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Comparisons of the Pestivirus Bovine Viral Diarrhoea Virus with Members of the Flaviviridae

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SUMMARY

The molecular features of bovine viral diarrhoea virus (BVDV), a member of the *Pestivirus* genus currently classified in the *Togaviridae*, were examined for characteristics resembling those of the *Flaviviridae* family. Like flaviviruses, BVDV possesses a single-stranded RNA genome (approx. $4{\cdot}3 \times 10^6$ M_r) deficient in a 3′ poly(A) tract. This RNA has a single open reading frame spanning the length of the genome in the viral RNA sense (positive polarity), implying an expression strategy involving the processing of a precursor polyprotein. With the exception of several short but significant stretches of identical amino acids within two non-structural proteins, no extended regions of nucleotide or amino acid sequence homology between BVDV and representatives of three serological subgroups of mosquito-borne flaviviruses were noted. However, comparison of the organization of protein-coding domains along the genomes and the hydropathic profiles of amino acid sequences revealed pronounced similarities. It is proposed that *Pestivirus*, of which BVDV is the prototype member, should no longer be grouped in the *Togaviridae* family, but rather be considered a genus of non-arthropod-borne viruses within the *Flaviviridae*.

Bovine viral diarrhoea virus (BVDV) is an economically important pathogen of cattle throughout the world, causing a wide range of clinical syndromes (Brownlie, 1985; Baker, 1987). It is a member of the *Pestivirus* genus, which also includes hog cholera virus and border disease virus of sheep. Pestiviruses are currently classified as non-arthropod-borne members of the *Togaviridae* family (Horzinek, 1981; Westaway *et al.*, 1985*a*). Also included in the *Togaviridae* are the genera *Alphavirus* and *Rubivirus*, as well as several unclassified viruses. *Flavivirus*, previously grouped within the *Togaviridae*, has been established as the single genus of the new family *Flaviviridae*. This reclassification was prompted by the appreciation of fundamental differences in genomic structure and organization and in expression strategy between alphaviruses and flaviviruses (Westaway *et al.*, 1985*b*; Schlesinger & Schlesinger, 1986). In the light of recent data on the genetic organization of BVDV, we present here molecular comparisons of BVDV with flaviviruses that have led us to reconsider the classification of pestiviruses.

Previous comparisons of pestiviruses with togaviruses and flaviviruses have involved morphological, physicochemical or serological characteristics. For example, the structural polymorphism observed for BVDV (Porterfield *et al.*, 1978; Horzinek, 1981; Gray & Nettleton, 1987) has been equated to that seen for rubella virus (Bielefeldt Ohmann & Bloch, 1982; Chu & Zee, 1984). The sensitivity of pestiviruses to inactivation by proteases is similar to that of flaviviruses (trypsin-sensitive), as opposed to alphaviruses (trypsin-resistant) (reviewed by Horzinek, 1981). Pestivirus assembly and maturation appear similar to flavivirus assembly at

the perinuclear endoplasmic reticulum and Golgi membranes in contrast to plasma membrane budding of togaviruses (Scherrer *et al.*, 1970; Horzinek, 1973; Bielefeldt Ohmann & Bloch, 1982; Gray & Nettleton, 1987). BVDV and HCV replication are uniquely sensitive to proflavine and acriflavine, while that of rubella virus and Sindbis virus is not (Dinter & Diderholm, 1971; Diderholm *et al.*, 1973). Although pestiviruses are serologically related, no antigenic relationships have been observed between pestiviruses and either togaviruses or flaviviruses (Horzinek, 1981; S. K. Belzer, M. S. Collett, T. J. Chambers & C. M. Rice, unpublished data).

Although important in the description of viruses, the taxonomic significance and relevance of the above observations become difficult to assess in the face of currently available molecular biological technologies. Recent considerations of virus classification have depended strongly on the molecular features of genome structure and genetic organization, and on the details of virus replication and expression. Unfortunately, there have been little molecular data on pestiviruses for such use, and this situation has only recently changed.

Molecular analyses of virus-specific intracellular RNA species have demonstrated that, as in flavivirus-infected cells, only a single genome-size RNA (approx. $4{\cdot}3 \times 10^6$ M_r) deficient in a 3′ poly(A) tract can be found in BVDV-infected cells (Purchio *et al.*, 1983; Renard *et al.*, 1985). This is in contrast to the viral genomic (49S) and subgenomic (26S) poly(A)-containing RNAs found in alphavirus-infected cells (Strauss & Strauss, 1986). The viral RNA of the NADL isolate of BVDV has recently been molecularly cloned and sequenced (Collett *et al.*, 1988*a*), and the genetic organization of this pestivirus has been described (Collett *et al.*, 1988*b*). These data were used to compare the molecular features of BVDV to flaviviruses.

For our comparisons, we have considered several representative flaviviruses. There are three serological subgroups within the *Flavivirus* genus, and at least one representative from each has been molecularly cloned and sequenced (Rice *et al.*, 1985; Castle *et al.*, 1985, 1986; Wengler *et al.*, 1985; Dalgarno *et al.*, 1986; Deubel *et al.*, 1986; Yaegashi *et al.*, 1986; Sumiyoshi *et al.*, 1987; Hahn *et al.*, 1988). Comparisons between them showed that 'as a general rule for flaviviruses examined to date, members of different serological subgroups demonstrate 50% or less amino acid homology, members of the same subgroup average 65–75% homology . . . .' (Hahn *et al.*, 1988). Due to these divergences, we have analysed one member from each flavivirus subgroup [yellow fever virus (YFV), dengue 2 virus and Japanese encephalitis virus].

The cloned sequence reported for BVDV is 12573 nucleotides in length (Collett *et al.*, 1988*a*). Although this is in agreement with size estimates of the viral RNA (Renard *et al.*, 1985; Collett *et al.*, 1988*a*), it most likely lacks sequences from the ends of the genome. Direct sequence determinations of the termini of the BVDV RNA have not yet been carried out and therefore these regions cannot be compared with those of flaviviruses. Regardless, the genome of BVDV is somewhat larger than those of flaviviruses (approx. 10703 to 10976 nucleotides). Within the cloned sequence of BVDV, there exists a single large open reading frame (ORF) in the positive RNA polarity consisting of 3988 codons, and capable of encoding a protein of M_r 449000 (449K). Flaviviruses also possess a single ORF extending the length of the genome and corresponding to between 3388 and 3432 codons. Implicit in these viruses having a single large ORF is an expression strategy involving co- and post-translational processing of a precursor polyprotein (Rice *et al.*, 1985; Collett *et al.*, 1988*b*).

Computer-assisted comparisons of the entire nucleotide sequence of BVDV with those of flaviviruses from each of the three subgroups were conducted. In no case was similarity involving extensive regions of sequence observed. The algorithm DNASEARCH (Altschul & Erickson, 1986*a*, *b*) was, however, able to detect several small tracts (17 to 30 nucleotides) of similarity between BVDV and YFV (data not shown). Use of the program HOMOLOGY (Lawrence *et al.*, 1986) with the BVDV sequences randomized 300% and 500% confirmed these limited similarities. Analogous although less extensive comparisons of the deduced amino acid sequence of the ORFs of BVDV and of flaviviruses also failed to reveal extensive tracts of similarity.

Comparison of the genetic organization of BVDV and flaviviruses, and detailed features of their protein-coding regions was more revealing. Fig. 1 shows hydropathic plots of the amino acid sequence of the ORFs of BVDV and the prototype flavivirus YFV. Also shown are the

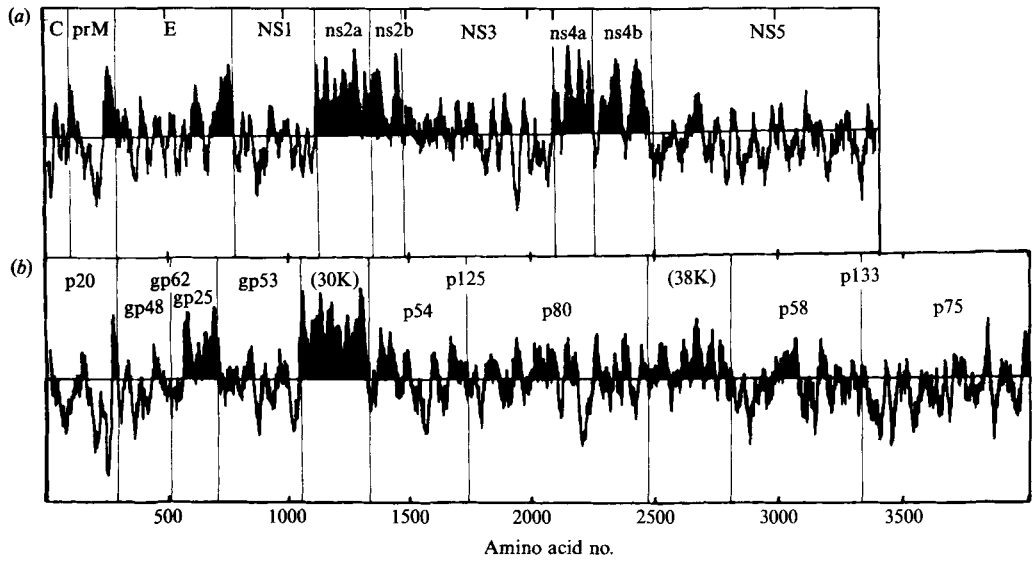

Fig. 1. Hydropathic plots of the amino acid sequence of the ORFs of (*a*) YFV and (*b*) BVDV. Calculations were based on the method of Kyte & Doolittle (1982) and are presented as averages over 25 amino acids. The blackened areas above the line denote regions of net hydrophobicity, and those below the line net hydrophilicity. The numbers represent amino acid positions beginning from the first methionine codon of each ORF. Vertical lines show the boundaries of specific protein-coding regions.

boundaries of the coding regions for individual gene products. Those for YFV are as originally reported (Rice *et al.*, 1985) with slight modifications (C. Rice, personal communication). Those for BVDV are considered tentative, being based on protein size estimates and characterizations employing sequence-specific antibody reagents (Collett *et al.*, 1988*b*). Currently, there are no protein sequence data for BVDV proteins to allow their precise positioning within the deduced amino acid sequence of the ORF. Notwithstanding, the overall similarity in hydropathic profiles of the BVDV and YFV amino acid sequences is impressive. Moreover, the organization of BVDV protein-coding domains along the ORF parallels that of the flavivirus.

The first protein product of the BVDV ORF is p20. The analogous region in flaviviruses encodes the nucleocapsid protein C, and the prM protein. Although p20 is predicted to be the most basic of all BVDV proteins (estimated pI 10·6), it is not as basic as the flavivirus C protein (estimated pI 13·1). The prM protein is a glycosylated putative precursor to the M protein. The p20 sequence lacks *N*-linked (Asn-X-Ser/Thr) glycosylation sites. Whether p20 functions in a capacity similar to either or both C and prM remains to be determined.

Following the p20 sequence is the region of the BVDV genome that encodes several glycoproteins. As shown elsewhere, a putative precursor gp116 gives rise to gp62 and gp53 (Collett *et al.*, 1988*b*). BVDV gp62 appears to be analogous to the flavivirus external envelope protein E, but can be processed further to yield the amino-terminal gp48 and the extremely hydrophobic carboxy-terminal gp25. Flavivirus E proteins possess from zero to two potential *N*-linked glycosylation sites which are not conserved and, when present, are not always used (Rice *et al.*, 1986). On the other hand, gp62 possesses 10 such sites (eight in gp48 and two in gp25), many apparently occupied with glycans (Collett *et al.*, 1988*b*). Protein gp53 corresponds to the first flavivirus non-structural protein, NS1. Both proteins are relatively hydrophilic and glycosylated: NS1 proteins possess from two to four sites, two of which are conserved among all flaviviruses, and gp53 contains four potential glycosylation sequences.

Both gp53 and NS1 abut an extremely hydrophobic amino acid stretch. The flavivirus proteins of this region, ns2a and ns2b, are as yet only poorly characterized. The polypeptide product(s) of this region of the BVDV genome remain to be identified.

The BVDV region encoding p125 seems to match that of flavivirus NS3. In cells infected with cytopathic BVDV, a portion of the p125 is cleaved to yield p54 and p80. This processing event appears to be deficient in cells infected with non-cytopathic BVDV (Donis & Dubovi, 1987; Pocock *et al.*, 1987).

Following the p125-coding sequences is a second area in the BVDV ORF for which no polypeptide products have been identified. This hydrophobic region aligns with the similarly hydrophobic ns4a- and ns4b-coding segment of the flavivirus ORF.

The final coding domain of BVDV is represented by p133, and corresponds to the NS5 region of the flavivirus genome. BVDV p133 is further processed to p58 and p75. Both p133 and NS5 are quite hydrophilic in character, but each displays a prominent hydrophobic segment toward its amino-terminal end.

Inspection of the amino acid sequences of the individual proteins of BVDV and their apparent flavivirus counterparts for very short stretches of homologous sequences revealed the following. Within p20, the glycoprotein gene group, and the extremely hydrophobic regions of BVDV (30K and 38K M_r unidentified proteins), no signficant homologies were found. However, within the p125/p54–p80 and p133/p58–p75 regions of BVDV and the corresponding flavivirus NS3 and NS5 proteins, several very similar runs of amino acids were identified. These are schematically presented in Fig. 2. In the BVDV p125-coding region, five short stretches, two in p54 and three in p80, aligned with similar sequences in YFV NS3. Within BVDV protein p133, three regions were detected, one in p58 and two in p75, that were found to be similarly situated in the NS5 protein of YFV. It is thought that NS5 may be a component of the flavivirus RNA-dependent RNA polymerase (Rice *et al.*, 1985). This suggestion was based largely on certain sequence homologies found among various known viral RNA polymerases (Kamer & Argos, 1984) and the NS5 sequence of flaviviruses. The sequence Gly-Asp-Asp (GDD) is common to all these polymerases. Within the entire BVDV ORF, this sequence appears only once, as it does in flavivirus sequences, and in the 3′-endmost gene product (Fig. 2). It is therefore likely that this region of the BVDV genome, represented by p133/p58–p75, encodes the pestivirus RNA-dependent RNA polymerase.

As indicated above, the molecular features of BVDV known to date are quite similar to those of flaviviruses: both BVDV and flaviviruses possess large positive-polarity RNA genomes that lack poly(A) tracts, no subgenomic RNA species exist and the genomes have a single ORF spanning the length of the RNA. These observations suggest that both the replication and expression strategies of BVDV and flaviviruses are similar. The organization of the protein-coding regions along the ORF of BVDV and flaviviruses is unquestionably alike. Furthermore, the hydropathic character of the amino acid sequences of the entire ORF and of the individual proteins is strikingly similar. With limited exception, these common features exist in the absence of extensive sequence similarity. The exception, however, is important. The several short, similarly aligned regions of highly homologous amino acids in two non-structural proteins may indicate that the respective proteins share similar functions, for which the conserved sequences may be essential.

With respect to virus classification, these data are sufficient to distinguish BVDV from members of the *Togaviridae*. Are they sufficient to consider BVDV and pestiviruses as part of the family *Flaviviridae*? The observation that BVDV and flaviviruses lack any substantial sequence identity should not disallow such consideration. Certainly, sequence homology may be used to assist in positive classification but sequence divergence should not be the sole criterion for exclusion from a family. Assessment of all the available data must be carried out. In view of these data, we propose that *Pestivirus*, of which BVDV is the prototype member, be considered a new genus of non-arthropod-borne viruses within *Flaviviridae*.

Undoubtedly additional molecular data for other BVDV isolates and for other members of the *Pestivirus* genus will be necessary to confirm this proposal. However, immediate consideration of the implications of our proposition may be useful.

Although there are many unanswered questions in flavivirology, there exists a large body of information relating to these viruses. Analysis of these data by pestivirologists may provide significant insight for understanding numerous issues involving pestiviruses. For example, we

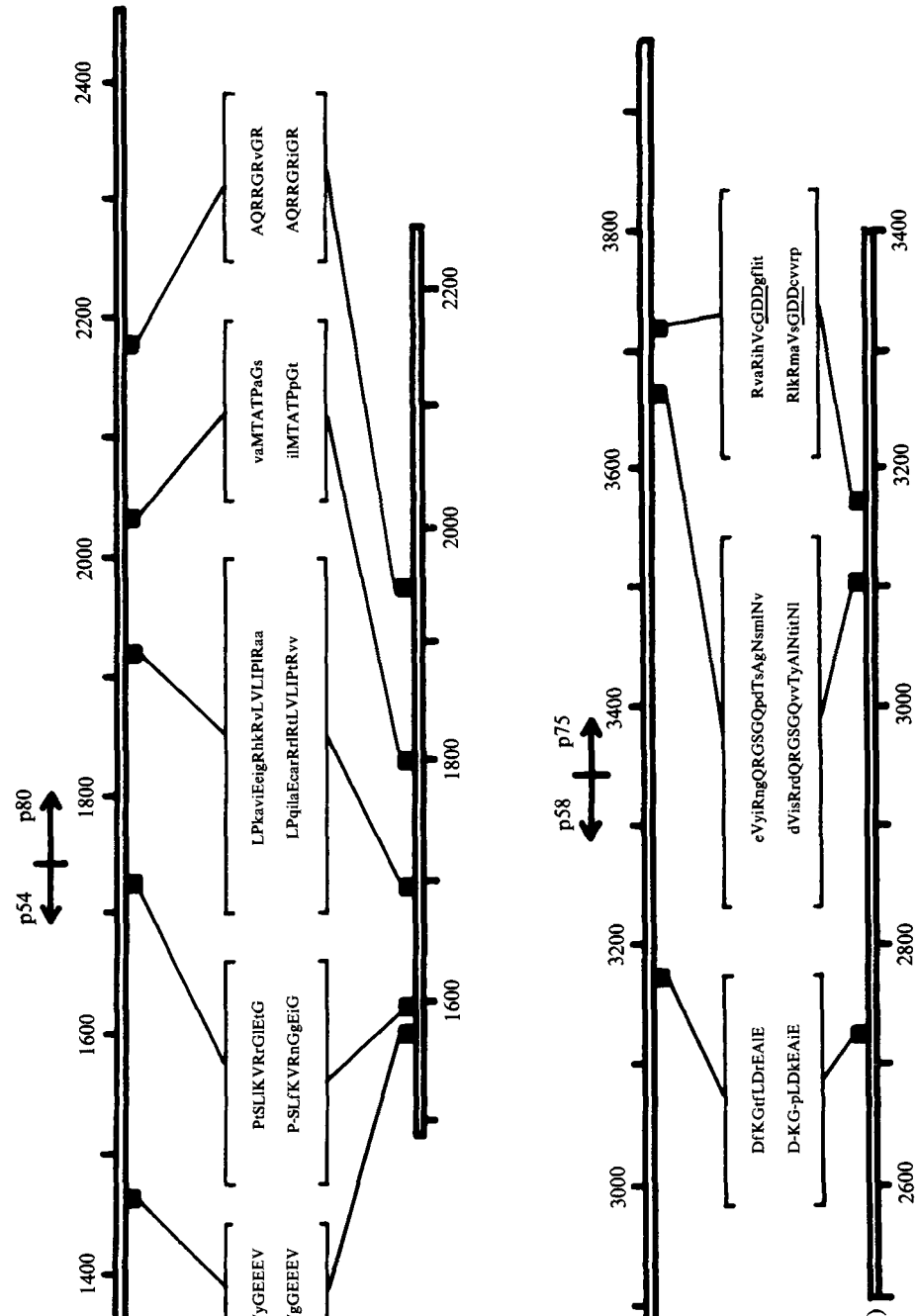

Fig. 2. Short segments of similarly aligned and highly homologous amino acid sequences in two proteins of BVDV and YFV. Schematically presented are the coding regions of BVDV p125 (*a*), YFV NS3 (*b*), BVDV p133 (*c*) and YFV NS5 (*d*). The numbers represent amino acid positions within the respective ORFs. The shaded blocks indicate the position of the displayed sequences within the coding region of each protein.

have already alluded to analogous proteins from the two virus groups potentially possessing the same biological functions. Currently, the nature of the actual virion structural polypeptides of BVDV remains obscure (Collett *et al.*, 1988*c*). Drawing analogies with the flavivirus system may aid in experimental designs to resolve the issue of structural and non-structural proteins for pestiviruses.

Beyond the similarities in molecular biology, possible parallels between pestiviruses and flaviviruses related to their immunobiology and viral pathogenesis might be considered. It is hoped that the issues raised here will stimulate fruitful discussions between workers in two areas of virology, and will help advance and accelerate our understanding of both.

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