

## MOLECULAR CLONING OF THE BOVINE VIRAL DIARRHEA VIRUS GENOMIC RNA

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### Résumé

CLONAGE MOLÉCULAIRE DE L'ARN GÉNOMIQUE DU VIRUS DE LA MALADIE DES MUQUEUSES. — L'ARN génomique du virus de la maladie des muqueuses (BVD) a été cloné chez *E.coli*. Sa séquence complète a été déterminée et l'organisation génomique en a été déduite. Plusieurs fragments d'ADNc, spécifique pour le BVD, ont été exprimés chez *E.coli*, dans les cellules eucaryotes et *in vitro*. Nous suggérons que ce virus appartient à une nouvelle famille différente des familles Togaviridae et Flaviviridae.

Two features of pestiviruses, the difficulty of growing large amount of virus and their sensitivity to manipulations, probably explain the wide gap in their molecular biology, and this despite the economical importance of the diseases they cause in cows (mucosal disease), pigs (hog cholera), goats and sheeps (Border disease) (reviewed by Horzinek 1981, and Nettleton *et al* 1985). Although this gap is being filled somewhat nowadays, as will be explained in other papers of this issue, it is still important in the characterization of the viral proteins and genome. Also much has still to be learned about the antigenic relationship between the three species of this genus and about the antigenic variations inside species. Of course, all these aspects have pertinent impacts on the diagnosis and prevention of these diseases.

With this in mind, we started some years ago the cloning of the bovine viral diarrhoea virus genome. We will summarize here some of the data we have obtained so far.

### Materials and Methods

The methods have been described in two recent papers (Renard *et al* 1985 a,b).

### Results and Discussion

#### *Growing and purification of the virus*

During this work, we have used the Osloss strain of BVDV, initially isolated in Dr Liess laboratory. This virus was grown in Bovine Embryonic

Kidney cells obtained from Dr Pastoret laboratory. The Osloss strain is cytopathic for these cells. After working on the conditions of the virus culture, we routinely obtained a titre of  $10^7$  pfu/ml and sometimes we were lucky to observe a  $10^8$  titre, which has never been reported before. This result explains in part our success in the cloning of the BVDV RNA.

We tried to purify the virus but we failed since the viral preparations are contaminated by a huge amount of cellular vesicles, of same size and properties as the virus, formed during the cell lysis. Consequently, in the rest of this paper, the words « virus » and « viral pellet » will refer to a pellet obtained by ultracentrifugation of a clarified infection medium.

#### *Characterization of the viral genome*

We have characterized the RNA present in the viral pellet. It was isolated by standard procedure, radioactively labeled and size fractionated by electrophoresis on a denaturing agarose gel. One high molecular weight band is readily detectable in viral pellets from  $10^8$  pfu/ml preparations, but barely visible in preparation with lower titres.

That this high molecular weight band is RNA is shown by its sensitivity to RNAase and alkali treatments and its resistance to DNAase and proteinase K treatments. This RNA, we hypothesized to be the viral genomic RNA, has a size of 12.5 kb on glyoxal gels; its size is greater and variable in formaldehyde gels, an indication of strong secondary structures. This RNA is not polyadenylated as judged by its non retention on an oligo-dT column. This last property is surprising for

Table 1 — Comparison of some molecular biology data for BVDV, Sindbis Virus, AEV and Yellow Fever Virus

	Togaviridae			Flaviviridae
	pestivirus BVD <sup>1</sup>	alphavirus Sindbis <sup>2</sup>	arterivirus EAV <sup>3</sup>	Yellow Fever <sup>4</sup>
gRNA size (kb)	12.5	11.7	± 12.4	10.2
Polyadenylation	—	+	+	—
Subgenomic RNA	0	1	5	0
Translation Strategy <sup>5</sup>	2 ORF/RNA Internal translation ?	1 ORF/RNA	1 ORF/RNA splicing ?	1 ORF/RNA
Genes order	sP — nsP	nsP — sP	(nsP — sP ?)	sP — nsP
Structural Proteins (kd)	(C : 28) (gE1 : 48) (gE2 : 55)	C : 30-34 gE1 : 50-60 gE2 : 50-60	C : 12 E1 : 14 gE2 : 21	M : 7-8 C : 13 gE1 : 51-59

1 : this work

2 : from Strauss *et al* (1984)

3 : from Van Berlo (1985)

4 : from Rice *et al* (1985)

5 : number of open reading frames (ORF) per messenger (RNA)

a togavirus RNA (Kennedy 1980) but not for a flavivirus RNA (Wengler and Wengler 1981).

A genomic like RNA, of same size and properties, is also detected, after tritiated uridine labeling, in the cells during an infection but not in mock infected cells. The high molecular weight RNA is visible at all times of infection; it is already present after 12 hrs and it is the main labeled species at 21 hours. In these experiments, we were never able to detect any specific subgenomic viral RNA.

#### Cloning of the viral RNA in *E. coli*

The RNA isolated from a viral pellet from a  $10^8$  pfu/ml preparation was size fractionated by a velocity centrifugation on a sucrose gradient.

The gradient was fractionated and the RNA from each fraction was analysed by electrophoresis on a denaturing agarose gel. The fractions containing the viral RNA were pooled and the RNA was precipitated with ethanol. This viral RNA, which is almost pure, was used for the cloning. Complementary DNA was prepared with reverse transcriptase and oligo-dT (after *in vitro* polyadenylation) or random oligonucleotide primers. The single stranded RNA and DNA were removed by treatment with ribonuclease A and S1 nuclease. The final population of double-stranded RNA-DNA hybrids was size fractionated by gel chromatography. The larger hybrids were dC tailed with the terminal transferase and annealed to pBR322 plasmid DNA digested with Pst1 and dG tailed. *E. coli* were directly transformed with this

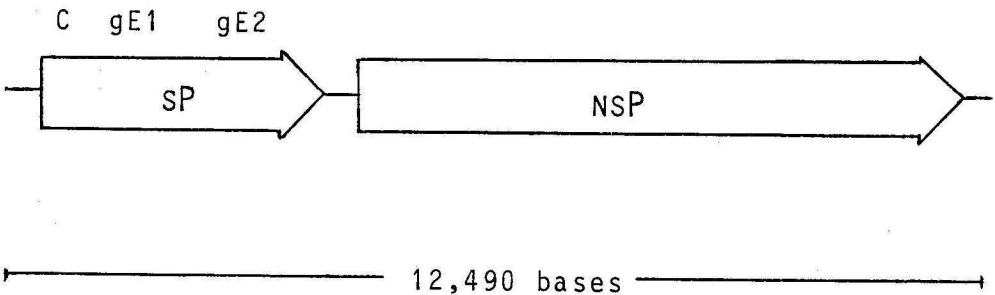


Fig. 1. — Genomic organization of the BVDV RNA.

mixture. Recombinant *E. coli* clones were selected by their resistance to tetracycline.

For the screening of BVD specific clones, we have used a plus and minus method. A viral specific labeled cDNA probe, or plus probe, was prepared with the reverse transcriptase on the purified viral RNA in presence of random oligonucleotides primers. This probe was prehybridized with a large excess of cellular RNA to lessen the background. The final probe did not hybridize with yeast RNA or RNA isolated from uninfected cells, but, as expected, the signal is strong with RNAs isolated from infected cells or from a viral pellet. The negative probe is labeled cDNA prepared by similar techniques from RNA isolated from uninfected

cells. For the screening, the recombinant *E. coli* colonies were replicated on nitrocellulose filters which were hybridized with the plus and minus probes. A BVDV specific clone is one which gave a strong signal with the plus probe and no signal, or a faint one, with the minus probe. With this technique, we isolated almost 300 positive colonies from a total of 4000.

The BVD-cDNA inserts from positive colonies were digested by restriction enzymes and cross-hybridized, experiments which allow a physical mapping of the inserts on the viral RNA. Eventually, we were able to cover the complete genome with only 10 different overlapping clones, the largest one having a 4 kb size. The orientation of this map was determined by hybridization of single stranded cDNA with viral RNA.

We performed some Northern blot experiments, where RNA isolated from infected cells was hybridized with labeled cDNA inserts which mapped on different parts of the viral genome, with the purpose to look for the presence of subgenomic RNA. In all these experiments, we detected only the 12.5 kb RNA and no specific subgenomic RNA. This result was obtained with all the inserts tried, thus confirming our precedent analysis of the synthesis of viral RNA in cells during an infection.

#### Sequencing and genomic organization

We sequenced the complete genome by the dideoxy technique as described in Sanchez-Pescador and Urdea (1984). The genomic organization deduced from this sequence, and confirmed by expression experiments described later, is schematized in figure 1.

The total length is 12 490 bases but we might be missing some non coding bases on the 3' end. There are two long non overlapping open reading frames, the second one on the 3' end being two times longer than the one on the 5' end. An analysis of the hydrophobicity plots of these two frames indicates that the one on the 5' end codes for the structural proteins. The order of the structural proteins on the first frame is the capsid protein followed by two glycosylated membranous proteins. The second open reading frame presumably codes for the non structural viral proteins.

#### Taxonomy of BVDV

Some of the data I have discussed so far are summarized in table 1 where they are compared with similar data from other togaviruses and from flaviviruses, a family formerly classified as Togaviridae (Westaway *et al* 1985).

The size of the viral RNA is typical for a togavirus and is greater than the viral RNA of flaviviruses.

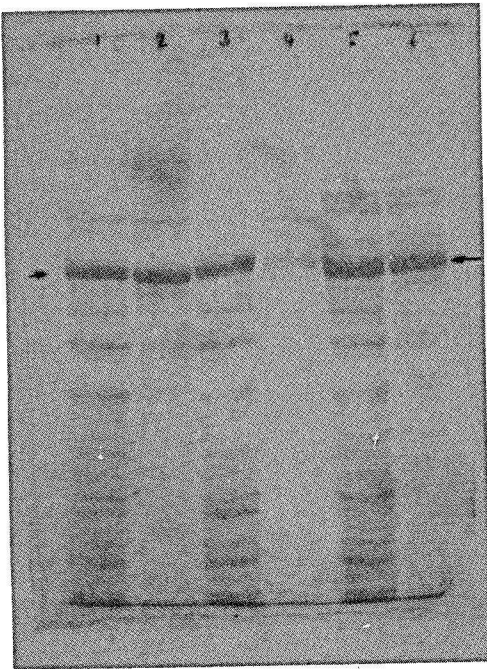


Fig. 2. — BVD.cDNA-lacZ fusions expressed in *E. coli*. Three different BVD-cDNA inserts were cloned in phase behind the lacZ gene (Rüther and Müller-Hill 1980). The expression was induced with IPTG and extracts of the cells were analysed by PAGE in presence of SDS (lanes 1, 3, 5). The position of the  $\beta$ -galactosidase is indicated by an arrow. The bands on the top of the  $\beta$ -galactosidase are fusion proteins. The presence of many bands indicates either a specific proteolysis or premature stops. The aggregates formed in *E. coli* were purified by the method of Klempnauer *et al* (1983). These purified aggregates were also analysed by PAGE (lanes 2, 4, 6). For each sample, we loaded proteins isolated from the same amount of *E. coli* cells.

The absence of polyadenylation and of subgenomic RNAs are features only shared by the flaviviruses.

The presence of two reading frames on the same RNA, a result which might indicate an internal initiation of translation, is a specific property of BVDV. The Equine Arthritis Virus has also a specific but different translation strategy with 5 subgenomic RNAs, presumably formed from the genomic RNA by a splicing-like mechanism and which code for distinct proteins.

The genomic organization is again flavivirus like, the structural proteins being on the 5' end and the non structural on the 3', the reverse order of the one observed in togaviruses, except maybe, the arteriviruses.

Finally, the four viruses of this table have different structural proteins.

From these data, we suggest that these four viruses belong to different families as they differ significantly in their translation strategies and their genomic organizations.

#### Expression experiments

We have expressed BVD-cDNA fragments, covering both open reading frames previously described, in three different systems: *E. coli*, mammalian cells and *in vitro*. We will only describe some expression experiments performed in *E. coli*.

We have used the expression vector of Rüter and Müller-Hill (1980). The BVD-cDNA fragment is inserted in phase behind the  $\beta$ -galactosidase gene, under the control of the lac promoter. After a specific induction of this promoter, there is a high synthesis level of a chimeric protein made of an active  $\beta$ -galactosidase and a BVD polypeptide. This chimeric protein is easily detected by SDS polyacrylamide gel electrophoresis. The expression is so high that the chimeric proteins precipitate inside the bacteria and form aggregates. These aggregates are quite insoluble in detergents, a property which can be used for their purification (fig 2).

The aggregates can be solubilized in urea and kept in solution by a careful dialysis. Disappointingly, none of the solubilized fusion proteins we have prepared so far was recognized by our anti-BVD antisera. We turn around this negative result by preparing rabbit antisera against these fusion proteins. These antisera were then analysed by different immunological techniques. The results for only one fusion protein with a BVD cDNA which mapped on the second open reading frame will be described below.

The rabbit antiserum against this chimeric protein is positive on ELISA with proteins isolated

from infected cells. It is also positive by immunofluorescence on fixed cells. The serum reacts with a 76 kd band by Western blotting. The immunoprecipitation is shown in figure 3 where it is compared with one anti-BVD antiserum. The two central lanes are labeled proteins from uninfected, on the left, and infected cells, on the right. They illustrate the difficulty to demonstrate the synthesis of specific viral proteins during an infection with BVD. The anti-BVD antiserum immunoprecipitates a 76 kd protein and a diffuse 55 kd band. We showed that the 76 kd band is identical to the one detected by the anti-fusion protein antiserum in Western blot experiment. The latter antiserum is immunoprecipitating a 36 kd protein which, we showed by other experiments, to be related to the 76 kd protein. The 76 kd protein is probably the soluble antigen described by different authors (Horzinek 1981). The 36 kd band has been miscalled the capsid band by comparison with other togaviruses.

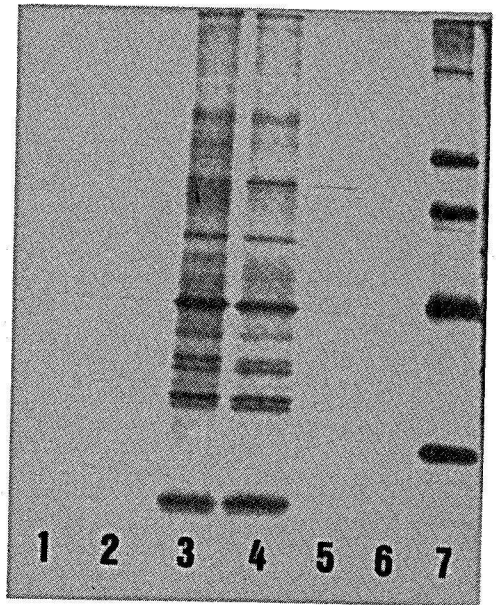


Fig. 3. — Immunoprecipitations.

Immunoprecipitations were performed as described in Martial *et al* (1977) with a rabbit antiserum prepared against a BVD-cDNA-lacZ fusion protein (lanes 1 and 6) or an anti-BVD hyperimmune rabbit antiserum (lanes 2 and 5). Lanes 1 to 3 contain labeled proteins isolated from mock infected cells, lanes 4 to 6, proteins from BVD infected cells. The proteins are immunoprecipitated and analysed by PAGE in presence of SDS. Lane 7 contains labeled marker proteins (from BioRad).

## Conclusions

We described here some of our results of the application of the recombinant DNA technology to pestivirology. We showed that these viruses belong to a specific family different from Togaviridae and Flaviviridae. We also showed how these techniques help us to solve the difficult problem, for these viruses, of the characterization of the viral proteins.

This approach, associated with works on monoclonal antibodies and biological typing as will be described in other papers of this issue, will lead to

a better understanding of the pathology and epidemiology of the pestiviruses and will open the way to better diagnosis and vaccines.

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## Abstract

The genomic RNA from Bovine Viral Diarrhea Virus (BVD) was cloned in *E. coli*. The complete sequence has been obtained and the genomic organization has been deduced. Some of the BVD specific cDNA have been expressed in bacteria, eucaryotic cells and *in vitro*. We suggest that BVDV belongs to a new family different from Togaviridae and Flaviviridae.

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