Nucleotide sequence of the bovine viral diarrhoea virus Osloss strain: comparison with related viruses and identification of specific DNA probes in the 5' untranslated region

L. De Moerlooze,1 C. Lecomte,2*, S. Brown-Shimmer,3 D. Schmetz,1 C. Guiot,1 D. Vandenbergh,2 D. Allaer,2 M. Rossius,2 G. Chappuis,4 D. Dina,3 A. Renard2 and J. A. Martial1

1Laboratoire de Biologie Moléculaire et de Génie Génétique, Université de Liège, Institut de Chimie B6, B-4000 Sart Tilman, 2Eurogentec s.a., Parc de Recherches de la Cense Rouge, Rue Bois Saint Jean 14, B-4102 Seraing, Belgium, 3Chiron Research Laboratories, Chiron Corporation, Emeryville, California 94608, U.S.A. and 4Rhône-Mérieux, Laboratoires IFFA, Rue Marcel Mérieux 254, B.P. 7009, 69342 Lyon, France

The nucleotide sequence of the cytopathic Osloss isolate of bovine viral diarrhoea virus (BVDV) was deduced from overlapping cDNA clones and from PCR products. The Osloss genome is an RNA molecule of positive polarity containing 12480 nucleotides and having the capacity to code for a polyprotein of 3975 amino acids. The presence of the previously described internal stop codon in this viral sequence was disproved after direct sequencing of the appropriate PCR-amplified fragment. Except for the previously reported insertion of a sequence coding for a ubiquitin-like protein, the viral genome shares great similarity with those of three other strains of the pestivirus genus. Computer-assisted sequence analyses and comparisons of known pestiviral genomic sequences led us to identify selected PCR primers in the 5' untranslated region. These primers were used successfully to amplify 18 distinct pestivirus isolates and potential DNA probes were noted from the deduced sequences. The possible use of a well conserved 26 base fragment as a diagnostic probe was confirmed in hybridization experiments. The 5' untranslated region was further studied and compared with those of other members of the Flaviviridae family, which includes the flaviviruses and the hepatitis C virus group. These sequence analyses support the possibility of discrimination amongst the closely related ruminant pestiviruses, border disease virus and BVDV.

Bovine viral diarrhoea virus (BVDV) is a positive-strand RNA virus of the pestivirus genus which also contains the border disease virus (BDV) and hog cholera virus (HCV). Previously classified in the Togaviridae family (Horzinek, 1981; Westaway et al., 1985), pestiviruses were later grouped within the Flaviviridae (Francki et al., 1991) because of their genomic sequence and organization (Renard et al., 1987a; Collett et al., 1988a). To date, three pestiviral sequences have been reported in the literature, one from a BVDV strain, NADL (Collett et al., 1988a) and two from the HCV strains Alfort (Meyers et al., 1989) and Brescia (Moorman et al., 1990). The nucleotide sequence of these three strains contains a single large open reading frame (ORF) encoding a polyprotein thought to be co- and post-translationally processed by either host- or virus-encoded proteases (Collett et al., 1988b; Wiskerchen et al., 1991; Wiskerchen & Collett, 1991). We previously described the identification of the RNA genome of the cytopathic BVDV Osloss strain (Osloss/c; Liess, 1967), its cloning from a cDNA library (Renard et al., 1985) and its sequencing from 10 overlapping cDNA clones (Renard et al., 1987a, b). The nucleotide (nt) sequence was then reported as containing two ORFs, with one translation termination codon located at nt 4242 to 4244. The singularity of this feature, the fact that from the cDNA library we were unable to select other clones encompassing this region except cDNAs with large deletions or insertions (probably of bacterial origin) and the inconsistencies between our data and other pestivirus sequences led us to examine this region using direct sequencing of PCR products.

A 353 nt fragment including the previously described stop codon was synthesized by PCR from total RNA extracts of Osloss/c-infected ovine kidney cells (Rhône-Mérieux). The amplifications were performed with one primer depleted to obtain single-stranded templates (100 pmol of one amplimer and 1 pmol of the other; De

The sequence data have been deposited with the EMBL/GenBank database under the accession no. M96687.

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The Osloss viral sequence were modified after their position 4224 and 4252. The insertion of the first of these nucleotide positions. Underlined regions, modified deduced amino acid sequences, compared with previously reported data (Renard et al., 1991; 1987b), two other regions of the Osloss/3 end, however, has an internal deletion of 50 nt in length.

The corrected BVDV Osloss polypeptide sequence was compared with that of the NADL, Alfort and Brescia pestiviruses. The four polypeptides were aligned using the CLUSTAL software package (Higgins & Sharp, 1988). This alignment is presented in Fig. 2, the degree of conservation between the four amino acid sequences being established from successive groups of 30 residues. Analysis of the similarity of the sequences confirmed that the p80 non-structural protein is an optimal target for the development of diagnostic tests based on the use of a well-conserved antigen, allowing the detection of infections caused by the three members of the pestivirus genus. Several assays were previously described that allowed the detection of specific anti-p80 antibodies in bovine sera (Kwang et al., 1991a; Lecomte et al., 1991; Paton et al., 1991a; Petric et al., 1992), or the detection of the pestivirus antigen itself using characterized antibodies (Fenton et al., 1990).

We also compared the Osloss/c, NADL, Alfort and Brescia sequences at the nucleotide level to determine regions of most identity. Computer-assisted analyses showed the most pronounced degree of conservation between the four genomes to be within the 5' untranslated region (64% identical nucleotides; Fig. 3a). Two locations were chosen for synthesis of PCR primers that could be useful to amplify other pestiviral genomes. The sequences of the primers are 5' ACGTGGACGAGGG-

one between nt 10470 and 10571, and a second between nt 11527 and 11705 (Fig. 1). The entire corrected sequence of the Osloss/c isolate of BVDV is 12480 nt in length. It has a coding capacity for a long polyprotein of 3975 amino acids, the methionine codon being positioned at nt 384 to 386. We did not perform direct sequencing, which would have allowed the complete sequence to be established in both untranslated regions, particularly in the 3' end of the genome. Concerning the 5' end, two distinct cDNA clones were sequenced and yielded identical termini. Moreover, Brock et al. (1992) recently reported the sequencing of both NADL 5' and 3' termini, which provides further evidence that our 5' sequence is complete. When compared with the NADL sequence, the Osloss 3' end, however, has an internal deletion of 50 nt in length.

Fig. 1. Corrected regions within the genomic sequence of the Osloss/c strain of BVDV.*. Positions of nucleotide modifications or insertions; underlined regions, modified deduced amino acid sequences, compared with previously reported data (Renard et al., 1987); dotted lines, primer positions.

Moerloose et al., 1990). After purification of the PCR products on a 30 Centricon column (Amicon), dideoxynucleotide sequencing reactions were carried out (T7 polymerase sequencing kit, Pharmacia) using as primers those depleted in PCR. From the sequencing of these templates, it appeared that the initial sequence deduced from a cDNA clone was missing two nucleotides, at position 4224 and 4252. The insertion of the first of these nucleotides allowed the stop codon to be eliminated, and the addition of the second transformed the two described ORFs into one single large ORF. The corrected sequence of this genome region is presented in Fig. 1. Compared with the sequence previously reported in a patent application (Renard et al., 1987b), two other regions of the Osloss viral sequence were modified after their sequencing on single-stranded PCR-amplified fragments, which provides further evidence that our 5' sequence is complete. When compared with the NADL sequence, the Osloss 3' end, however, has an internal deletion of 50 nt in length.

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detailed in a previous report (Lecomte et al., 1990). All the RNAs led to specific PCR products of the correct size. A comparison of the 18 resulting nucleotide sequences is presented in Fig. 3 (b).

This alignment revealed a separation of the 18 pestivirus sequences into two unexpected groups, one containing the BVDV strains, and the other containing the BDV and HCV ones. This observation conflicted with the usual subdivision of the pestivirus species into either HCV type or ruminant BDV/BVDV type, made on the basis of their respective pathology, biotype and antigenic relationships. Indeed, it was observed elsewhere that although the differentiation of the HCV isolates from the two other pestiviruses was easily feasible using monoclonal antibodies, the separation of BVDVs from BDVs was much more problematical (Paton et al., 1991 b; Moennig & Plagemann, 1992). Our analysis of a number of pestiviral 5' untranslated regions thus suggests an easy method of differentiating BVDVs from BDVs was more problematical (Paton et al., 1991 b; Moennig & Plagemann, 1992). Our analysis of a number of pestiviral 5' untranslated regions thus suggests an easy method of differentiating BVDVs from BDVs: the observed sequence dissimilarities can be exploited to design useful probes discriminating between the two types of ruminant pestiviruses.

This comparative study of various 5' non-coding sequences also highlighted a particularly well conserved region from which we deduced the position of a common 26 base probe. The probe sequence is 5' GTGGGCCTCTGCAGCACCTATCAGG 3' and is complementary to nucleotides 322 to 347 of the BVDV Osloss/c 5' region. This sequence is perfectly conserved among 16 of the 18 strains analysed, and one isolate (Lamspringe) has only one mutation. The Singer/nc sequence, however, contains five mutations in this region and Singer/c strain also appeared different from the other pestiviruses examined in hybridization experiments performed with DNA probes complementary to other parts of the Osloss genome (data not shown).

The selected 26 base sequence might be used as a probe for the diagnosis of pestiviral infections. Its ability to detect pestiviruses was confirmed in hybridization experiments carried out on total RNA extracted from cells infected with BVDV, BDV and HCV isolates. It was also noted that this sequence constitutes one of the four regions of similarity found between hepatitis C virus type 1 and two pestiviruses, NADL and Alfort (Han et al., 1991). Our study reveals that this block is also conserved in a series of other pestiviral strains. The 26 base probe described above might therefore be a useful diagnostic tool for the detection of pestivirus as well as hepatitis C.
Fig. 3. (a) Alignment of the 5’ untranslated sequences of the four known pestivirus genomes. *, Conserved nucleotides; * position of the conserved blocks of the NADL and Alfort strains and hepatitis C virus (Han et al., 1991); PCR primers are underlined. (b) Comparison of the 18 amplified pestiviral sequences using primers underlined in (a).
virus infections. Our data support the idea of a close relationship between these two groups although Han et al. (1991) reported, as well as the four similar sequences in the 5' termini of pestivirus and hepatitis C virus genomes, the presence of two ORFs that we were unable to find in the Osloss/c sequence; the ATG codon that starts the second ORF of NADL is a GTG codon in Osloss/c.

Further comparisons between pestiviruses and the other members of the Flaviviridae family were carried out, for example to investigate the presence of conserved RNA secondary structures in the 5' non-coding region. The prediction of such stable structures previously led Strauss & Strauss (1983) to suggest that these sequences were recognition sites for replicase components, and consequently played an essential role in the regulation of viral RNA synthesis. More recently, stable and well conserved secondary structures were also described in flaviviruses (Brinton & Dispo, 1988), as well as in members of the hepatitis C virus group (Tsukiyama-Kohara et al., 1992). We failed to find any striking homology between the predicted secondary structures for the four pestiviral regions, however, despite the high conservation observed in the corresponding nucleotide sequences. No more homology was found between these structures and those reported for either the flavivirus or the hepatitis C virus genomic RNA.

In conclusion, the pestivirus 5' untranslated region appears to be an effective target for diagnostic methods based on the detection of viral nucleotide sequences, using both PCR and hybridization. Other reports have described similar procedures but with primers (Schroeder & Balassu, 1990; Lopez et al., 1991; Ward & Misra, 1991) or probes (Kwang et al., 1991b; Ridpath & Bolin, 1991; Lewis et al., 1991) located in the coding part of the genome. The choice of the 5' non-coding region has, however, been described for detecting hepatitis C viruses by PCR (Bukh et al., 1992) and for pestiviruses (Boye et al., 1991). Here we propose a set of specific primers for detecting pestiviral sequences, and a 26 base DNA probe for both pestiviruses and hepatitis C viruses. Moreover, sequence dissimilarities observed in the 5' untranslated regions of 18 pestiviral strains suggest that probes could distinguish BDV from BVDV isolates within the predominant pestivirus group.

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References


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