is common[10,11]. A carrier state exists in seropositive stallions in which EAV is produced in semen[12]. These 'shedding stallions' may consequently infect broodmares by a venereal route. Field isolates are rare, may be difficult to propagate in cell culture, and consequently the biology of EAV is poorly understood. The biological and clinical properties of EAV have been reviewed recently[13].

The molecular characterization of lactate dehydrogenase-elevating virus (LDV) of mice[13,14] and swine infertility and respiratory syndrome virus[15] (SIRSV or 'Lelystad virus'), which has been reported recently, has revealed that these two viruses are closely related to EAV. Though it has not yet been characterized at the molecular level, simian hemorrhagic fever virus (SHFV) may be the fourth member of this virus group[13].

The morphological characteristics and genome size of EAV (12.7 kb) are most comparable to those of togaviruses. However, as will be described below, the arterivirus replication strategy[16] is similar to that of coronaviruses and toroviruses, which possess 25-31 kb genomes.

VIRION ARCHITECTURE

Torovirions are pleiomorphic particles which measure 120-140 nm in their largest diameter[3]. Spherical, oval, elongated, and kidney-shaped virions have been observed. Their two most striking morphological features are the spikes on the viral envelope, which resemble the peplomers of coronaviruses, and the tubular nucleocapsid of helical symmetry, which seems to determine the shape of the virion[3]. The presence of nucleocapsids in the form of a doughnut, a shape described in Latin by the word 'torus', led to the proposal of the name toroviruses.

The morphogenesis of BEV has been studied by EM methods[17]. Preformed, tubular nucleocapsids were found to bud at intracellular membranes, predominantly those of the Golgi system. A morphological change seems to take place during virus maturation: prior

to budding the nucleocapsids are straight, but in extracellular virus the characteristic torus-shape is prevalent.

Three structural BEV proteins have been identified and characterized (see also below): an 18K nucleocapsid (N) protein, a 26K transmembrane protein (initially named E), and a 180K spike glycoprotein (initially named P). In view of the recent classification of the toroviruses as a *genus* in the coronavirus family, the E and P protein will from now on be referred to as M and S protein, respectively. The current structural model of the BEV particle is shown in Fig. 1.

Equine arteritis virus was initially classified as a member of the togavirus family[18].

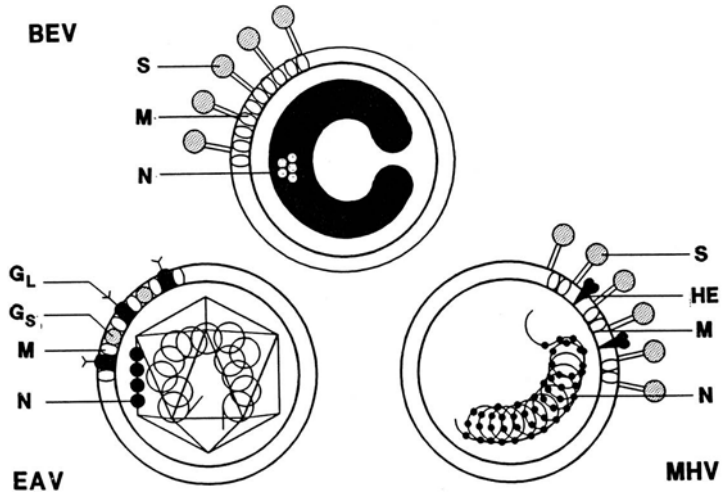

Fig. 1. Schematic representation of the structure of BEV (torovirus), EAV (arterivirus), and MHV (coronavirus). The structural proteins of each virus group are included.

The spherical enveloped equine arteritis virus particle has a diameter of 50-70 nm[19]. It consists of an icosahedral core structure of 35 nm surrounded by an envelope carrying ring-like structures with a diameter of 12-15 nm[20]. The identification and characterization of four virion proteins have been reported recently[21]: a 12K nucleocapsid (N) protein, an unglycosylated 18K transmembrane protein (M), a 25K glycoprotein G_S, and a second glycoprotein, G_L, which, due to heterogeneous glycosylation, gives rise to proteins with sizes between 30K and 42K. The current model of the EAV particle is shown in Fig. 1.

In summary, nucleocapsid architecture - a classic trait for viral taxonomy, with the same ranking as nucleic acid type or the presence of an envelope - is icosahedral in EAV, helical in coronaviruses, and tubular in toroviruses. An important additional difference at

the structural level is the fact that the EAV envelope does not bear the elongated spikes which are so characteristic for both coronaviruses and toroviruses. However, as will be discussed in the next paragraph, the genome organization and expression of the three virus groups are strikingly similar and evidence for common ancestry was obtained by comparison of replicase amino acid sequences.

GENOME ORGANIZATION AND EXPRESSION

The BEV genome (probably) contains six open reading frames (ORFs). As in coronaviruses, the two most 5' reading frames (ORF1a and 1b) are expressed from the genomic RNA and constitute the replicase gene. Assuming that the 5' part of the BEV replicase gene, which has yet to be sequenced, contains a single open reading frame (ORF1a) of about coronaviral size, the toroviral genome measures approximately 25 kb.

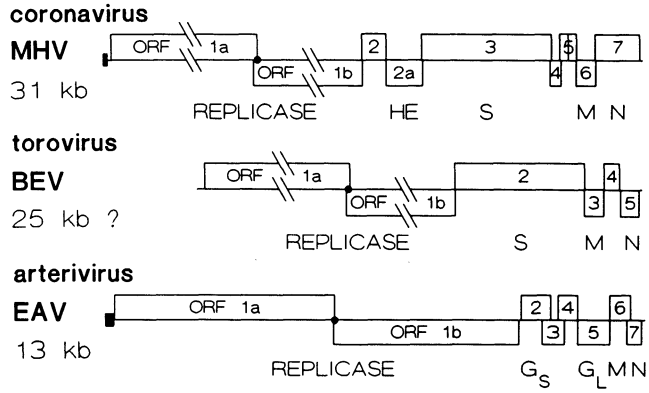

Fig. 2. Genome organization of MHV (coronavirus), BEV (torovirus), and EAV (arterivirus). The genes encoding the viral replicases and structural proteins are indicated.

The other reading frames are expressed by the generation of a 3'-coterminal nested set of four mRNAs[22]. ORFs 2, 3, and 5 encode the viral structural proteins S[23], M[24], and N[25], respectively. In view of the observed sequence similarities with other viral surface proteins, the apparently truncated ORF4[26] should probably be considered a (structural) pseudogene. The BEV genome organization is summarized in Fig 2.

Also during EAV replication a 3'-coterminal nested set of viral mRNAs is produced, ranging in size from genome length (12.7 kb) to 0.8 kb[16,27]. Viral subgenomic (sg) RNAs are composed of leader and body sequences which are not contiguous on the

EAV genome, the 207 nt leader sequence being derived from the extreme 5' end[16,27]. Again two replicase ORFs (1a and 1b) are expressed from the genomic RNA. Of the reading frames from the 3' end of the genome, ORFs 2, 5, 6, and 7 have now been shown to encode the G_S, G_L, M and, N proteins, respectively. The characteristics of the products of ORFs 3 and 4 are typical of membrane proteins, but no information on the function of these proteins has been obtained until this moment. The EAV genome organization is shown in Fig. 2.

Unlike most other ORFs of coronavirus-like genomes, ORF1b is not expressed from a separate sgRNA but by ribosomal frameshifting during the translation of genomic RNA[16,28,29]. The ORF1b product contains a number of domains which are likely to be indispensable during the early stages of viral replication, e.g. synthesis of negative-stranded RNA and the onset of sgRNA synthesis. The ORF1a/ORF1b frameshift mechanism and the RNA structures involved in this process are remarkably conserved in corona-, toro-, and arteriviruses. This indicates that translational frameshifting is an ancient and probably essential regulating step in replicase gene expression. Apparently the respective levels of ORF1a- and ORF1b-derived proteins in infected cells, and possibly also the level of ORF1a/ORF1b fusion product(s), have to be regulated. Nevertheless, the frameshifting efficiency in a reporter gene varies: figures of 25-40%, 20-30%, and 15-20% have been reported for corona-[28,30], toro-[29], and arteriviruses[16], respectively.

RNA SYNTHESIS

The generation of an extensive 3'-coterminal nested set of mRNAs from an unsegmented genome distinghuishes the members of the coronaviruslike superfamily from the viruses from most other groups of positive-stranded RNA viruses. The ancestral relationship between corona-, toro-, and arteriviral replicases[16,29] (see below) suggests that this replication strategy is dictated by the properties of the coronaviruslike replicase.

Since both corona- and arteriviral mRNAs contain a common leader sequence at their 5' end, the absence of such a leader in BEV RNAs[22,31] would form a conspicuous difference. For all three virus groups conserved sequences which are assumed to be involved in sgRNA transcription have been described. For the coronavirus MHV this is the intergenic 5' AAUCuAuAC 3' motif which has been identified as the site of leader to body fusion[32]. A similar though shorter junction sequence (5' UCAAC 3') has been reported for the arterivirus EAV[16,27]. Though also the genome of the torovirus BEV contains conserved intergenic sequences (5' uaUcUUUAGa 3'), no evidence for the presence of a common leader sequence has been obtained[22,31]. BEV mRNAs appear to terminate at or just upstream of the conserved intergenic sequence. In terms of replication however, the consequences of this dissimilarity could be limited to the initiation of positive-stranded RNA transcription only: direct binding of the polymerase to the various BEV 'core promoters' on a negative-stranded template may simply replace the leader-primed initiation of this transcription process which is used by coronaviruses and EAV.

Recent findings suggest that coronaviruses may utilize the fact that their sgRNAs contain 5'- and 3'-terminal sequences which are identical to those of the genomic RNA: transcriptionally active negative-stranded sgRNAs have been detected in infected cells[33,34]. This implies that coronaviral sgRNAs may function as replicons. Until now, attempts to demonstrate negative-stranded viral RNA in torovirus-infected cells have remained unsuccessful, but from EAV-infected cells subgenomic RF RNAs can be isolated[35]. In view of the similar replication strategies, analogous transcription mechanisms, possibly with minor variations, may very well be used by corona-, toro-, and arteriviruses.

STRUCTURAL PROTEINS

The nucleocapsid (N) protein

In view of the very different nucleocapsid structures which have been described for corona-, toro-, and arteriviruses, it is not surprising that their N proteins have little in common. No sequence similarities were detected except for the fact that all three N proteins are rich in basic amino acid residues. The N protein sizes are very different: the coronavirus N protein has a characteristic molecular weight of 45K-55K[32], BEV contains an 18K N protein[23], and the EAV N protein is only 12K in size[16,21].

The membrane (M) protein

Structurally similar transmembrane proteins are found in corona-, toro-, and arterivirus particles. These membrane (M) proteins all lack an N-terminal signal sequence. Instead they contain three membrane-spanning domains in their N-terminal half which are attached to a rather amphipathic C-terminal part. The topological model which has been proposed for the coronavirus M protein[36], has been found to apply to the orientation of the BEV M protein in the membrane as well[24]. In addition to these structural similarities, the MHV and BEV M proteins are about the same size (26K) and contain a small box of sequence similarity[24]. In our opinion, these similarities and the linkage of the coronaviral and toroviral M proteins to homologous replicase genes (see below) indicate that these proteins are homologous and not analogous.

For the EAV triple-spanning M protein the evidence for common ancestry is less convincing: the protein is smaller (18K) and no obvious sequence similarities have been detected. However, whether homologous or not, the role of the EAV M protein may be similar to that of its coronaviral and toroviral equivalents. An interesting common feature of these three virusgroups is their intracellular maturation. The M protein of coronaviruses has been implicated to play a crucial role in the budding process[37] and its localization. It was shown to accumulate in intracellular membranes[38], which is probably also true for the BEV M protein[24]. Therefore, the M proteins of corona-, toro-, and possibly arteriviruses may contain properties which are essential for virus assembly and which have been conserved during the evolution of these intracellularly budding RNA viruses.

The surface glycoproteins

The surface glycoproteins of arteriviruses are clearly different from those of corona- and toroviruses. The characterization of the small (G_S) and large (G_L) EAV glycoproteins was reported recently[21]. The 25K G_S protein is a minor protein in virus particles (1-2 %). The G_L protein, which is observed as a 30K-42K smear due to heterogeneous N-acetyllactosamine addition, is about equally abundant in virions as the M and N proteins.

The envelopes of both corona- and torovirus particles are studded with drumstick-shaped projections of similar size. Heterogeneous 75K-100K protein material from BEV particles was recognized by both neutralizing and hemagglutination-inhibiting monoclonal antibodies and was therefore assumed to represent the spike (S) protein[39]. This N-glycosylated protein is derived from processing of a 200K precursor which is found in infected cells, but not in virions. However, size, post-translational cleavage, and extensive N-glycosylation are not the only similarities between toro- and coronaviral S proteins. Both glycoproteins also form oligomers and contain hydrophobic domains and heptad repeat sequences at corresponding positions in their sequence[25]. Therefore, the coiled-coil

structural model, which has been put forward to explain the elongated shape of the coronavirus spike[40], probably also applies to the torovirus surface projections.

In conclusion, the BEV envelope proteins (formerly named E and P) have been shown to be structurally similar to the coronaviral M and S proteins. This phenomenon could possibly be explained by invoking convergent evolution. However, in view of the evolutionary relationship between toro- and coronaviruses (see below), we postulate that these similarities reflect common ancestry. The absence of antigenic relationships and amino acid sequence homologies is indicative for the large evolutionary distance between both virus groups. The arteriviruses clearly contain a completely different set of envelope proteins, with the possible exception of the M protein.

THE CORONAVIRUSLIKE REPLICASE

Genome replication and assembly of new virions are generally considered to be the two fundamental processes in the viral life cycle. The replicase gene is the best candidate for the title 'core of the virus' since replicase proteins (and the replication strategy which they impose upon a viral genome) are conserved among seemingly disparate groups of plant and animal viruses[1,2]. This is (again) exemplified by the replicases of the members of the coronaviruslike superfamily, which have been discussed extensively elsewhere[16,29].

The evolutionary relationship between corona- and toroviruses was first recognized during the sequence analysis of the BEV ORF1b region. Conserved domains (up to 50% amino acid sequence identity) were identified[29] which are present in the same order in the ORF1b sequences of the coronaviruses MHV and IBV. The importance of these homologous domains was underlined by their subsequent detection in the replicase sequence of EAV[16], which is, however, much shorter and more distantly related (up to 30% amino acid sequence identity in the most conserved regions). The organization and conserved regions of the various coronaviruslike replicases are shown in Fig. 3. Of course, the conservation of two domains (polymerase and helicase) which are common to all positive-stranded RNA viruses is not very surprising; their presence indicates that all these viruses may have descended from the same RNA virus prototype. It is remarkable, however, that only in coronaviruslike replicases the helicase domain is located downstream of the

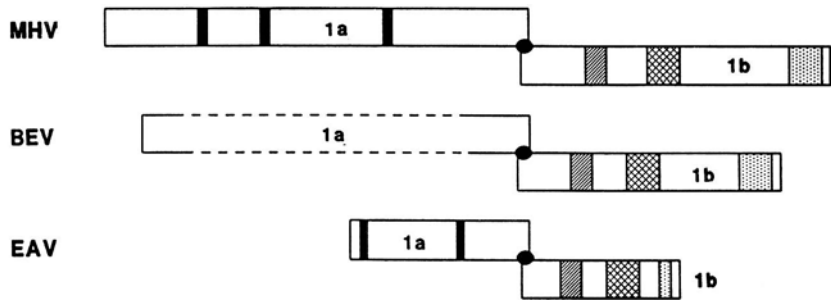

Fig. 3. Schematic representation of coronaviruslike replicases. Filled, hatched, cross-hatched, and dotted boxes represent protease, polymerase, helicase and C-terminal ORF1b domains, respectively.

polymerase motif. Also the conservation of additional replicase domains, for which no homologue can be found in other viral replicases, clearly indicates that the coronaviruslike replicases are more related to each other than to any other group of positive-stranded RNA viruses.

Ribosomal frameshifting during coronaviruslike replicase gene expression produces a large ORF1a/ORF1b fusion product (345K for EAV, 741k for IBV, 810K for MHV). As described for other viral replicases, these large replicase proteins are proteolytically cleaved into smaller active units. The proteases responsible for this posttranslational processing are thought to be located in the ORF1a protein (it should be noted that the torovirus ORF1a sequence is not yet available). A number of replicase cleavage products has been detected in coronavirus-[41,42] and arterivirus-infected[43,44] cells. However, the characterization of the viral proteases involved, which are thought to belong to the papainlike, trypsinlike, and picornavirus 3C-like classes of proteolytic enzymes, has only just begun.

THE EVOLUTION OF THE CORONAVIRUSLIKE SUPERFAMILY

Features which are shared by the members of the coronaviruslike superfamily are: the basic genome organization replicase-envelope proteins-nucleocapsid protein, the production of 3'-coterminal nested sets of mRNAs, and the conserved organization of the replicase gene (homologous replicase domains at comparable positions in the protein; two reading frames connected by a frameshift site). Noticeable differences are the dissimilar N proteins and nucleocapsid structures, the (probable) absence of a common 5' leader sequence in the BEV mRNAs, the much smaller genome size of arteriviruses, and the absence of a large spike glycoprotein in this same virus group.

The common ancestry of the coronaviruslike replicase proteins (and probably also the replication strategy connected with it) is evident. Since viral structural proteins are known to evolve at a higher rate than nonstructural proteins, it is likely that the structural similarities of coronavirus and torovirus S and M proteins also reflect common ancestry, despite the absence of convincing sequence similarities. Similar observations can be made for the EAV M protein. This leaves the different glycoproteins of arteriviruses and the diverse N proteins of coronaviruslike viruses to account for.

The coupling of different sets of structural genes to the same replicase gene has been explained by recombination of complete genes or gene sets (modules)[1,2,45]. The RNA recombination frequency during coronavirus replication has been shown to be remarkably high[32,46], which is thought to be determined by the replication strategy and replicase properties. The different sets of structural genes (and the varying number of 'additional' genes) which are now known to be linked to the coronaviruslike replicase gene indicate that also this property may be shared by all members of the superfamily ('modular' evolution). Direct evidence for multiple recombination events during BEV evolution has already been obtained[26]. Together with divergent evolution, a high recombination frequency can account for the diverse composition of coronaviruslike genomes.

THE TAXONOMY OF CORONAVIRUSLIKE VIRUSES

A useful taxonomic system should allow us to organize our information about viruses. Such a framework should (among other things) show evolutionary relationships between *species*. Until recently, phylogeny has not been an important criterion in animal virus taxonomy[47]. The taxonomic system discriminated only three hierarchical levels:

families (sometimes subfamilies), *genera*, and *species*[48]. This explains why we have so gratefully utilized the unofficial 'superfamily' or 'supergroup' category which was introduced by Strauss & Strauss[2] and Goldbach & Wellink[1].

Our increasing knowledge of viral genomic sequences and expression strategies will necessitate the introduction of additional higher taxonomic categories to permit a true phylogenetic classification of all viruses. At the VIIIth International Congress of Virology (ICV) in Berlin (august 1990) the International Committee on the Taxonomy of Viruses (ICTV) has recognized the common ancestry of *Rhabdoviridae*, *Paramyxoviridae*, and *Filoviridae* (which incidentally also display diverse nucleocapid morphologies) by bringing them together in a new (higher) taxonomic category: the order of the *Mononegavirales*.

Although a number of *species* may have to be reclassified, the introduction of phylogenetic taxonomy does not have to cause a revolution in virus systematics: many of the existing virus families and groups can probably be maintained. However, their correct organization into higher categories will require some serious consideration.

A meaningful classification of the members of the coronaviruslike superfamily clearly requires four hierarchical levels: the coronavirus and torovirus *species* have now been classified into two *genera* which belong to the *family Coronaviridae*. The obvious evolutionary link of this family to the arteriviruses would be reflected most accurately by promoting the present *genus* arterivirus to the family status and by establishing an '*order*' (to replace the 'superfamily') comprising the *Coronaviridae* and *Arteriviridae* families. Classification of the arteriviruses as a third genus of the coronavirus family is a less attractive alternative, because this would not recognize the more distant position of the arteriviruses (which do not carry a 'corona', anyway). However, this problem could be circumvented by establishing two subfamilies (*Coronavirinae* and *Arterivirinae*) and changing the family name *Coronaviridae* into something more appropriate.

The recently proposed polythetic definition of virus *species*[49] could also be applied to higher order taxa and would be flexible enough to accomodate the observed differences and similarities between the various members of the coronaviruslike superfamily. A polythetic class is defined by a large number of properties, each of which might also be absent in a member of the class or present in a member of another class[49]. The description of a virus *species* as 'a polythetic class of viruses constituting a replicating lineage and occupying a particular ecological niche' incorporates aspects from previous *species* definitions, but it can accommodate biological variability and genetic recombination more easily. From this *species* definition it is clear that, like in other areas of biology, the taxonomy of viruses should have a genetic basis[50]: as exemplified by the members of the coronaviruslike superfamily, virus evolution is governed by heredity (the passing of genetic information from parent to progeny) and the processes of mutation, recombination, and selection.

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