The Coronaviruslike Superfamily

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I. INTRODUCTION

Until recently, the *Coronaviridae* was classified as a monogeneric family of closely related viruses. However, in the past four years, it has become evident that similarities in the genome organization, replication strategies, and nucleotide sequences of coronaviruses, toroviruses, and arteriviruses, require a revision of this taxonomy. The "superfamily" concept (Strauss and Strauss, 1988; Goldbach and Wellink, 1988), which is based on evolution and phylogeny and which has already closed the gaps between other virus groups (e.g., the alphaviruslike and picornaviruslike superfamilies), can now also be applied to a group of "coronaviruslike viruses."

The sequence analysis of the genomes of the coronaviruses infectious bronchitis virus (IBV) (Bournsnell *et al.*, 1987) and mouse hepatitis virus (MHV) (Bredenbeek *et al.*, 1990; Lee *et al.*, 1991), the Berne torovirus (BEV) (Snijder *et al.*, 1990a,b), and the (at the time) "unclassified togavirus" equine arteritis virus (EAV) (Den Boon *et al.*, 1991b) revealed unexpected evolutionary links. The common features of these viruses are centered around the coronaviruslike replicase gene and its associated replication and expression strategy. Consequently, the corona- and toroviruses have now been formally joined together in the family *Coronaviridae* (Chapter 11, this volume), and the International Committee on the Taxonomy of Viruses has recently created a new study group to establish a virus family that will comprise the members of the presently free-floating genus arterivirus: EAV (the prototype of the genus), lactate dehydro-

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genase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV), and the recently discovered porcine reproductive and respiratory syndrome virus (PRRSV; also known as Lelystad virus). The question of how the evolutionary links between the *Coronaviridae* and arteriviruses should be reflected in their taxonomic status is still open to discussion (Snijder and Horzinek, 1993; Cavanagh *et al.*, 1994).

The biological properties of EAV, LDV, and SHFV have recently been reviewed by Plagemann and Moennig (1992). The morphological characteristics and genome size of arteriviruses (12–15 kb) are most comparable to those of the *Togaviridae*. However, as will be described, the arterivirus replicase and replication strategy are strikingly similar to those of the *Coronaviridae*. In this short comparative chapter, we will briefly summarize the molecular characteristics of the arteriviruses and focus on the most important similarities and differences with the *Coronaviridae*.

II. ARTERIVIRUSES

EAV was first isolated from a fetus aborted during an endemic disease outbreak in pregnant mares (Doll *et al.*, 1957). The sequence of the 12.7-kb positive-stranded, polyadenylated EAV genome was published in 1991 by Den Boon *et al.* (1991b). Two years later, the molecular characterization of PRRSV of swine (Meulenberg *et al.*, 1993a) and LDV of mice (Godeny *et al.*, 1993a) revealed slightly larger genome sizes: 15.1 and 14.2 kb, respectively. At the IXth Congress of Virology in Glasgow, 1993, the first sequence data from the SHFV genome were reported and confirmed its close relationship to the other three arteriviruses (Godeny *et al.*, 1993b).

Due to their morphology, EAV and LDV were initially classified as members of the togavirus family. The spherical arterivirus particle has a diameter of 50 to 70 nm (Hyllseth, 1973) and is comprised of an icosahedral core structure of 35 nm surrounded by an envelope carrying ringlike structures with a diameter of 12 to 15 nm (Horzinek *et al.*, 1971). The identification and characterization of four structural EAV proteins has been reported recently (de Vries *et al.*, 1992): a 12-kDa nucleocapsid (N) protein, an unglycosylated 18-kDa transmembrane protein (M), a 25-kDa glycoprotein G_s , and a second glycoprotein, G_v , which, due to heterogeneous glycosylation, has sizes between 30 and 42 kDa. The current model of the EAV particle is shown and compared with the *Coronaviridae* in Fig. 1. It is clear that the nucleocapsid architecture (a classic trait for viral taxonomy, with the same ranking as nucleic acid type or the presence of an envelope), is different for arteriviruses (icosahedral) and *Coronaviridae* (helical). Another important difference is the fact that the EAV envelope does not bear the elongated spikes which are so characteristic of the *Coronaviridae*.

III. ARTERIVIRUS GENOME ORGANIZATION AND EXPRESSION

The arterivirus genome is a polycistronic RNA that contains 8 open reading frames (ORFs) in the case of EAV, PRRSV, and LDV (Fig. 2). Like *Corona-*

FIGURE 1. Schematic representation of the structure of BEV (a torovirus), EAV (an arterivirus), and MHV (a coronavirus). The major structural proteins of each virus group are illustrated.

viridae, arteriviruses produce a 3'-coterminal nested set of viral mRNAs in the infected cell. These mRNAs range in size from genome length to about 0.7 kb and are used to express internally located ORFs (de Vries *et al.*, 1990; Kuo *et al.*, 1991; Meulenberg *et al.*, 1993a). Viral subgenomic (sg) RNAs are composed of leader and body sequences that are not contiguous on the genome, another striking similarity with coronaviruses (but possibly not with toroviruses; see Chapter 11, this volume).

The EAV ORFs 2, 5, 6, and 7 have been shown to encode the structural G_s , G_L , M, and N proteins, respectively (de Vries *et al.*, 1992). The structural 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 so far.

The arterivirus replicase gene is between 9.7 (EAV) and 11.6 (PRRSV) kb in

FIGURE 2. Genome organization of MHV (a coronavirus), BEV (a torovirus), and EAV (an arterivirus). The genes encoding the viral replicases and major structural proteins are illustrated.

size and is comprised of two ORFs (1a and 1b) that are expressed from the genomic RNA. As in the case of *Coronaviridae*, the arterivirus ORF 1b is probably expressed by ribosomal frameshifting during the translation of genomic RNA (den Boon *et al.*, 1991b). "Shifty" sequences (5' UUUAAAC 3' for LDV and PRSSV; 5' GUUAAAC 3' for EAV) and a downstream RNA pseudoknot structure are thought to form the frameshift signals (Jacks *et al.*, 1988; Brierley *et al.*, 1989; Ten Dam *et al.*, 1990). The ORF 1a/ORF 1b frameshift mechanism and the RNA structures involved in this process are remarkably conserved in corona-, toro-, and arteriviruses, indicating that translational frameshifting is an ancient and probably essential regulatory step in replicase gene expression.

IV. mRNA SYNTHESIS

The generation of a 3'-coterminal nested set of mRNAs from a nonsegmented genome distinguishes the members of the coronaviruslike superfamily from other groups of positive-stranded RNA viruses. The common ancestry of corona-, toro-, and arteriviral replicases (see Section V) suggests that this expression strategy is related to the properties of the coronaviruslike replicase and that similar (in fact, homologous) transcription mechanisms may be used by these viruses.

An early analysis of EAV mRNA transcription by means of UV transcription mapping suggested that all sg RNAs are derived from the processing of a genome-length precursor molecule (van Berlo *et al.*, 1982), a situation that would be very different from the independent transcription of coronavirus and torovirus mRNAs, which was deduced using the same experimental approach (Jacobs *et al.*, 1981; Snijder *et al.*, 1990b). To resolve this point, the UV transcription mapping analysis of both EAV and MHV at a late time point in infection has recently been repeated (Den Boon *et al.*, 1995b), and in contrast to the observations of van Berlo *et al.* (1982), a clear correlation between UV target size and physical size for the sg RNAs of EAV was established. However, a quantitative analysis indicated that for both arteriviruses and coronaviruses this correlation does not justify the conclusion that the sg RNA transcription is fully independent from the synthesis of the genomic RNA (Den Boon *et al.*, 1995b). Nevertheless, splicing can be ruled out as the major mechanism for arterivirus sg RNA production late in infection.

The comparative analysis of mRNA transcription of corona- and arteriviruses was extended by preparing replicative form (RF) RNA in order to study the EAV minus strands (Den Boon *et al.*, 1995c). As in coronavirus-infected cells (Sethna *et al.*, 1989; Sawicki and Sawicki, 1990), sg RFs were detected, suggesting that complements of both genomic and sg positive strands are generated. However, it remains to be excluded that the sg negative strands of EAV are derived from cleavage of RNase-sensitive sites in a genome-length RF. For a discussion on the possible role of the sg minus strands of coronaviruses, the reader is referred to Chapter 2 (this volume).

Both corona- and arteriviral mRNAs contain a leader sequence at their 5' end that is identical to the 5' end of their respective genomes (de Vries *et al.*,

1990). Thus the generation of corona- and arterivirus subgenomic mRNAs must involve a process of discontinuous transcription. The arterivirus leader RNA has between 170 and 210 nucleotides and is, therefore, two to three times larger than that of coronaviruses. For EAV, LDV, and PRRSV, conserved AU-rich sequences have been identified at the leader-to-body junction (LBJ) sites of the mRNAs (Fig. 3) (de Vries *et al.*, 1990; Den Boon *et al.*, 1995c; Chen *et al.*, 1993; Meulenberg *et al.*, 1993b). Coronavirus genomes contain similar conserved AU-rich boxes (known as intergenic sequences) at the fusion sites of leader and mRNA bodies (reviewed by Spaan *et al.*, 1988; Lai, 1990). However, since most of the arterivirus ORFs 2 to 6 overlap to some extent, the coronavirus term "intergenic sequence" is inappropriate for arteriviruses. Although no evidence for a common leader sequence in torovirus mRNAs has been obtained, a conserved AU-rich sequence, which is thought to function as a promoter sequence for mRNA transcription, is also found upstream of ORFs 2 to 5 of the Berne virus (Fig. 3) (Chapter 11, this volume).

In view of its relatively small size, it is, perhaps, not surprising that the arterivirus LBJ sequences are found at sites not involved in mRNA synthesis. The EAV 5' UCAAC 3' LBJ motif, for example, occurs 18 times in the genomic sequence. Thus, it is evident that this sequence element cannot be the sole determinant of mRNA transcription.

The role of the LBJ motif in mRNA synthesis has not yet been fully elucidated. As in coronaviruses, the extreme 3' end of the arterivirus leader sequence is identical to the LBJ sequence. Thus, it is possible that, in the arterivirus minus strand, the complements of LBJ sites may function as mRNA promoters in a discontinuous leader-primed transcription mechanism involving basepairing between the leader and the promoter region. This process may correspond to the model that has been put forward for coronavirus mRNA transcription (Baric *et al.*, 1983; Lai *et al.*, 1983; Spaan *et al.*, 1983). A basepairing interaction between leader and mRNA promoter sequences on the negative

strand is still the most attractive explanation for the discontinuous step during coronavirus mRNA transcription. Since the coronavirus system has been studied more extensively, we would refer the reader to Chapter 2 (this volume) for a more detailed discussion of this subject.

V. THE CORONAVIRUSLIKE REPLICASE

Genome replication is a fundamental process in the viral life cycle and viral replicase genes are at the hub of viral biology. Replicase proteins are conserved among seemingly disparate groups of plant and animal RNA viruses (Strauss and Strauss, 1988; Goldbach and Wellink, 1988), and this evolutionary link is clearly apparent in the replicases of the coronaviruslike (CVL) superfamily. The comparison of the replication strategy and replicase properties of corona-, toro-, and arteriviruses has clearly distinguished the CVL replicase module from those of alpha, picorna, and flaviviruses. The organization of the CVL replicase in two ORFs, expression of the gene by ribosomal frameshifting, and the arrangement of conserved domains within the gene product are unique.

Although arterivirus replicase genes are considerably smaller (9.5–12 kb) than their toro- and coronavirus counterparts, they contain a number of conserved domains that are present in the same relative positions (Den Boon *et al.*, 1991b). The sequence alignments (Fig. 4) of these domains reveal up to 30% amino acid sequence identity in the most conserved regions, a percentage that cannot be due to convergent evolution. The conservation of two of these domains (polymerase and helicase), which are common to all positive-stranded RNA viruses (Poch *et al.*, 1989; Gorbalenya and Koonin, 1993), is not very surprising; their presence indicates that these viruses have probably all descended from the same RNA virus prototype. It is remarkable, however, that only in CVL replicases the helicase domain is located downstream of the polymerase motif. The polymerase motif also carries another CVL replicase trademark: the substitution of the classic GDD in the core of the motif by an SDD. Also, the conservation of additional replicase domains, for example, the carboxyl-terminal ORF 1b domain, for which no homologue can be found in other viral replicases, clearly indicates that the CVL replicases are more related to each other than to any other group of positive-stranded RNA viruses.

VI. PROTEOLYTIC PROCESSING OF THE CVL REPLICASE

Proteolytic processing of nonstructural proteins fulfills a key role in the life cycle of most viruses. In the course of virus evolution, highly specific virus-encoded proteases have evolved, and their importance for the regulation of virus replication is becoming more and more evident. For detailed information on this topic, the reader is referred to an excellent review by Dougherty and Semler (1993).

CVL replicase gene expression leads to the production of an ORF 1a/1b fusion protein that is large in the case of arteriviruses (345–420 kDa) (Den Boon

et al., 1991b; Meulenberg *et al.*, 1993a; Godeny *et al.*, 1993a) and extremely large in coronaviruses (740–810 kDa) (Bournsnel *et al.*, 1987; Lee *et al.*, 1991; Herold *et al.*, 1993) and probably also in toroviruses. The presence of multiple putative protease domains in the ORF 1a proteins of both coronaviruses (Gorbalenya *et al.*, 1989a; Lee *et al.*, 1991; Herold *et al.*, 1993) and arteriviruses (Den Boon *et al.*, 1991b; Meulenberg *et al.*, 1993a; Godeny *et al.*, 1993a) suggests that extensive proteolytic processing is involved in the regulation of CVL replicase function. This assumption is supported by the fact that the characterization of temperature-sensitive mutants of MHV revealed the presence of at least five RNA⁻ complementation groups (Schaad *et al.*, 1990; Baric *et al.*, 1990).

A number of replicase cleavage products have recently been detected in MHV-infected cells (Denison *et al.*, 1992) and in *in vitro* translation reactions programmed with genomic RNA (Denison *et al.*, 1991). The preliminary processing scheme of the EAV ORF 1a protein comprises at least five proteolytic cleavages carried out by three different viral proteases (Snijder *et al.*, 1994a). Combined with cleavage site predictions based on the putative protease types encountered in corona- and arteriviruses (Gorbalenya *et al.*, 1989a; Lee *et al.*, 1991; Godeny *et al.*, 1993a), these experimental data forecast the generation of at least 10 (and possibly up to 20) cleavage products from the CVL ORF 1a/1b protein. If this estimate is correct, the number of intermediary processing products could be very large. This implies that the CVL replicase processing may be very complex, especially since the processing analysis of other viral replicases has revealed that intermediary products can be functional subunits themselves, e.g., in the replication of poliovirus (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988) and Sindbis virus (de Groot *et al.*, 1990; Strauss and Strauss, 1990; Shirako and Strauss, 1994).

The functional characterization of the proteases in the CVL ORF 1a sequences, which belong to different protease supergroups, has only just begun. However, a general pattern for coronaviruses and arteriviruses seems to emerge from comparative replicase sequence analysis. In comparable positions, the ORF 1a proteins of both virus groups contain a domain belonging to the protease superfamily that comprises the chymotrypsinlike and picornavirus 3C-like proteolytic enzymes (Fig. 5) (Gorbalenya *et al.*, 1989b; Bazan and Fletterick, 1988). Although the predicted catalytic nucleophile of the coronavirus protease (Cys) differs from that in the arterivirus proteases (Ser), this domain may still be a remnant from a common ancestor of both virus groups. The exchange of Cys for Ser at the active site of the enzyme is considered to be feasible (Gorbalenya *et al.*, 1989b; Bazan and Fletterick, 1988, 1990; Dougherty and Semler, 1993).

The functions encoded from the central region of ORF 1a to the 3'-end of ORF 1b appear to be the core of the CVL replicase polyprotein: the well-conserved domains (protease–polymerase–helicase–C-terminal “unique” ORF 1b domain) are within this area, and only small insertions and deletions in this part of the replicase can be detected within the coronavirus or arterivirus groups (Fig. 5). The chymotrypsin/3C-like protease is most likely to be responsible for the processing of the core replicase.

The N-terminal half of the ORF 1a protein, on the other hand, is quite variable: the variability of the replicase gene in this area is largely responsible

Polymerase domain

MHV	576	VIGTTKfYGGWdMLrr-9-LMGWDPKCDRAMPnIIR-21-YRLaNE-12-GcyYvKPGGTsSGDATTafANSVFN-57-FSmiLSDdgvVCYN-42-MEFCSQH-12-LPYDPSPSRIL
IBV	582	VIGTTKfYGGWdMLrn-9-LMGWDPKCDRAMPnIIR-21-YRLyNE-12-GgiYvKPGGTsSGDATTAYANSVFN-57-FSiMiLSDdgvVCYN-42-MEFCSQH-12-LPYDPSPSRIL
HCV	575	VIGTTKfYGGWdMLkn-9-LMGWDPKCDRAMPsmIR-21-YRLsNE-12-GgyfYKPGGTsSGDATTAYANSVFN-57-FSmiLSDdsvVCYN-42-MEFCSQH-12-LPYDPSPSRIL
		** * ** **** * * * * * **** * * * * *
BEV	513	lIGvsKyglkfskfLkd-9-vfGsDYtKCDRtPfIsfR-17-Y-L-NE-12-GmllnKPGGTsSGDATTAhShtfYN-51-yfInflSDDsfi-fs-37-eEFCsah-10-L--Psrgrll
		* ** * * * * * * * * * * * * * * *
PRRSV	367	w-ksPIaLG--knKfke-9-LEa-DLascDRSTPaIVR-19-YVL-N--9-dgaFtKRGGlSSGDPVtSVsNTVYS-38---mLVsDDlVL-ya-34--sFlGCR-6-LV-pnrDRil
LDV	365	i-gSPiYLG--nnKfTp-9-LEa-DLascDRSTPaIR-19-YVL-N--9-sgcFdKRGGlSSGDPVtSVsNTVYS-38---lLVsDDWL-yd-34--qFpGCR-6-LV-pqrDRil
EAV	369	kdsPIYLG--ksKfdp-8-LEt-DLesCDRSTPaIVR-19-YVL-N--9-svaFtKRGGlSSGDPtSiSNTiYS-39---vyiYsDDdWL-tt-35--sFlGCR-9-La-slqDrvt

Helicase domain

MHV	1199	VQPPPGtGKSHl-66-tTfINALPElvtDIiVDEVSML-16-vYvIGDPaqlPAPRVL-25-FLgtCYRCPKEIV-75-VLGLqtqTVDsSaGS-18-vMRFNVAiTRAKKGIc
IBV	1206	VQPPPGtGKSHf-66-stfINALPEVscDIiVDEVSML-16-vYvGDPaqlPAPRtL-24-FLakCYRCPKEIV-78-mLGLnvqTVDsSaGS-18-inRFNVAiTRAKtGIv
HCV	1200	iQPPPGtGKSHc-66-stVNALPEVnabIvVDEVSMc-16-iVvGDPqqlPAPRVL-25-FLhkCYRCPaEIV-75-LLGLqtqTVDsSaGS-18-amRFNVAiTRAKKGIc
		**** * * * * * **** * * * * *
BEV	1097	vMPPPGtGKtttf-61-cThNtLPfiksavliadEVSLi-15-wlIGDPfQL-SP--v-23-yLtaCYRCPpql-67-gLG-dvtiIDSsQgt-18-vMrvIvgcsRst-thlv
		* ** * * * * * * * * * * * * * * *
PRRSV	788	iVcPGSGKtTw-51-RLias-GhvpGrvsyIDEagYc-13-lvcIGDLqL-----19--LttiYRFgpnic-40-igs--aiTIDSsQGa-16-ksRALVAiTRArhqlfi
LDV	785	itGaPGtGKtCy-51-RLira-GfIpGrvsyIDEaYc-13-lvcvGDLnL-----19--LievRFgpsiv-40-vgg--aiTIDSsQgc-16-saRALVAiTRArfyvfv
EAV	801	veGPGSGKtTh-58-RLpqv-GtseGe-tfVDEvayf-13-vkyGDLnL-----22--LrvchRfGaaVc-40-glg--hrTiDSiQgc-16-rpRAVVAiTRASqelyi

Carboxyl-terminal ORF 1b domain

	2315	2282	2281	1933	1225	1208	1248	11-Nalv
MHV	GGUH-39-KsVCTviD-18-SKVvnvNd-16-TfYP-31-NYG-11-NvAKYtQLC-12-NHRV	GGUH-39-KqCtVtYD-21-SKVtvsID-16-TcYP-29-NYG-11-NvAKYtQLC-12-NHRV	GGUH-40-KVCTYmD-18-SKvheviD-16-TfYP-30-NYG-11-NvAKYtQLC-12-NHRV	GGVH-31-Krt-TlVd-21-SKVifvniD-17-TfYP-25-NYG-10-NfakYtQic-11-Nalv	GGCH-28-KavCttID-15-SkcwklID-12-TaY--12-dya-7-davvYfdbc-6-NrkV	GGCH-28-KelCtVtD-15-SmdYkVlVd-12-TaY--13-sms-7-eegYfvd---6-NakV	GGSH-28-KaaCtsVD-15-SrvqYimID-12-TfY--9-avs-7-nepYsfvd--6-NalV	
IBV								
HCV								
BEV								
PRSV								
LDV								
EAV								

FIGURE 4. Amino acid sequence comparisons of the three most conserved domains of CVL ORF 1b proteins: polymerase, helicase, and the C-terminal domain, respectively. Amino acids identical in all three coronaviruses are shown in capitals. Amino acids in the BEV sequence that are identical to the amino acid at that position in all three coronaviruses are also shown in capitals and indicated with asterisks on the line between the coronavirus and BEV sequences. Within the arterivirus group, capitals again indicate absolutely conserved amino acids. The asterisks on the line between the sequences of the arteriaviruses and BEV indicate the amino acids conserved in all available CVL ORF 1b sequences.

FIGURE 5. Scaled representation of the replicase gene organization of coronaviruses, toroviruses, and arteriviruses. The filled circle indicates the ORF 1a/1b frameshift site. The largest replicase gene (MHV) is about 22 kb, the smallest one (EAV) approximately 9.5 kb. Boxes represent conserved domains: PCP, papainlike cysteine protease; 3CCP, 3C-like cysteine protease; CSP, chymotrypsinlike serine protease; POL, polymerase; HEL, helicase; CTD, carboxyl-terminal ORF 1b domain.

for the size differences in corona- or arterivirus genomes. A comparison between the coronavirus and arterivirus N-terminal ORF 1a protein sequences does not yield any significant similarities, and even within the coronavirus and arterivirus groups there is little conservation in this region.

However, one striking observation can be made. Both corona- and arteriviruses contain putative protease domains in the amino-terminal half of their ORF 1a proteins. These proteases have been shown, or are predicted, to belong to the papainlike cysteine protease superfamily (Fig. 5) (Gorbalenya *et al.*, 1991). A number of these proteases have recently been characterized. In EAV (Snijder *et al.*, 1992) and MHV (Baker *et al.*, 1993), a papainlike protease is responsible for the production of an amino-terminal ORF 1a cleavage product. Furthermore, the production of the next amino-terminal cleavage product from the EAV ORF 1a protein has now been shown to be mediated by an autoproteolytic activity that is most similar to papainlike proteases (Snijder *et al.*, 1995). Two other arteriviruses, PRRSV and LDV, have been found to use even a third, papainlike protease in the processing of the N-terminal region of the ORF 1a protein (Den Boon *et al.*, 1995a). During arterivirus evolution, EAV appears to have lost the protease function that is responsible for the production of an additional amino-terminal cleavage product in PRRSV and LDV (Fig. 5).

Unfortunately, only the 5'- and 3'-sequences of the Berne torovirus ORF 1a region (about 1 kb from each end) have been determined so far and no putative proteases have been identified in these regions. Their future identification would be very useful, especially for a comparative analysis of the CVL chymotrypsin/3C-like proteases.

VII. STRUCTURAL PROTEINS

Viral structural proteins are known to diverge at a higher rate than non-structural proteins. This is exemplified by the relationship between the corona- and torovirus replicase, S, and M proteins (Chapter 11, this volume), a set of genes that is thought to be related by common ancestry. Whereas, undisputable sequence homologies are present in the replicase proteins of these viruses, these are absent in the S and M protein sequences, which only display structural similarities. The corona- and torovirus N proteins, on the other hand, are so different in size and sequence that common ancestry is highly unlikely. Also, the nucleocapsid structures that they form together with the genomic RNA are significantly different. It therefore has been proposed that during evolution either the coronavirus or the torovirus group may have acquired a novel N protein gene (Snijder and Horzinek, 1993).

When the arteriviruses are included in the comparison of the structural proteins of CVL replicase-containing viruses, it is hard to detect any similarity at all, either in size, sequence, or structural characteristics (Den Boon *et al.*, 1991b; de Vries *et al.*, 1992). The N protein (12kDa) is even smaller than that of toroviruses and gives rise to an icosahedral nucleocapsid. The presently known surface glycoproteins G_s and G_t are small compared to the S proteins of corona- and toroviruses. Only the presence of a triple-spanning membrane (M) protein, encoded by arterivirus ORF 6, is similar to the *Coronaviridae*, even though this protein (18kDa) is also smaller than its coronaviral counterparts. The triple-spanning membrane proteins of corona- and toroviruses have been implicated to play a crucial role in determining the intracellular budding process of these viruses (Dubois-Dalcq *et al.*, 1982; Holmes *et al.*, 1981; Rottier *et al.*, 1981; Den Boon *et al.*, 1991a). Since arteriviruses also assemble at intracellular membranes, the conservation or acquisition of an M protein with similar structural properties may have been expected during arterivirus evolution (de Vries *et al.*, 1992).

VIII. THE EVOLUTION OF THE CVL SUPERFAMILY

The common ancestry of the CVL replicase proteins and the associated replication strategy are evident. The features that are shared by the members of the CVL superfamily are the basic genome organization, namely, "replicase-envelope protein-nucleocapsid protein," the production of 3'-coterminal nested sets of mRNAs, and the conserved organization of the replicase gene. However, the N proteins and nucleocapsid structures of the three CVL genera are apparently unrelated. Furthermore, the arterivirus surface proteins show no evidence of a common ancestry.

The coupling of different sets of structural genes to the same replicase gene has been explained by the recombination of complete genes or gene sets (modules) (Zimmern, 1987; Strauss and Strauss, 1988; Goldbach and Wellink, 1988). The RNA recombination frequency during MHV replication has been shown to be remarkably high (reviewed by Spaan *et al.*, 1988; Lai, 1992), a characteristic

that may be determined by their replicase properties. The different sets of structural genes (and the varying number of "additional" genes) that are now known to be linked to the CVL replicase gene indicate that this characteristic may be shared by other members of the superfamily ("modular" evolution). Direct evidence for multiple recombination events during torovirus evolution has already been obtained (Snijder *et al.*, 1991). Together with divergent evolution, a high recombination frequency can account for the diverse composition of CVL genomes. It has been hypothesized that the acquisition of a novel nucleocapsid protein gene, giving rise to a helical virus core rather than an icosahedral one, may have created the possibility for the corona/torovirus branch to diverge from the arterivirus branch (Godeny *et al.*, 1993a). The increased genome size of the corona- and toroviruses could in this case be explained by the more relaxed packaging constraints of a helical nucleocapsid structure.

Arteriviruses and, especially, coronaviruses possess exceptionally large genomes in comparison with other positive-stranded animal RNA viruses. The apparent gap between the genome sizes of arteriviruses (EAV:12.7 kb) and coronaviruses (MHV:31.5 kb) is slowly being reduced now that the sequences of larger arterivirus genomes (PRRSV:15.1 kb) and smaller coronavirus genomes [human coronavirus (HCV) 229E:27.3 kb] have been determined. A group of positive-strand RNA viruses that almost bridge the remaining gap are the plant closteroviruses, with genome sizes between 7 and 20 kb. The molecular biology properties of this virus group has recently been reviewed by Dolja *et al.* (1994). A number of remarkable similarities with the CVL group is found in the closterovirus group: e.g., polycistronic genomes, frameshifting during replicase expression, and the generation of a 3'-coterminal nested set of mRNAs to express the 3' proximal genes. Although phylogenetic studies, including closterovirus and CVL replicase sequences, indicated that these similarities are analogies rather than homologies (Koonin and Dolja, 1993), this example of convergent evolution may elucidate some general principles governing the evolution of viruses with large RNA genomes. The size variation and plasticity of both closterovirus and CVL genomes is remarkable, suggesting again the frequent involvement of recombination during the evolution of these virus groups (Dolja *et al.*, 1994). The process of recombination could also partially compensate for the general high error frequency of viral RNA-dependent RNA polymerases, a feature that will greatly influence the stability and evolution of large RNA genomes.

IX. THE TAXONOMY OF CVL VIRUSES

The "split personality" of the arteriviruses, a CVL replicase gene coupled to a non-CVL set of structural genes, is an interesting test for viral taxonomists. Using traditional taxonomic criteria, e.g., the virion and nucleocapsid structure, there is no reason to propose a taxonomic relationship between arteriviruses and *Coronaviridae*. At the same time, certain characteristics, such as genome organization, replication strategy, and sequence homologies, provide a solid basis for a formal taxonomic status of the CVL superfamily. Any meaning-

ful classification of the members of the CVL group clearly requires four hierarchical levels (Fig. 6) (Snijder and Horzinek, 1993; Cavanagh *et al.*, 1994): the coronavirus and torovirus species have now been classified into two genera that belong to the *Coronaviridae* family. The obvious evolutionary link of this family to the arteriviruses would be reflected most accurately by promoting the present arterivirus genus 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. However, this problem could be circumvented by establishing two subfamilies (*Coronaviridae* and *Arteriviridae*) (Cavanagh *et al.*, 1994) and changing the family name of the *Coronaviridae*.

The taxonomic fate of the "CVL superfamily" will depend on the future weighing of both old (structural) and new (genetic) criteria for virus classification. The balance between these criteria will decide whether the genetic similarities between coronaviruses and arteriviruses outweigh their structural dissimilarities. If, as in other areas of biology, the taxonomy of viruses should have a genetic basis and should reflect phylogenetic relationships, the creation of an order (Fig. 6) for the *Coronaviridae* and *Arteriviridae* would be the most elegant solution to recognize both their similarities and differences (Snijder and Horzinek, 1993; Cavanagh *et al.*, 1994). A taxonomic system that is unable to ac-

FIGURE 6. Pedigree of the CVL superfamily. The distances between the various CVL viruses and groups are shown arbitrarily. The four hierarchical (taxonomic) levels that can be discerned are indicated. Abbreviations: BEV, Berne virus; BRV, Breda virus; EAV, equine arteritis virus; HCV 229E, human coronavirus 229E; IBV, infectious bronchitis virus; LDV, lactate dehydrogenase-elevating virus; MHV, mouse hepatitis virus; PRRSV, porcine reproductive and respiratory syndrome virus; SHFV, simian hemorrhagic fever virus. For simplicity, the coronavirus clusters that include MHV and HCV 229E are shown as a single species.

commodate the results of virus evolution (mutation, recombination, and selection) will have no future.

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X. REFERENCES

- Baker, S. C., Yokomori, K., Dong, S., Carlisle, R., Gorbalenya, A. E., Koonin, E. V., and Lai, M. M. C., 1993, Identification of the catalytic sites of a papain-like cysteine proteinase of murine coronavirus, *J. Virol.* **67**:6056.
- Baric, R. S., Stohlman, S. A., and Lai, M. M. C., 1983, Characterization of replicative intermediate RNA of mouse hepatitis virus: Presence of leader RNA sequences on nascent chains, *J. Virol.* **48**:633.
- Baric, R. S., Fu, K., Schaad, M. C., and Stohlman, S. A., 1990, Establishing a genetic recombination map for murine coronavirus strain A59 complementation groups, *Virology* **177**:646.
- Bazan, J. F., and Fletterick, R. J., 1988, Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: Structural and functional implications, *Proc. Natl. Acad. Sci. USA* **85**:7872.
- Bazan, J. F., and Fletterick, R. J., 1990, Structural and catalytic models of trypsin-like viral proteases, *Semin. Virol.* **1**:311.
- Boursnell, M. E. G., Brown, T. D. K., Foulds, I. J., Green, P. F., Tomley, F. M., and Binns, M. M., 1987, Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus, *J. Gen. Virol.* **68**:57.
- Bredenbeek, P. J., Pachuk, C. J., Noten, J. F. H., Charité, J., Luytjes, W., Weiss, S. R., and Spaan, W. J. M., 1990, The primary structure and expression of the second open reading frame of the polymerase gene of the coronavirus MHV-A59, *Nucleic Acids Res.* **18**:1825.
- Brierley, I., Diggard, P., and Inglis, S., 1989, Characterization of an efficient coronavirus ribosomal frameshifting signal: Requirement for an RNA pseudoknot, *Cell* **57**:537.
- Cavanagh, D., Brian, D. A., Brinton, M., Enjuanes, L., Holmes, K. V., Horzinek, M. C., Lai, M. M. C., Laude, H., Plagemann, P. G. W., Siddell, S., Spaan, W. J. M., Taguchi, F., and Talbot, P. J., 1994, Revision of the taxonomy of the Coronavirus, Torovirus and Arterivirus genera, *Archives Virology* **135**:227.
- Chen, Z., Kuo, L., Rowland, R. R. R., Even, C., Faaberg, K. S., and Plagemann, P. G. W., 1993, Sequence of 3'-end of genome and 5'-end of ORF 1a of lactate dehydrogenase-elevating virus (LDV) and common junction motifs between 5'-leader and bodies of seven subgenomic mRNAs, *J. Gen. Virol.* **74**:643.
- de Groot, R. J., Hardy, W. R., Shirako, Y., and Strauss, J. H., 1990, Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing *in vivo*, *EMBO J.* **9**:2631.
- Den Boon, J. A., Snijder, E. J., Krijnse Locker, J., Horzinek, M. C., and Rottier, P. J. M., 1991a, Another triple-spanning envelope protein among intracellularly budding RNA viruses: The torovirus E protein, *Virology* **182**:655.
- Den Boon, J. A., Snijder, E. J., Chirnside, E. D., De Vries, A. A. F., Horzinek, M. C., and Spaan, W. J. M., 1991b, Equine arteritis virus is not a togavirus but belongs to the coronavirus-like superfamily, *J. Virol.* **65**:2910.
- Den Boon, J. A., Faaberg, K. S., Meulenberg, J. J. M., Wassenaar, A. L. M., Plagemann, P. G. W., Gorbalenya, A. E., and Snijder, E. J., 1995a, Processing and evolution of the N-terminal region of the arterivirus ORF1a protein: Identification of two papainlike cysteine proteases, *J. Virol.*, in press.
- Den Boon, J. A., Spaan, W. J. M., and Snijder, E. J., 1995b, manuscript in preparation.
- Den Boon, J. A., Kleijnen, M. F., Spaan, W. J. M., and Snijder, E. J., 1995c, manuscript in preparation.

- Denison, M. R., Zoltick, P. W., Leibowitz, J. L., Pachuk, C. J., and Weiss, S. R., 1991, Identification of polypeptides encoded in open reading frame 1b of the putative polymerase gene of the murine coronavirus mouse hepatitis virus A59, *J. Virol.* **65**:3067.
- Denison, M. R., Zoltick, P. W., Hughes, A., Giangreco, B., Olsen, A. L., Perlman, S., Leibowitz, J. L., and Weiss, S. R., 1992, Intracellular processing of the N-terminal ORF1a proteins of the coronavirus MHV-A59 requires multiple proteolytic events, *Virology* **189**:274.
- de Vries, A. A. F., Chirnside, E. D., Bredenbeek, P. J., Gravenstein, L. A., Horzinek, M. C., and Spaan, W. J. M., 1990, All subgenomic mRNAs of equine arteritis virus contain a common leader sequence, *Nucleic Acids Res.* **18**:3241.
- de Vries, A. A. F., Chirnside, E. D., Horzinek, M. C., and Rottier, P. J. M., 1992, Structural proteins of equine arteritis virus, *J. Virol.* **66**:6294.
- Dolja, V. V., Karasev, A. V., and Koonin, E. V., 1994, Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes, *Annu. Rev. Phytopathol.* (in press).
- Doll, E. R., Bryans, J. T., McCollum, W. H. M., and Wallace, M. E., 1957, Isolation of a filterable agent causing arteritis of horses and abortion by mares. Its differentiation from the equine abortion (influenza) virus, *Cornell Vet.* **47**:3.
- Dougherty, W. G., and Semler, B. L., 1993, Expression of virus-encoded proteinases: Functional and structural similarities with cellular enzymes, *Microbiol. Rev.* **57**:781.
- Dubois-Dalcq, M. E., Doller, E. W., Haspel, M. V., and Holmes, K. V., 1982, Cell tropism and expression of mouse hepatitis virus (MHV) in mouse spinal chord cultures, *Virology* **119**:317.
- Godeny, E. K., Chen, L., Kumar, S. N., Methven, S. L., Koonin, E. V., and Brinton, M. A., 1993a. Complete genomic sequence and phylogenetic analysis of the lactate dehydrogenase-elevating virus, *Virology* **194**:585.
- Godeny, E. K., Zeng, L., Smith, S. L., and Brinton, M. A., 1993b, Simian haemorrhagic fever virus: Another member of the coronavirus-like superfamily, in: *Abstracts of the IXth International Congress of Virology*, p. 22, Glasgow, Scotland.
- Goldbach, R., and Wellink, J., 1988, Evolution of plus-strand RNA viruses, *Intervirology* **29**:260.
- Gorbalenya, A. E., and Koonin, E. V., 1993, Helicases: Amino acid sequence comparisons and structure-function relationships, *Curr. Opin. Struct. Biol.* **3**:419.
- Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M., 1989a, Coronavirus genome: Prediction of putative functional domains in the non-structural polyprotein by comparative amino acid sequence analysis, *Nucleic Acids Res.* **17**:4847.
- Gorbalenya, A. E., Donchenko, A. P., Blinov, V. M., and Koonin, E. V., 1989b, Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases: A distinct protein superfamily with a common structural fold, *FEBS Lett.* **243**:103.
- Gorbalenya, A. E., Koonin, E. V., and Lai, M. M. C., 1991, Putative papain-related thiol proteases of positive-stranded RNA viruses, *FEBS Lett.* **288**:201.
- Herold, J., Raabe, T., Schelle-Prinz, B., and Siddell, S. G., 1993, Nucleotide sequence of the human coronavirus 229E RNA polymerase locus, *Virology* **195**:680.
- Holmes, K. V., Doller, E. W., and Sturman, L. S., 1981, Tunicamycin resistant glycosylation of a coronavirus glycoprotein: Demonstration of a novel type of viral glycoprotein, *Virology* **115**:334.
- Horzinek, M. C., Maess J., and Laufs, R., 1971, Studies on the structure of togaviruses. II. Analysis of equine arteritis, rubella, bovine viral diarrhoea, and hog cholera viruses, *Arch. Gesamte Virusforsch.* **33**:306.
- Hyllseth, B., Structural proteins of equine arteritis virus, 1973, *Arch. Gesamte Virusforsch.* **40**:177.
- Jacks, T., Madhani, H. D., Masiarz, F. R., and Varmus, H. E., 1988, Signals for ribosomal frameshifting in the Rouse sarcoma virus gag-pol region, *Cell* **55**:447.
- Jacobs, L., Spaan, W. J. M., Horzinek, M. C., and van der Zeijst, B. A. M., 1981, Synthesis of subgenomic mRNAs of mouse hepatitis virus is initiated independently: evidence from UV transcription mapping, *J. Virol.* **39**:401.
- Jore, J., de Geus, B., Jackson, R. J., Pouwels, P. H., and Enger-Valk, B. E., 1988, Poliovirus 3CD is the active protease for processing of the precursor protein P1 *in vitro*, *J. Gen. Virol.* **69**:1627.
- Koonin, E. V., and Dolja, V. V., 1993, Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences, *Crit. Rev. Biochem. Mol. Biol.* **28**:375.

- Kuo, L. L., Harty, J. T., Erickson, L., Palmer, G. A., and Plagemann, P. G. W., 1991, A nested set of eight RNAs is formed in macrophages infected with lactate dehydrogenase-elevating virus, *J. Virol.* **65**:5118.
- Lai, M. M. C., 1990, Coronavirus—organization, replication and expression of genome, *Annu. Rev. Microbiol.* **44**:303.
- Lai, M. M. C., 1992, RNA recombination in animal and plant viruses, *Microbiol. Rev.* **56**:61.
- Lai, M. M. C., Patton, C. D., Baric, R. S., and Stohlman, S. A., 1983, Presence of leader sequences in the mRNA of mouse hepatitis virus, *J. Virol.* **46**:1027.
- Lee, H. J., Shieh, C. K., Gorbalenya, A. E., Koonin, E. V., Lamonic, N., Tuler, J., Bagdzhadzhuyan, A., and Lai, M. M. C., 1991, The complete sequence (22 kilobases) of murine coronavirus gene-1 encoding the putative proteases and RNA polymerase, *Virology* **180**:567.
- Meulenbergh, J. J. M., Hulst, M. M., De Meijer, E. J., Moonen, P. L. J. M., Den Besten, A., De Kluyver, E. P., Wensvoort, G., and Moormann, R. J. M., 1993a, Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV, *Virology* **192**:62.
- Meulenbergh, J. J. M., De Meijer, E., and Moormann, R. J. M., 1993b, Subgenomic RNAs of Lelystad virus contain a conserved leader-body junction sequence, *J. Gen. Virol.* **74**:1697.
- Plagemann, P. G., and Moennig, V., 1992, Lactate dehydrogenase-elevating virus, equine arteritis virus and simian haemorrhagic fever virus, a new group of positive strand RNA viruses. *Adv. Virus Res.* **41**:99.
- Poch, O., Sauvaget, I., Delarue, M., and Tordo, N., 1989, Identification of four conserved motifs among the RNA dependent polymerase encoding elements, *EMBO J.* **8**:3867.
- Rottier, P. J. M., Horzinek, M. C., and van der Zeijst, B. A. M., 1981, Viral protein synthesis in mouse hepatitis virus strain A59-infected cells: effect of tunicamycin, *J. Virol.* **40**:350.
- Sawicki, S. G., and Sawicki, D. L., 1990, Subgenomic mouse hepatitis virus replicative intermediates function in RNA synthesis, *J. Virol.* **64**:1050.
- Schaad, M. C., Stohlman, S. A., Egbert, J., Lum, K., Fu, K., Wei, T., and Baric, R. S., 1990, Genetics of mouse hepatitis virus transcription: Identification of cistrons which may function in positive and negative strand RNA synthesis, *Virology* **177**:634.
- Sethna, P. B., Hung, S. L., and Brian, D. A., 1989, Coronavirus subgenomic minus-strand RNAs and the potential for mRNA replicons *Proc. Natl. Acad. Sci. USA* **86**:5626.
- Shirako, Y., and Strauss, J. H., 1994, Regulation of Sindbis virus RNA replication: Uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand synthesis, *J. Virol.* **68**:1874.
- Snijder, E. J., and Horzinek, M. C., 1993, Toroviruses: Replication, evolution and comparison with other members of the coronavirus-like superfamily, *J. Gen. Virol.* **74**:2305.
- Snijder, E. J., den Boon, J. A., Bredenbeek, P. J., Horzinek, M. C., Rijnbrand, R., and Spaan, W. J. M., 1990a, The carboxyl-terminal part of the putative Berne virus polymerase is expressed by ribosomal frameshifting and contains sequence motifs which indicate that toro- and coronaviruses are evolutionarily related, *Nucleic Acids Res.* **18**:4535.
- Snijder, E. J., Horzinek, M. C., and Spaan, W. J. M., 1990b, A 3'-coterminal nested set of independently transcribed messenger RNAs is generated during Berne virus replication, *J. Virol.* **64**:331.
- Snijder, E. J., den Boon, J. A., Horzinek, M. C., and Spaan, W. J. M., 1991, Comparison of the genome organization of toro- and coronaviruses: Both divergence from a common ancestor and RNA recombination have played a role in Berne virus evolution, *Virology* **180**:448.
- Snijder, E. J., Wassenaar, A. L. M., and Spaan, W. J. M., 1992, The 5' end of the equine arteritis virus genome encodes a papainlike cysteine protease, *J. Virol.* **66**:7040.
- Snijder, E. J., Wassenaar, A. L. M., and Spaan, W. J. M., 1994a, Proteolytic processing of the equine arteritis virus replicase ORF1a protein, *J. Virol.* **68**:5755.
- Snijder, E. J., Wassenaar, A. L. M., Spaan, W. J. M., and Gorbalenya, A. E., 1995, The arterivirus nsp2 protease: an unusual cysteine protease with similarities to both papainlike and chymotrypsinlike proteases, *J. Biol. Chem.*, in press.
- Spaan, W. J. M., Delius, H., Skinner, M., Armstrong, J., Rottier, P. J. M., Smeekens, S., van der Zeijst, B. A. M., and Siddell, S. G., 1983, Coronavirus mRNA synthesis involves fusion of non-contiguous sequences, *EMBO J.* **2**:1839.
- Spaan, W. J. M., Cavanagh, D., and Horzinek, M. C., 1988, Coronaviruses: Structure and genome expression, *J. Gen. Virol.* **69**:2939.

- Strauss, J. H., and Strauss, E. G., 1988, Evolution of RNA viruses, *Annu. Rev. Microbiol.* **42**:657.
- Strauss, J. H., and Strauss, E. G., 1990, Alphavirus proteinases, *Semin. Virol.* **1**:347.
- Ten Dam, E. B., Pleij, C. W. A., and Bosch, L., 1990, RNA pseudoknots; translational frameshifting and read-through on viral RNAs, *Virus Genes* **4**:121.
- Van Berlo, M. F., Horzinek, M. C., and van der Zeijst, B. A. M., 1982, Equine arteritis virus-infected cells contain six polyadenylated virus-specific RNAs, *Virology* **118**:345.
- Ypma-Wong, M. F., Dewalt, P. G., Johnson, V. H., Lamb, J. G., and Semler, B. L., 1988, Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor, *Virology* **166**:165.
- Zimmern, D., 1987, Evolution of RNA viruses, in *RNA Genetics*, vol. 2 (J. J. Holland, E. Domingo, and P. Ahlquist, eds.), pp. 211–240. Boca Raton, FL, CRC Press.