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REVIEW ARTICLE

Recent Advances in Pestivirus Research

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INTRODUCTION

The term 'pestivirus' was coined in 1973 to group together two antigenically related enveloped RNA viruses: hog cholera virus (HCV) and bovine viral diarrhoea virus (BVDV; Horzinek, 1973). A third animal pathogen, the border disease virus (BDV) of sheep, was found later to be a close relative of BVDV. Pestiviruses are among the smallest enveloped animal RNA viruses (about 40 nm in diameter) and possess a nucleocapsid of non-helical, probably icosahedral symmetry (Horzinek *et al.*, 1967); they share these traits with the numerous flaviviruses, of which the arthropod-borne yellow fever virus is the prototype. The pestiviruses are not arthropod-borne and currently hold generic status in the family Togaviridae. Previously, flaviviruses also held generic status in this family. However, when details of flavivirus molecular structure, replication strategy and gene sequence became known in the early 1980s, the Togaviridae Study Group recognized the fundamental differences and proposed the creation of the new family Flaviviridae with Flavivirus as the only genus (Westaway *et al.*, 1985). As will be explained in this review, a similar reallocation of the pestiviruses is now inevitable.

Non-arthropod-borne togaviruses were last reviewed in 1981, when limited data from studies at the molecular level were available (Horzinek, 1981). Meanwhile, expanding knowledge has made another earlier classification unjustifiable: equine arteritis virus, which fulfils the structural criteria of a togavirus and has been listed as a possible member of the family (Matthews, 1982), replicates via multiple subgenomic mRNAs (van Berlo *et al.*, 1987) which form a 3′-coterminal nested set, not unlike coronaviruses (W. J. M. Spaan, unpublished observations).

Although not covered in the present review, recent advances have also been made in approaching the pathogenesis of pestivirus infections. It was an old and enigmatic finding that fatal mucosal disease (MD), one of the consequences of BVDV infection of cattle, could not be reproduced experimentally. The conditions which must be met for MD to develop have now been defined. Cows infected during the first 4 months of gestation with BVDV can give birth to healthy, persistently viraemic calves (Orban *et al.*, 1983; Leiss, *et al.*, 1984; McClurkin *et al.*, 1984). When this virus is of a non-cytopathogenic (ncp) biotype, superinfection with a 'matching' cytopathogenic (cp) strain of BVDV will result in the severe MD condition (Brownlie *et al.*, 1984; Bolin *et al.*, 1985*a*, *b*). It has been suggested that MD may be a consequence of the *in vivo* conversion of the ncp strain causing the persistent infection to cytopathogenicity (Brownlie *et al.*, 1986; Howard *et al.*, 1987), which would explain the erratic and sporadic occurrence of MD in a cattle population. As will be discussed below, the cp and ncp strains differ in the expression of at least one polypeptide.

THE MOLECULAR BIOLOGY OF PESTIVIRUSES

Only a small number of investigations detailing the molecular features of pestiviruses has been published. A constant difficulty has been the inability to distinguish unequivocally viral

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components, particularly less abundant ones, from host cell contaminants. This problem is a direct consequence of the low level of pestivirus growth in cultured cells. Another complicating factor is that pestiviruses often contaminate cell cultures undetectably and are present in tissue culture supplements (especially bovine sera) (King & Harkness, 1975; Kniazeff *et al.*, 1975). Consequently, the low sensitivity and selectivity of the earlier methods has led to a less than clear picture of pestivirus molecular biology (Horzinek, 1981). With the application of modern techniques to research on pestiviruses, however, their molecular portrait is gradually becoming more distinct.

The genome of pestiviruses

The nature of the genetic material of pestiviruses has long been known to be RNA (see Horzinek, 1981). Furthermore, the viral RNA was shown early on to be infectious, establishing pestiviruses as positive-strand RNA viruses (Diderholm & Dinter, 1966). However, the size and physical features of the RNA genome remained unclear since sedimentation values between 24S and 40S had been reported; RNA infectivity sedimented at the higher values (Moennig, 1971). The large variation is most probably due to differences in analytical methods and measurements, although strain variation has not been excluded. Using denaturing gel electrophoresis and appropriate molecular size standards, the size of the pestivirus genome has recently been more accurately estimated. Working with the Osloss strain of BVDV, Renard *et al.* (1985) measured the viral RNA to be 12·5 kb in length. Collett *et al.* (1988*c*) reported a similar size for the RNA of the NADL strain. The previously reported size of 8·2 kb for the NADL RNA (Purchio *et al.*, 1983) is an incorrect value based on an erroneous measurement. Comparison of the length of viral RNA from additional BVDV isolates, of both cp and ncp biotypes, with that of the NADL virus revealed their sizes to be indistinguishable (M. S. Collett, unpublished observations). However, the resolution of these analyses would not have permitted detection of small (5%) differences. The size of HCV RNA (Brescia strain), as measured on a denaturing gel, also appears to be in the 12 kb range (R. Moormann, personal communication).

The above-mentioned sedimentation data hinted that pestivirus RNA might have substantial secondary structure. Indeed, it was found that BVDV (NADL strain) RNA behaved similarly to double-stranded RNA by virtue of its ability to bind to CF-11 cellulose in the presence of 15% ethanol (Purchio *et al.*, 1984*b*) and to remain soluble in 2 M-LiCl (Collett *et al.*, 1988*a*). It even exhibited resistance to hydrolysis by low concentrations of RNase A, but could be distinguished from true duplex (replicative form) RNA by its sensitivity to higher enzyme concentrations (Purchio *et al.*, 1983). A high degree of secondary structure was also indicated during attempts to translate BVDV RNA in a reticulocyte *in vitro* system: it was unable to serve as an efficient message unless it had been denatured immediately before use (Purchio *et al.*, 1984*b*). Early observations by Dinter *et al.* (Dinter & Diderholm, 1971; Diderholm *et al.*, 1973) regarding the unique sensitivity of pestivirus replication to proflavine and acriflavine might be interpreted as a consequence of the unusual secondary structure of pestivirus genomic RNA.

Analyses of the termini of pestivirus RNA have been superficial to date. The viral RNA lacks significant polyadenylation as indicated by its inability to bind to oligo(dT)–cellulose (Purchio *et al.*, 1983; Renard *et al.*, 1985; R. Moormann, personal communication). Attempts to modify the RNA by addition of AMP residues to its 3′ end using *Escherichia coli* poly(A) polymerase have been unsuccessful (Renard *et al.*, 1985; Collett *et al.*, 1988*c*). However, the 3′ end of the RNA was a suitable substrate for the addition of cytidine 3′,5′-bisphosphate (pCp) by T4 RNA ligase (Renard *et al.*, 1985; R. Larson & M. S. Collett, unpublished observations), indicating the availability to this enzyme of a 3′ hydroxyl group. Why the 3′ end of the BVDV RNA is accessible to one enzyme and not to another remains unclear. However, the fact that BVDV RNA may be labelled at its 3′ end with [^{32}P]pCp using RNA ligase indicates that it should be possible to sequence this end of the genome. At the time of writing, such data have not been reported. Essentially nothing is known regarding the 5′ terminus of pestivirus RNA. BVDV RNA failed to be radiolabelled with [^{32}P]ATP using polynucleotide kinase with or without prior phosphatase treatment (R. Larson & M. S. Collett, unpublished observations). Whether this

indicates that the 5′ terminus possesses a cap must await direct demonstration of such a structure.

There are no reports describing the molecular features of pestivirus RNA replication. Infection of cells results in the appearance of a single viral RNA of about 12·5 to 13 kb. Replicative form (RF) or replicative intermediate (RI) RNA species in pestivirus-infected cells have not been described. Although the RNA found in BVDV (NADL strain)-infected cells had features similar to RF RNA (sedimentation characteristics, Purchio *et al.*, 1983; binding to CF-11 cellulose, Purchio *et al.*, 1984*b*; solubility in 2 M-LiCl, Collett *et al.*, 1988*c*), it could be distinguished from RF by its sensitivity to RNase (Purchio *et al.*, 1983). A small proportion (approx. 5 to 10%) of the BVDV RNA was insoluble in LiCl (R. Larson & M. S. Collett, unpublished observations). Whether this fraction represents RI RNA must await its further characterization. Due to the apparent high degree of secondary structure exhibited by pestivirus RNA, it may be inherently difficult to distinguish genomic from RF and RI RNA species.

Production of pestivirus RNA is unaffected by concentrations of actinomycin D that completely shut down host cell RNA synthesis (Horzinek, 1981; Purchio *et al.*, 1983). This observation implies that pestiviruses possess their own enzyme for RNA synthesis, presumably produced by direct translation of the incoming RNA. The identity of a BVDV gene product that may be a component of the viral RNA-directed RNA polymerase is discussed below.

The proteins of pestiviruses

Numerous reports have appeared describing pestivirus-specific polypeptides (summarized in Table 1); only the more recent data will be discussed. BVDV purified by Coria *et al.* (1983) revealed four major proteins upon Coomassie Brilliant Blue staining of gel-resolved material; two were found to be glycosylated (Table 1, fourth column). Purchio *et al.* (1984*a*) found three major and two less abundant BVDV-specific proteins (Table 1, fifth column) by radioimmuno-

Table 1. *Pestivirus-specific polypeptides**

	BVDV isolates†						
HCV‡	NADL§	NADL C24V‖	Singer¶	NADL**	NADL††	Singer‡‡	Proposed name§§
						165	
						135	
	93–110			115	120	(gp118)‖‖	p125
				80	87	80	p80
	70		gp75			(gp75)	
(65)			66		gp69	(gp65)	gp62
gp54–56	50–59	gp57	gp54	gp55	gp57	gp57	gp53
gp45–47		gp44		(45)	gp49	gp48	gp48
35–37		34		(38)	37	37	
(32)					33	32	
	25		26		gp23	(gp25)	gp25
(21)						19	p20

* The displayed values represent M_r estimates ($\times 10^{-3}$). The prefix 'gp' indicates glycoprotein.

† With the exception of the first column (HCV), each column represents data relating to BVDV with the isolates indicated.

‡ Enzmann & Rehberg, (1977); Enzmann & Weiland (1978).

§ Pritchett & Zee (1975).

‖ Matthaeus (1979).

¶ Coria *et al.* (1983).

** Purchio *et al.* (1984*a*).

†† Pocock *et al.* (1987).

‡‡ Donis & Dubovi (1987*a*, *b*).

§§ To establish consistency in the description of pestivirus proteins, a proposed nomenclature is presented.

‖‖ Numbers in parentheses represent polypeptides noted as minor components by the respective authors.

precipitation using convalescent bovine serum in both 'enriched' virus preparations and infected cell lysates. More recently, Pocock *et al.* (1987) described eight BVDV-specific polypeptides, including four glycoproteins, in cell lysates using radioimmunoprecipitation with BVDV antisera prepared in gnotobiotic calves (Table 1, sixth column). Finally, by combining radiolabelling of virus-infected cells in the presence of hypertonic translation initiation blockage and immunoprecipitation, Donis & Dubovi (1987*a*, *b*) succeeded in identifying 12 BVDV-specific polypeptides (Table 1, seventh column). The apparent discrepancies among these results may be due to a number of reasons, e.g. the low level production of many of the BVDV polypeptides, the varied experimental approaches, the different immunological reagents used, etc. The inability in any of these studies to distinguish between 'virus-encoded', 'virus-induced' and 'virus-associated' polypeptides has also contributed to the confusion. Although quite suggestive, immunoprecipitation of polypeptides with polyvalent antisera cannot establish the observed proteins as virus-encoded. The coprecipitation of host proteins with BVDV components has been observed (Matthaeus, 1979; M. S. Collett, unpublished observations). Furthermore, most studies have failed to explore the structural relatedness among the putative BVDV-specific polypeptides. Only Purchio *et al.* (1984*a*) showed by tryptic peptide analysis that the M_r 115K and 80K polypeptides of BVDV are structurally related, and unrelated to the 55K and 38K proteins.

For further progress to be made in clarifying the polypeptide components of pestiviruses emphasis must shift from descriptive studies to molecular genetic analyses. To introduce a consistent nomenclature for pestivirus proteins, we propose to use the M_r value labels listed in Table 1 and explained in Fig. 1, e.g. the M_r 115K polypeptide will hereafter be referred to as p125; this nomenclature will be used throughout this review.

Antigenic variation and epitope mapping

Since the historical observations by Darbyshire (1960) that HCV and BVDV are antigenically related, numerous attempts have been made to elucidate the degree of and the basis of serological cross-reactions. Although most workers agree that each pestivirus is antigenically homogeneous, i.e. that serotypes of HCV, BVDV or BDV do not exist (Carbrey, 1988), analyses using polyclonal antisera have shown strain variations detectable by cross-neutralization tests within each pestivirus (Aynaud *et al.*, 1974; Neukirch *et al.*, 1980). Neutralization assays are also able to distinguish between HCV and BVDV, but not between BVDV and BDV. This latter distinction must be based on biological and epidemiological data (Laude & Guelfi, 1979).

Monoclonal antibodies (MAbs) now provide the tools for a more detailed analysis. These MAbs have been characterized in respect of their spectrum of reactivity with different pestivirus strains and isolates, their virus-neutralizing capacity and their protein specificity.

Preparation of the first MAbs to HCV was reported by Wensvoort *et al.* (1986) and to BVDV by Peters *et al.* (1986). The latter antibodies were found to be specific for p125 of ncp BVDV. When analysed with cp BVDV, they reacted with both the p125 and p80 found in cells infected with this biotype (S. R. Bolin & V. Moennig, unpublished observations). When tested against other pestiviruses using indirect immunofluorescence and peroxidase-linked antibody tests, some of these MAbs recognized only cp BVDV, while others reacted broadly with all BVDV isolates and/or with both BDV and HCV (Greiser-Wilke *et al.*, 1987). One antibody (BVD/C16) was pestivirus-specific, reacting with all 50 pestivirus isolates tested so far. These results suggest that sequences within the p125 are highly conserved among pestiviruses. The limited genomic sequence data available indicate that the conserved component is p80 (see below). This protein probably represents the soluble antigen forming the 'single line of identity' observed by Darbyshire (1960) in agar gel immunodiffusion tests with HCV- and BVDV-specific antisera. E. J. Dubovi (personal communication) has characterized a MAb specific for a minor glycoprotein (gp48) of BVDV which reacted with all pestiviruses tested, including one strain of HCV. Additional MAbs with broad anti-pestivirus activity have been generated (G. Chappuis, S. Edwards & P. F. Nettleton, unpublished results) their protein specificity is not yet known.

A number of MAbs directed against the major glycoprotein of BVDV and HCV (gp50–59; referred to hereafter as gp53, see Table 1) were shown to possess virus-neutralizing activity.

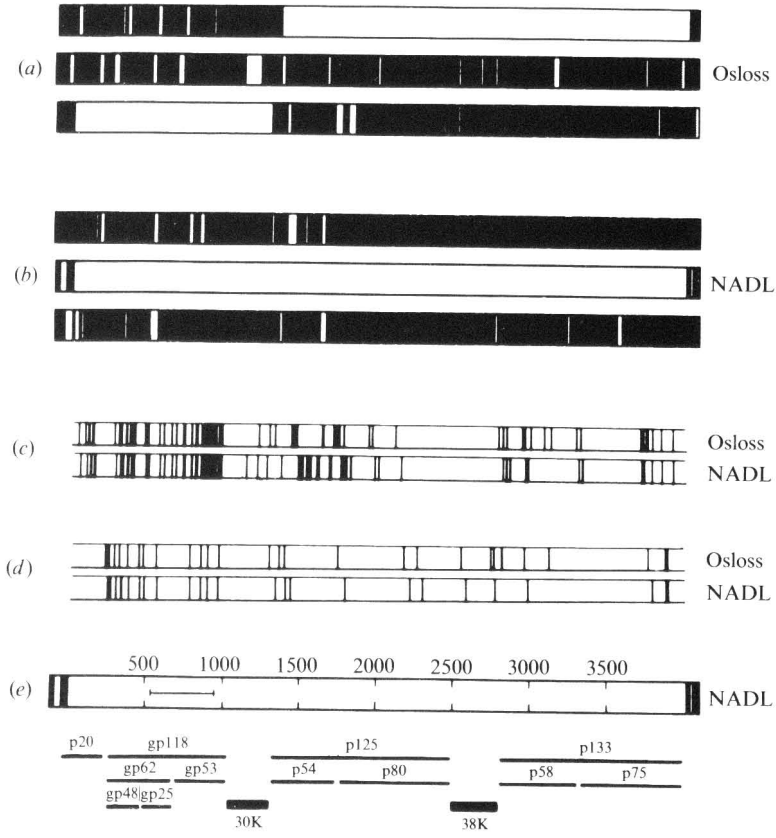

Fig. 1. Protein-coding regions of BVDV. Potential protein-coding regions, shown as ORFs that begin with an ATG codon, are displayed in all three phases in the positive polarity for the sequence of the Osloss (*a*) and NADL (*b*) isolates of BVDV. (*c*) Distribution along the ORFs of cysteine residues. (*d*) Distribution along the ORFs of potential *N*-linked glycosylation sites (N-X-S/T). (*e*) Positioning of BVDV-encoded polypeptides along the ORF of the NADL virus (for details see Collett *et al.*, 1988*b*). Numbers along the ORF indicate amino acid position. The line within the ORF between amino acids 500 and 1000 indicates the genomic region of the Singer isolate of BVDV that has been cloned. The blocked areas (M_r 30K and 38K) represent regions of the indicated coding potential for which no polypeptides have yet been identified.

MAbs lacking neutralizing activity and specific to gp53 of BVDV have also been described (Donis *et al.*, 1988; V. Moennig & S. R. Bolin, unpublished observations). Of the neutralizing MAbs, most neutralized several isolates of the homologous virus, but did not neutralize other pestiviruses (Bolin *et al.*, 1988; Donis *et al.*, 1988; C. Coulibaly, unpublished observations; G. Wensvoort, unpublished observations). Interestingly, however, some MAbs which neutralized one isolate did bind to another virus without neutralizing its infectivity (A. Mateo & V. Moennig, unpublished observations). A similar phenomenon was recently described for Sindbis virus and some of its variants (Olmsted *et al.*, 1986). Thus, the significance of conserved epitopes for virus neutralization differs among pestiviruses. Using neutralizing MAbs, G. Wensvoort *et al.* (unpublished observations) have identified three antigenic domains with a total of eight epitopes on the major glycoprotein of HCV. Three additional domains on the same glycoprotein comprising five epitopes were not involved in neutralization. Extending the results of Bolin *et al.* (1988) by using 47 pestiviruses in competitive binding studies, A. Mateo & V. Moennig (unpublished observations) identified 10 epitopes on gp53 of BVDV relevant for neutralization. Eight of them were clustered in one domain whereas one epitope, which thus far could be identified only on the homologous virus, was located outside this domain. In these studies,

binding of a single MAb was sufficient for virus neutralization. However, a synergistic effect of MAbs directed against different domains was observed with HCV (G. Wensvoort, unpublished observations); in contrast, there was no such effect with anti-gp53 MAbs to BVDV (A. Mateo & V. Moennig, unpublished observations).

In 1987, a workshop was held at the Hannover Veterinary School to compare 50 MAbs against 43 pestivirus isolates. Materials were contributed by 13 European laboratories (Moennig, 1988; tables summarizing the data will be provided by V.M. upon request). It soon became clear that the ability of MAbs to discriminate between antigenic variants was very powerful: numerous differences were found between strains bearing the same name but coming from different laboratories and having distinct passage histories. These results emphasize the need for cautious interpretation when comparing results obtained with the 'same' viruses in different laboratories (Moennig, 1988).

VIRUS–CELL INTERACTIONS

All pestiviruses possess a similar host spectrum. HCV is transmissible to cattle and small ruminants and BVDV naturally infects pigs, sheep, goats and a wide range of wild ruminants (Nettleton *et al.*, 1980; Doyle & Heuschele, 1983; Dahle *et al.*, 1987; Moennig *et al.*, 1988). Pestivirus host range for cultured cells is even broader (Horzinek, 1981). However, despite their ability to cross species barriers, pestiviruses in general replicate inefficiently in heterologous hosts. In most cases they cause neither c.p.e. in cell culture nor clinical disease. BVDV-induced disease in sheep seems to be an exception.

Recently, Teyssedou *et al.* (1987) described a MAb against a receptor molecule on bovine cells for bovine enterovirus 3 which partially protected cells against infection with cp BVDV. In the Hannover laboratory, a MAb directed against a bovine cell surface protein was shown to interfere specifically with the infectivity of a number of cp BVDV strains while leaving infection with HCV and BDV, as well as with parainfluenza virus type 3 and infectious bovine rhinotracheitis virus, unimpaired (Moennig *et al.*, 1988). Although a comparison of the protein specificity of these two MAbs has not yet been made, these findings suggest that a specific cell surface receptor mediates entry of BVDV into bovine cells. Furthermore, it appears that different pestiviruses may not share the same receptor, at least in bovine cells. However, when studied more closely using immunoperoxidase techniques, the inhibition of infection by the Hannover MAb was not always complete; a few foci of infected cells were detectable in monolayers pretreated with this MAb. Therefore, either multiple receptors for BVDV may exist on cells or a less efficient, receptor-independent mechanism of virus internalization may be operative, as has been described for other viruses (Minor *et al.*, 1984; Markwell *et al.*, 1985).

The biological significance of receptor molecules for the histotropism of pestiviruses is not yet understood. It has been shown that the receptor specificity of viruses can be altered upon passage in cultured cells (Reagan *et al.*, 1984). The ability of pestiviruses to adapt to heterologous hosts by expressing new attachment sites on the virion needs to be investigated. Thus, pestiviruses infecting bovine, ovine and porcine cells alike have been identified, but so have strains infecting only one or two of the above species (V. Moennig, unpublished observation). The variation may even be greater when wild ruminants are taken into account (Nettleton *et al.*, 1980; Doyle & Heuschele, 1983).

Cytopathogenicity of pestiviruses is a property which depends on genetic factors of the virus as well as on the type of cell culture used (Horzinek, 1981). In general, HCV does not produce c.p.e. in porcine cell cultures; only a few exceptions have been reported (Gillespie *et al.*, 1960; Laude, 1978). BDV and BVDV strains behave differently with respect to cytopathogenicity and numerous cp isolates exist. The recent appreciation of the significance of ncp and cp biotypes for the pathogenesis of MD (see Introduction) has focused attention on the determinants of cytopathogenicity. The fact that pairs of ncp and cp BVDV isolates from MD-affected animals are serologically indistinguishable (but distinct from other pairs) suggests that only minor alterations have occurred. Comparison of the viral polypeptides of the two BVDV biotypes has revealed a difference: whereas cp BVDV isolates possess both p80 and p125 (among other proteins), ncp viruses display only p125 (Akkina, 1982; Donis & Dubovi, 1987*c*; Pocock *et al.*,

1987). Earlier peptide mapping data have shown p80 and p125 to be structurally related (Purchio *et al.*, 1984*a*). More recently, the genetic relationship between these polypeptides has been proven, and an M_r 54K protein related to p125 but not to p80 has been identified (Collett *et al.*, 1988*b*; see below). With this difference between the cp and ncp biotypes of BVDV identified, questions arise concerning the function of p125 and its cleavage products, p54 and p80, during viral replication and in the origin of biotypes. It has been suggested that cp biotypes arise by mutation from ncp BVDV in persistently infected animals (Howard *et al.*, 1987). It is tempting to speculate that a mutation affecting a virus-encoded protease or a protein cleavage site might be responsible for the conversion.

MOLECULAR CLONING OF PESTIVIRUSES

Recent work on the molecular cloning and sequencing of pestivirus genomes has greatly advanced our understanding of these viruses. Renard *et al.* (1985) first reported five non-overlapping, non-aligned families of cDNA clones obtained from the genome of their Osloss strain of BVDV. A more complete description of their cloning efforts appeared subsequently in a European patent application (Renard *et al.*, 1987). The Osloss DNA clones spanned the length of the RNA and yielded a sequence of 12492 nucleotides. Dale *et al.* (1987) reported, also in a European patent application, the cloning and sequencing of a 1149 nucleotide segment of the Singer strain of BVDV. Collett *et al.* (1988*c*) recently published the nucleotide sequence of the NADL strain of BVDV which was 12573 nucleotides in length. Finally, a large portion of the genome of HCV (Brescia strain) has been cloned (R. Moormann, personal communication), but the sequence is not yet published.

The methods used to obtain cDNA clones for the Osloss and NADL viruses would not have enabled the cloning of the genomic ends, particularly of the 3′ terminus. Therefore, the sequence of a complete pestivirus genome is not yet at hand; direct sequencing of the ends of the viral RNA must be undertaken in order to reach this goal.

Comparison of the nucleotide sequences of the Osloss and NADL strains of BVDV shows that they are quite closely related: aligned sequence identity was approximately 74% as determined using the algorithms and programs of Pustell & Kafatos (1982, 1984). Their base compositions are nearly identical; that for the NADL RNA is A 31·7%, G 25·7%, U 22·2%, C 20·4%.

Analysis of the nucleotide sequence for potential protein-coding regions revealed significant open reading frames (ORFs) in the positive polarity only. Renard *et al.* (1987) reported two large ORFs for the Osloss strain (Fig. 1*a*); in contrast, Collett *et al.* (1988*c*) found only one ORF extending the length of the RNA (Fig 1*b*). Comparison of the deduced amino acid sequences of the two ORFs of the Osloss virus with those of the single NADL virus ORF corroborated the close relationship between the viruses (see below; Table 2). Furthermore, the alignment of cysteine residues and sites of potential *N*-linked glycosylation (Asn-X-Ser/Thr) along the ORFs of the two viruses are nearly identical (Fig. 1*c* and *d*).

It is unlikely that the Osloss and NADL viruses actually differ in their organization of potential protein-coding regions. The possibility of a mistake in the Osloss sequence is suggested by the fact that an insertion of two nucleotides (or the deletion of one) at or just before Osloss nucleotide 4241 transforms the sequence to a single ORF, with the resultant amino acid sequence in the previously untranslated region between ORFs largely homologous to and in register with that of the NADL strain (M. S. Collett, unpublished observations). Finally, there appears to be only a single large ORF in the genome of HCV; sequencing of this RNA, however, is not yet completed (R. Moormann, personal communication).

Given the single ORF of the NADL virus sequence, translation initiation would be expected to occur at the first methionine codon at the 5′ end of the ORF. This codon occurs at nucleotide positions 386 to 388. From there, the ORF extends uninterrupted to nucleotide 12349, where a TGA termination codon is encountered (Collett *et al.*, 1988*c*). The NADL virus ORF consists of 3988 codons capable of encoding protein with a total M_r of 449K. Such a genomic arrangement suggests that pestivirus protein biogenesis proceeds in a manner similar to that of picornaviruses and flaviviruses, with mature viral proteins being generated by proteolytic processing of a polyprotein precursor. Analyses of the polypeptides encoded within the NADL virus ORF

Table 2. *Amino acid sequence homologies between the proteins of the NADL and Osloss isolates of BVDV*

Region*	Homology (%)†
p20	90·9
gp62	87·6
gp48	89·0
gp25	85·9
gp53	78·5
(30K)	72·4‡
p125	86·1§
p54	68·0§
p80	96·3§
(38K)	95·0
p133	79·0¶
p58	86·6
p75	77·0¶

* Regions correspond to those depicted in Fig. 2(*e*).

† Percentage homology is the number of identical amino acids per total number compared.

‡ In this portion of the ORF, the reported Osloss sequence is terminated 46 amino acids before the proposed end of the 30K protein-coding region, resulting in a homology value of 42·9%. If two nucleotides are inserted appropriately within the 19 nucleotides preceding the reported termination codon of the first Osloss ORF, a single ORF results and the amino acid sequence comes in register with the NADL sequence, yielding the displayed homology value.

§ The reported Osloss second ORF begins eight amino acids downstream of the NADL sequence for this protein. The NADL sequence has an 89 amino acid addition relative to the Osloss sequence in one region of p54. In an adjacent region, the Osloss sequence possesses a non-homologous 76 amino acid insertion.

‖ A region of low homology exists near the carboxy terminus of p80 (18 of the last 56 amino acids are mismatches); otherwise the homology is 98·8%.

¶ A region of 40 amino acids near the amino terminus of p75 is non-homologous (34 out of 40 mismatches). Within the carboxy terminus of p75, the NADL sequence has a 56 amino acid insertion relative to the Osloss sequence. Disregarding these differences, homology of p75 is 91·6%, and that of p133 is 87·1%.

suggest that polyprotein processing does indeed occur (see below). Since regulation of gene expression at the level of transcription is excluded (in contrast to the situation in alphaviruses, arteriviruses and coronaviruses, for example), differences in the abundance of individual polypeptides may be the result of dissimilar protein stability.

In further support of this suggested expression strategy is the observation that subgenomic RNA species have never been found during BVDV infection (Purchio *et al.*, 1983; Renard *et al.*, 1985). Purified genomic RNA can serve as a template for viral protein synthesis, but only upon denaturation immediately before addition to the translation reaction (Purchio *et al.*, 1984*b*). Genome size viral RNA has also been found associated with polyribosomes in infected cells (Purchio *et al.*, 1984*b*). The viral RNA obtained from polysomes and that purified by CF-11 cellulose chromatography are probably identical, but this has yet to be established. If the BVDV genomic RNA serves as a message *in vivo*, the putative 5′ untranslated region preceding the large ORF would be at least 385 nucleotides in length. However, upstream of this initiation position lie two methionine codons near the 5′ ends of two small ORFs, one capable of encoding 45 amino acids, the other 48 amino acids (Collett *et al.*, 1988*c*). Whether these small ORFs are functional remains to be determined.

GENOMIC ORGANIZATION OF PESTIVIRUSES

Cloned sequences have been used to identify the virus-encoded gene products and to elucidate the genomic organization of BVDV (Collett *et al.*, 1988*b*). Short segments of the cloned BVDV genome were engineered into an *E. coli* protein expression system and the resulting BVDV-specific polypeptides were used to generate a panel of sequence-specific antisera. These sera were then used in immunoprecipitation analyses to identify authentic BVDV-encoded proteins. Results from this work allowed the identification and positioning along the genome of BVDV gene products, accounting for about 83% of the coding capacity of the virus (Collett *et al.*,

1988*b*). A preliminary map of the genetic organization of BVDV is given in Fig. 1(*e*). The boundaries of the coding regions for individual gene products are considered tentative, since they are based only on protein size estimates and sequence-specific antiserum reactivity. Currently, there are no sequence data for BVDV proteins to allow their precise positioning within the deduced amino acid sequence of the ORF.

Most of the BVDV-specific polypeptides described in previous studies (Table 1) are accounted for in the gene map. An exception appears to be the M_r 32K to 38K proteins described in several studies (Table 1), but not yet established as virus-encoded. There remain two regions (30K and 38K) of the genome for which protein products have yet to be identified (Fig. 1*e*). Whether the 32K to 38K proteins are viral proteins and are encoded in either of these sequences remains to be established. Several precursor–product relationships are suggested among the proteins described in the gene map. However, proof of such relationships will require appropriate pulse–chase experiments.

Of particular interest is the clustering of the coding sequences for all viral glycoproteins to a single region near the 5′ end of the genome. The putative precursor gp116 spans this area and apparently gives rise to gp62 and gp53; gp62 in turn yields gp48 and gp26 (Fig. 1*e*). The entire region possesses 14 sites (Asn-X-Ser/Thr) for potential *N*-linked glycosylation. Ten sites are found in gp62, of which eight reside in gp48 and two in gp25. Glycoprotein gp53 contains four such sites, which are conserved between the Osloss and NADL viruses (Fig. 1*d*) as mentioned earlier. The extent to which these glycan attachment sites are used is not known; indirect information suggests that most may be occupied with oligosaccharide chains (Collett *et al.*, 1988*b*).

The localization of the BVDV glycoprotein genes suggests that the viral structural proteins are encoded in the 5′-terminal region of the genome. However, currently the identity of most pestivirion polypeptides is unclear. Donis & Dubovi (1987*b*) have obtained functional evidence to identify gp53 as one of the envelope glycoproteins. This appears logical since neutralizing MAbs raised against BVDV and HCV recognized this major glycoprotein. In all probability, some (but not necessarily all) of the glycoproteins in the gene segment encompassed by gp116 (Fig. 1*e*) are viral structural proteins. Based on the assumption that the virion proteins are coded for by the 5′ end of the genome, p20, the first protein of the BVDV ORF (Collett *et al.*, 1988*b*), also may be a structural polypeptide, perhaps the nucleocapsid protein.

If the virion proteins are positioned within the 5′-terminal portion of the pestivirus genome, it follows that the remainder of the ORF programmes 'non-structural' proteins involved in virus replication and maturation. The products of the p125 (38K) and p133 regions (Fig. 1*e*) might represent such proteins. Interestingly, the p80 protein (related to p125) is an immunodominant polypeptide. The p133/p58:p57 proteins may be components of the viral replicase. Clearly, more work in the area of pestivirus protein function is needed to clarify which are structural and which are non-structural.

Using the protein boundaries described in the BVDV gene map, we can compare in more detail the amino acid sequence homologies between the individual polypeptides of the Osloss and NADL viruses. Such a comparison may indicate which polypeptides possess variable sequences and which are conserved. If correction is made for the shift in the ORFs of the Osloss sequence (see Table 2 footnotes), protein sequence homology (identity) ranges from 68% for p54 to almost 100% for p80. Since a short segment of the genomic sequence of the Singer strain of BVDV is available (Dale *et al.*, 1987), further comparison of the relevant area can be made. The region encompassed by the Singer sequence includes the carboxy-terminal 88% of gp25 and the amino-terminal 58% of gp53 (Fig. 1*e*). From this limited three-way comparison of amino acid sequences (Fig. 2), it appears that the NADL and Singer viruses are more closely related (93·2%) than either is to the Osloss strain (77·8% and 76·0% respectively).

PESTIVIRUS CLASSIFICATION REVISITED

In view of the recent progress in the description of the molecular features of pestiviruses, the discussion of virus classification must be re-opened. The first molecular data which suggested that pestiviruses are distinct from members of the Togaviridae family relate to characteristics of

```
  530       540       550       560       570       580
   *         *         *         *         *         *
KIVGPGKFGTNAEDGKILHEMGGHLSEVLLLSLVVLSDFAPETASVMYLILHFSIPQSHV

KIVGPGKFdTNAEDGKILHEMGGHLSEVLLLSLVVLSDFAPETASaMYLILHFSIPQSdV

KIVGPGrFdTNAEDGKILHEMGGHLSEVLLLSvVVLSDFAPETASVvYLILHFSIPQgHt

  590       600       610       620       630       640
   *         *         *         *         *         *
DVMDCDKTQLNLTVELTTAEVIPGSVWNLGKYVCIRPNWWPYETTVVLAFEEVSQVVKLV

DiMeCDKTQLNLTVELTTAdVIPGSVWNLGKWVCIRPNWWPYETTVVLAFEEVSQVVKLV

DihDCDKnQLNLTVgLTTAEVIPGSVWNLGKYVCIRPdWWPYETatFLVFEEVgQVirlV

  650       660       670       680   gp25◄····►gp53
   *         *         *         *         *         *
LRALRDLTRIWNAATTTAFLVCLVKIVRGQMVQGILWLLLITGVQGHLDCKPEFSYAIAK

LRALRDLTRIWNAATTTAFLiCLVKIVRGQMVQGILWLLLITGVQGdLhCKPEFSYAIAr

LRALRDLTRIWtAATTTAFLVCLVKvVRGQvlQGILWLiLITGaQGlpvCKPgFyYAIAK

  710       720       730       740       750       760
   *         *         *         *         *         *
DERIGQLGAEGLTTTWKEYSPGMKLEDTMVIAWCEDGKLMYLQRCTRETRYLAILHTRAL

DERIGQLGAEGLTTTWKdYSPeMKLEDTMVIAWCkDGKftYLpRCTRETRYLAILHTRAL

nneIGpLGAtGLTTqWyEYSdGMrLqDTgVvvWCkgGeikYLitCeREaRYLAILHTRAL

  770       780       790       800       810       820
   *         *         *         *         *         *
PTSVVFKKLFDGRKQEDVVEMNDNFEFGLCPCDAKPIVRGKFNTTLLNGPAFQMVCPIGW

PTSVVFKKLFDGRKQEDVVEMdDNFEFGLCPCDAKPIVRGKFNTTLLNGPAFQMVCPIGW

PTSVVFeKiiDGkeQEDVVEMdDNFElGLCPCDAKPlVRGKFNTTLLNGPAFQMVCPIGW

  830       840       850       860       870       880
   *         *         *         *         *         *
TGTVSCTSFNMDTLATTVVRTYRRSKPFPHRQGCITQKNLGEDLHNCILGGNWTCVPGDQ

TGTVSCmSFNMDTLATTViRTYRRSKPFPHRQGCITQKtLGEDLHNCILGGNWTCVPGDm

TGTVSChwsNkDTLAmTVVRTYkRhrPFPfRQGCITQKviGgDLydCaLGGNWTCVPGDi

  890       900
   *         *
LLYKGGSIESCKWCGYQFKESEG   NADL

LLYKGGSIESCKWCGYQFfhpss   Singer

LrYvdGpvESCKWCGYkFhkSEG   Osloss
```

Fig. 2. Comparison of a 383 amino acid sequence from the ORFs of three BVDV isolates. Numbering is according to that of the NADL sequence (Collett *et al.*, 1988*a*). The proposed junction between gp25 and gp53 (amino acid 691) is shown. Vertical lines indicate amino acids (in lower case) not identical to those in the NADL sequence. Three sites for potential *N*-linked glycosylation are shaded. Homologies of the Singer and Osloss sequences relative to the NADL sequence are 93·2% and 77·8% respectively.

the virus-specific RNA. In infected cells, only a single high M_r RNA species was found, which lacked a 3′ poly(A) tract (Purchio *et al.*, 1983; Renard *et al.*, 1985; R. Moormann, personal communication). No subgenomic RNA was detected at any time after infection. These features not only distinguish pestiviruses from togaviruses but, more importantly, also suggest a similarity of pestiviruses to flaviviruses. With much of the nucleotide sequence and genetic organization of BVDV known, further comparisons of this prototype pestivirus to members of Flaviviridae have been made (Collett *et al.*, 1988*a*). With the exception of several short but significant stretches of identical amino acids within two of the putative non-structural proteins, no extended regions of nucleotide or amino acid sequence homology exist between BVDV and representatives of the three antigenic subgroups of mosquito-borne flaviviruses. Nevertheless, the molecular layout of the BVDV and flavivirus genomes is strikingly similar. Comparison of the arrangement of the protein-coding domains along both genomes and the hydropathic features of their amino acid sequences revealed pronounced similarities. Based on these comparisons, it was proposed that the Pestiviruses no longer be grouped in the Togaviridae family, but rather should be considered a genus within the Flaviviridae (Collett *et al.*, 1988*a*).

For such a proposal to be accepted, additional molecular data for other BVDV isolates and other pestiviruses will be required. However, the implications of this proposition are immediately provocative. Considering the parallel organization of their genomes, analogous polypeptides may be predicted to possess similar biological functions. For example, the several short, similarly aligned regions of highly homologous amino acids in p125 of BVDV and NS3 of flaviviruses, and in the respective p133 and NS5 proteins, may indicate that they are essential for some common function (Collett *et al.*, 1988*a*). The NS5 protein may be a component of the flavivirus RNA-dependent RNA polymerase (Rice *et al.*, 1985), a suggestion based largely on sequence homologies found with various viral RNA polymerases (Kamer & Argos, 1984). The sequence Gly-Asp-Asp (GDD) is common to these polymerases; it appears only once in the NADL and Osloss virus ORFs, in the 3′-terminal gene product analogous to the NS5 of flaviviruses, p133. Therefore it is likely that this polypeptide represents a component of the pestivirus RNA-dependent RNA polymerase. Drawing analogies to the flaviviruses may help in experimental designs to resolve the issue of structural versus non-structural proteins for pestiviruses. Certainly insights beyond those obtained in molecular biology may be gained from further comparisons between these two groups of viruses.

PROGRESS ON PRACTICAL PROBLEMS

The diagnosis of pestivirus infections is beset with several problems. Current routine procedures are tedious, expensive and often inaccurate. Discrimination between pestivirus strains by virological and serological means is difficult due to extensive serological cross-reactions (Carbrey, 1988; Liess, 1988). Simple procedures for the detection of antibodies to pestiviruses in field sera have been lacking. However, all this is changing. The availability of MAbs, nucleic acid probes and synthetic peptides has opened new diagnostic avenues.

Broadly reactive MAbs to HCV are currently used in routine diagnostic procedures for the differentiation of HCV and BVDV infections (Wensvoort *et al.*, 1986; Hess *et al.*, 1988). In addition, some MAbs are able to discriminate the Chinese vaccine strain of HCV from field isolates (Wensvoort *et al.*, 1986). BVDV-specific MAbs with broad reactivity offer the opportunity for development of an antigen capture ELISA. Such an assay, in a field-compatible format, will be valuable for the detection of persistently infected animals within herds. Although yet to be explored, nucleic acid probes offer the potential for highly specific and sensitive detection of virus directly in blood cells.

A problem of economic significance is the lack of simple procedures to detect antibodies against pestiviruses in field sera. Present serological techniques do not discriminate between antibodies produced in pigs after infection with HCV and BVDV. Competition ELISA procedures using MAbs as described by Juntti *et al.* (1987) can be implemented to approach this problem. Synthetic peptides reactive exclusively with antibodies to specific pestiviruses offer another diagnostic possibility.

Understanding the pathogenesis of the MD syndrome represents a major objective of future work. Important to this goal will be the elucidation of the molecular differences between biotype pairs of BVD viruses. Currently, two cp virus genomes (Osloss and NADL) have been largely cloned and sequenced, and others will follow. However, if we are to gain insight into the cooperation between cp and ncp BVDV, it will be essential to analyse naturally occurring virus pairs capable of inducing MD.

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