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Molecular Cloning of Bovine Viral Diarrhea Viral Sequences

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ABSTRACT

Bovine viral diarrhea virus (BVDV) genomic RNA was identified as a 12.5-kb single-stranded RNA molecule in both infected bovine embryonic kidney cells (BEK-1) and partially purified virions. BVD virion RNA was partially purified and used as a template for cDNA synthesis. BVDV-specific cDNA sequences were molecularly cloned and shown to hybridize to infected cell RNA but not to uninfected cell RNA or DNA. A single RNA species of 12.5 kb, representing the viral RNA genome, was detected in infected cells. A preliminary map of the BVDV specific cDNA clones was constructed and five major, nonoverlapping families were observed, accounting for approximately one-half of the viral genome.

INTRODUCTION

BOVINE viral diarrhea virus (BVDV) is a non-arthropod-borne Togavirus belonging to the pestivirus genus. The virus causes an economically important disease in cattle of worldwide distribution, associated with an enteric and/or respiratory syndrome, especially in calves, and characterized by lesions of the mucous membranes in the digestive and respiratory tracts. Abortion, stillbirth, and/or congenital birth defects are commonly attributed to infection of pregnant cows.

Limited and partially contradictory information is available concerning the genetic and physical structure of the viral RNA, perhaps due to difficulties associated with *in vitro* generation of adequate material for analysis. Early studies reported a single-stranded RNA genome of positive polarity of 9–12 kb (for review, see Horzinek, 1981), while a more recent study determined that the prevalent RNA species in BVDV-infected cells was 8.2 kb (Purchio *et al.*, 1983).

During the present work we defined a new cell-virus strain pair, the use of which permits routine *in vitro* production of high titre progeny virus in amounts suitable for biochemical manipulations. In this study we describe the identification of a 12.5-kb viral RNA species, its partial purification, and initial molecular characterization. Using viral RNA as a template for reverse transcription, 95 recombinant DNA plasmids carrying BVDV-specific information were obtained and a partial map of the BVDV RNA genome was derived.

MATERIALS AND METHODS

Cells and viruses

Bovine embryonic kidney cells (BEK-1) were grown in MEM (Earl's) containing 0.85 g/liter $NaHCO_3$ and 10% γ-irradiated fetal calf serum. The biologically cloned Osloss strain of BVDV was obtained from RIT, Belgium.

Virus production

Virus was amplified from stocks through four passages of increasing size on BEK-1 cells at a multiplicity of infection of 0.1. For a typical virus production experiment, 150 plastic flasks (175 cm^2) of freshly confluent BEK-1 cells were used for a fifth and last passage. The cells were washed three times with infection buffer (MEM [Earl's] containing 2.2 g/liter of $NaHCO_3$ pH 7.6) and then infected with 2 ml of medium containing BVDV at a multiplicity of infection of 0.5 pfu/cell. After 1 hr at 35°C, 18 ml of medium were added

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and the cells were incubated for 4–5 days at 35°C. Cytopathic effects (CPE), as evidenced by vacuolation followed by cell lysis, was consistently 80%, usually ≥90%. The infected cell medium (about 3 liters) was collected and stored at 4°C. The remaining cells were harvested by scraping in 2 ml of infection buffer per flask, subjected to three cycles of freezing and thawing, and the final suspension was added to the collected infected cell medium. Yields of virus were consistently $\geq 10^7$ pfu/ml while our best preparations gave titers of 10^8 pfu/ml. After centrifugation at 10,000 × g for 30 min, the supernatant was concentrated tenfold by ultrafiltration on a millipore PTHK 10,000 membrane and the virus was collected by ultracentrifugation at 100,000 × g for 4.5 hr at 4°C.

Extraction and purification of viral RNA

Total RNA was isolated from the virus pellet by the CsCl/guanidinium thiocyanate method (Chirgwin *et al.*, 1979) and stored in 70% ethanol at −20°C.

To separate high-molecular-weight viral RNA from contaminating cellular RNAs in the preparation, and aliquot containing approximately 5μg of total RNA was centrifuged at 10,000 × g for 15 min at 4°C. The pellet was washed with 80% ethanol, denatured by incubation in 375 μl of 99% dimethylsulfoxide, 5 m*M* Tris-HCl pH 7.5 for 5 min at 37°C. After addition of 1.125 ml of 5 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA, and 1% Sarkosyl, the solution was heated for 2 min at 70°C and quickly cooled on ice. This solution was distributed onto five 15–30% linear sucrose gradients in 5 m*M* Tris-HCl pH 7.5, 10 m*M* EDTA, 0.1 *M* NaCl, 1% Sarkosyl prepared in sterile, siliconized Beckman SW41 tubes. A sixth gradient was similarly loaded with an aliquot of the same RNA previously 3′-labeled (see below) for use as a marker. After centrifugation for 16 hr at 19,000 rpm (20°C) in a Spinco SW41 rotor, the gradients were collected (1 ml fractions), and the RNA from each fraction of marker gradients was subjected to formaldehyde agarose gel electrophoresis to detect those fractions containing high-molecular-weight BVDV-specific RNA. Fractions from the five sample gradients equivalent to those containing labeled marker RNA were separately precipitated with 2.5 volumes of ethanol in the presence of carrier yeast RNA (10μg).

Intracellular RNA labeling

BEK-1 cells grown in 75-cm^2 plasstic flasks were washed 3 times with infection buffer and infected with BVDV at multiplicities of infection of 50–100 pfu/cell in 1 ml of infection buffer. After 1 hr at 35°C, an additional 4 ml of infection buffer was added and the incubation continued. At selected intervals 100 μCi aliquots of [^{3}H]uridine (30–40 Ci/mmol, Amersham) were added and after 30 min of incorporation, cellular RNA was extracted by the CsCl/guanidinium thiocyanate method (Chirgwin *et al.*, 1979). The resultant pellet of RNA, obtained after ultracentrifugation through a 5.7 *M* CsCl cushion, was directly analyzed by formaldehyde agarose gel electrophoresis. After the gel was dried bands were seen by fluorography and autoradiography.

In vitro *RNA labeling*

Labeling of RNA (3′ terminus) with [^{32}P]$_p$C$_p$(Amersham) using T4 RNA ligase (P-L Biochemicals) was performed as described by England *et al.*, (1980). To polyadenylate RNA *in vitro*, the method of Sippel (1973) was used with some modifications. An amount of of purified BVDV RNA estimated to be 0.7 μg was incubated in 5 μl of 5 m*M* methylmercury hydroxide for 10 min at room temperature. Following denaturation, the sample was incubated for 6 min at 37°C with 20 units of poly(A) polymerase (BRL) and 500 μCi of [^{3}H]ATP (36 Ci/mmol, Amersham) in 50 μl of 50 m*M* Tris-HCl pH 7.5, 10 m*M* $MgCl_2$, 2.5 m*M* $MnCl_2$, 0.3 *M* NaCl, 1.5 m*M* 2-mercaptoethanol, containing 2.5 μg of RNase free bovine serum albumin (BSA) and 5 units of human placental ribonuclease inhibitor (BRL). (BSA was made RNAse-free by chromatography on UMP-agarose [Maniatis *et al.*, 1982].) The sample was then deproteinized with phenol/chloroform and the RNA purified by chromatography on Sephadex G50 followed by precipitation with 2.5 volumes of ethanol.

Synthesis of labeled cDNA probes

One microgram of partially purified BVDV genomic RNA was initially incubated for 10 min at room temperature in 5 μl of 10 m*M* methylmercury hydroxide and then an additional 45 min at 37°C with 40 units of AMV reverse transcriptase (Life Sciences) in 100 μl of 50 m*M* Tris-HCl pH 8.3, 10 m*M* $MgCl_2$, 1.5 m*M* 2-mercaptoethanol, 1 m*M* dATP, dGTP, and dTTP, 10 μ*M* dCTP, 0.2 mg/ml of actinomycin D, containing 5 units of human placental ribonuclease inhibitor, 500 μCi of [α^{32}P]dCTP (3000 Ci/mmol, Amersham) and 20 μg of calf thymus DNA random primers (prepared as described by Maniatis *et al.*, 1982). At 15 and 30 min of incubation, an additional 10 units of reverse transcriptase were added. At the end of the reaction period, the sample was deproteinized with phenol/chloroform and the labeled cDNA purified by chromatography on a Sephadex G50 column. RNA was hydrolyzed with 0.1 *M* NaOH (1 hr at 65°C) and the cDNA sample then neutralized with 0.1 *M* acetic acid and added directly to hybridization buffer.

Synthesis of cDNA for cloning

Approximately 1 μg of BVDV-specific RNA (partially purified viral genome) was incubated (10 min, room temperature) with 10 m*M* methylmercury hydroxide in a volume of 10 μl and the excess methylmercury hydroxide then titrated by addition of 1 μl of a 3 *M* β-mercaptoethanol solution. The denatured RNA sample was used immediately for the synthesis of cDNA by incubation at 37°C in the presence of 50 m*M* Tris-HCl pH 8.0, 1 m*M* dATP, dCTP, dGTP, and dTTP, 2.5 μg/ml $dT_{12\text{-}18}$, 10 m*M* $MgCl_2$, 10 μg/ml actinomycin D, containing 100 units of placental ribonuclease inhibitor and 60 units of reverse transcriptase in a total volume of 100 μl.

At the end of the reaction (45 min), the sample was diluted to 400 μl with a buffer containing 10 m*M* Tris-HCl pH 7.0, 100 m*M* NaCl, 10 m*M* EDTA, and 0.2% $NaDodSO_4$. After phenol/chloroform extraction, the sample was freed of

dNTPs by Sephadex G50 chromotography and ethanol precipitated.

Purification of high-molecular-weight cDNA:RNA hybrids

The resultant mixture of RNA and RNA:cDNA hybrids was treated sequentially with pancreatic ribonuclease A and nuclease S_1 as follows. The sample (10 μl) was diluted into 50 μl buffer S_1 (50 m*M* Na acetate pH 4.5, 500 m*M* NaCl, 1 m*M* $ZnCl_2$) and digested for 15 min at room temperature with 20 units of S_1 nuclease (Sigma). The reaction was stopped by diluting the sample to 500 μl with a buffer containing 50 m*M* Tris-HCl pH 7.0, 50 m*M* NaCl, and 10 m*M* EDTA. RNase A was added to a final concentration of 20 μg/ml and digestion was continued for 15 min at room temperature. After phenol/chloroform extraction, the RNA:cDNA hybrids were concentrated by ethanol precipitation and fractionated on a Sepharose CL4B column prepared in a 1-ml plastic pipette. The fractions containing the excluded material (cDNA:RNA hybrid molecules greater than 800_{bp} in length) were then pooled and ethanol precipitated.

Tailing of cDNA:RNA hybrids and cloning into Escherichia HB101

The final recovery of RNA:cDNA hybrid in the excluded fraction for the above dT-primed reaction was approximately 50 ng. This material was then tailed with dC residues under conditions yielding 15–25 residues per DNA or RNA terminus (Maniatis *et al.*, 1982) and inserted into a pBR322 vector linearized at the *Pst* I site and tailed with dG (NEN). After annealing of the above RNA:cDNA hybrid preparation at a vector DNA concentration of 0.1 μg/ml, the samples were used to tranform *E. coli* HB101 under standard conditions.

Preparation of labeled inserts

Recombinant plasmids were isolated in alkaline NaDodSO$_4$ (Maniatis *et al.*, 1982) and digested with *Pst* I and the resultant inserts were purified on 5% polyacrylamide gels. One microgram of each insert was then further digested with *Dde* I and *Mbo* I and labeled by end-filling using the Klenow fragment of DNA polymerase I and the four [α-^{32}P]dNTPs (3,000 Ci/mmol, Amersham). (The restriction enzymes employed were obtained from New England Biolabs.)

Gel electrophoresis and hybridizations

RNA agarose gel electrophoresis in the presence of 2.2 *M* formaldehyde was carried out as described in Lehrach *et al.*, (1977). Hybridizations with RNA on agarose gel were done as described in Smiley *et al.*, (1983). Prehybridizations were performed overnight at 42°C in a large volume of 5× SSC (1× SSC is 0.15 *M* NaCl, 15 m*M* sodium citrate pH 7.0), containing 50% formamide, 0.1% PVP, 0.1% Ficoll, 1 m*M* EDTA, 0.2% NaDodSO$_4$, and 100 μg/ml of denatured salmon sperm DNA. Hybridizations were performed overnight at 42°C in 5× SSC, containing 50% formamide, 0.1% PVP, 0.1% Ficoll, 1 m*M* EDTA, 10% dextran sulfate, 0.2% NaDodSO$_4$, and 100 μg/ml of denatured salmon sperm DNA. Washing was initially at 65°C with 2× SSC, 0.1% NaDodSO$_4$, and then with 0.2× SSC and 0.1% NaDodSO$_4$.

RESULTS

Production of BVDV

Bovine embryonic kidney cells (BEK-1) and BVDV-Osloss strain were selected for virus production because of our observation of the capacity of this cell-virus pair to produce a high progeny titer (approximately 10^8 pfu/ml) under the conditions described in Materials and Methods. Five passages of the virus on BEK-1 cells were required to achieve amplification to this titer, but cytopathic effects, consisting of clustering of cells followed by vacuolation and cell lysis, were readily observable from the first passage. Although BVDV infections usually produced 90% lysis of the cell monolayer, only partial recovery of infectious virus was obtained from the medium. Freezing and thawing was necessary to disrupt cells further for marginal recovery of progeny virus with titers between 10^7 and 10^8 pfu/ml.

Characterization of BVDV

Infectious virus had a density of 1.12 g/ml as measured by isopycnic banding in a sucrose density gradient and appeared as 45- to 55-nm diameter spherical particles by electron microscopy (Fig. 1A, B). The virus preparations were neutralized by several bovine anti-BVDV antisera, kindly provided to us by M. Lobman (RIT-Smith & Kline, Rixensart, Belgium) and B. Lansival (Institut Provincial Veterinaire, Marloie, Belgium). Table 1 shows data from a typical virus neutralization experiment performed with the Marloie antiserum. Complete neutralization could be obtained only with relatively low dilution of the serum, but some neutralizing activity was evident at dilutions as high as 1:500. From these neutralization tests we conclude that the high-titer virus obtained by passage of the biologically cloned Osloss BVDV strain in BEK-1 cells is serologically indistinguishable from BVDV field isolates.

Intracellular RNA synthesis during the infection

To investigate the properties of virus-specific RNA present in the high-titer virus population, we monitored intracellular RNA synthesis at intervals during BVDV infection. BEK-1 cells were infected with BVDV at a multiplicity of infection greater than 1. After 12, 15, 18, and 21 hr, newly synthesized RNA was pulse-labeled with [^{3}H]uridine for 30 min before extraction. As a control, uninfected BEK-1 cellular RNA, identically pulse-labeled and harvested at 18 hr following mock infection of cells, was also analyzed. Infected and uninfected cellular RNAs were fractionated on 0.9% agarose gels. As shown in Fig. 2, at each postinfection period analyzed, a virus-specific RNA band of approximately 12.5 kb, not present in uninfected cells, could be observed. No subgenomic length RNA was detected in this experiment.

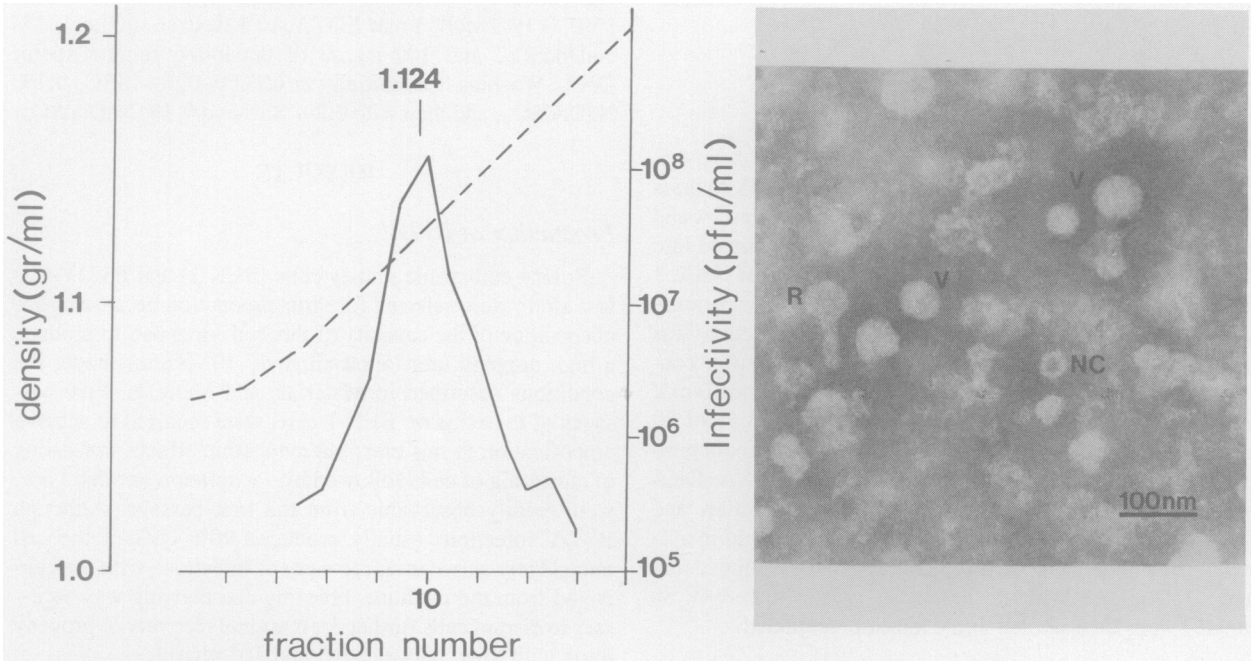

FIG. 1. A. Purification of BVD virus by isopycnic centrifugation. Two milliliters of concentrated virus (see Material and Methods) were loaded on a linear sucrese gradient (15–45%). The sample was centrifuged 15 hr at 27,000 rpm in a Spinco SW28 rotor. Twenty one fractions were collected and individually titered for virus infectivity. B. Electron micrograph of purified BVDV preparations. V, Typical virus particles; N, permeabilized virions with visible nucleocapsid; R, raquet forms.

TABLE 1. SERUM NEUTRALIZATION OF BVDV PREPARATIONS

Antiserum Dilution	*pfu/ml*
1/16	80
1/128	479
1/512	5,200
1/1024	35,000

Medium (100 μl) containing 5×10^3 infectious virions was incubated for 1 hr at 37°C with 100 μl of calf antiserum preabsorbed on BEK-1 uninfected cell extract. At the end of the incubation, the treated samples were inoculated onto subconfluent BEK-1 cultures for plaque titration assays.

Characterization of the viral RNA

To verify that the 12.5-kb RNA found exclusively in infected cells was also associated with viral particles, we pelleted virions from infected cell lysates and purified total RNA from these pellets as described in Materials and Methods. RNA obtained from pelleted virions was labeled at the 3′-terminus with [^{32}P]-pCp and RNA ligase and analyzed by agarose gel electrophoresis in the presence of 2.2 *M* formaldehyde (Fig. 3). Most of the radioactivity was associated with low-molecular-weight RNA (<2 kb), but a high-molecular-weight band of approximately 12.5 kb was consistently observed. Its rapid labeling kinetics with RNA ligase provided an initial indication that the 12.5-kb band was in fact RNA, and not contaminating DNA derived from lysed cells. To confirm this conclusion a sample was treated with either RNase A or alkali before electrophoretic analysis. In both cases the 12.5-kb band was completely eliminated. Treatment with either DNase or Proteinase K did not affect the migration properties of the 12.5-kb material (data not shown).

As indicated in Fig. 3, the BVDV RNA band represents a minor proportion of the total RNA extracted from virions. In a first set of experiments, we attempted to enrich the genomic viral RNA by chromatography on oligo(dT)-cellulose. BVDV RNA failed to bind to the column and was quantitatively recovered in the flow-through fractions together with the majority of the contaminating low-molecular-weight RNA. The same result was obtained with pulse-labeled intracellular viral RNA (data not shown).

As an alternative purification method, we then used sucrose velocity gradient sedimentation to fractionate BVDV genomic RNA. As shown in Fig. 4, the 12.5-kb RNA was readily separated from smaller RNA species, yielding a final pool of BVDV RNA estimated to be 50% pure. (This estimate is based on the percentage of total incorporation of labeled pCp present in BVDV 12.5-kb RNA.) The final purified BVDV RNA pools obtained from five sucrose gradients contained approximately 10 μg of total RNA, or 5 μg of BVDV-specific RNA.

Preparation of BVDV-specific probes

As indicated above, our partially purified BVDV genomic

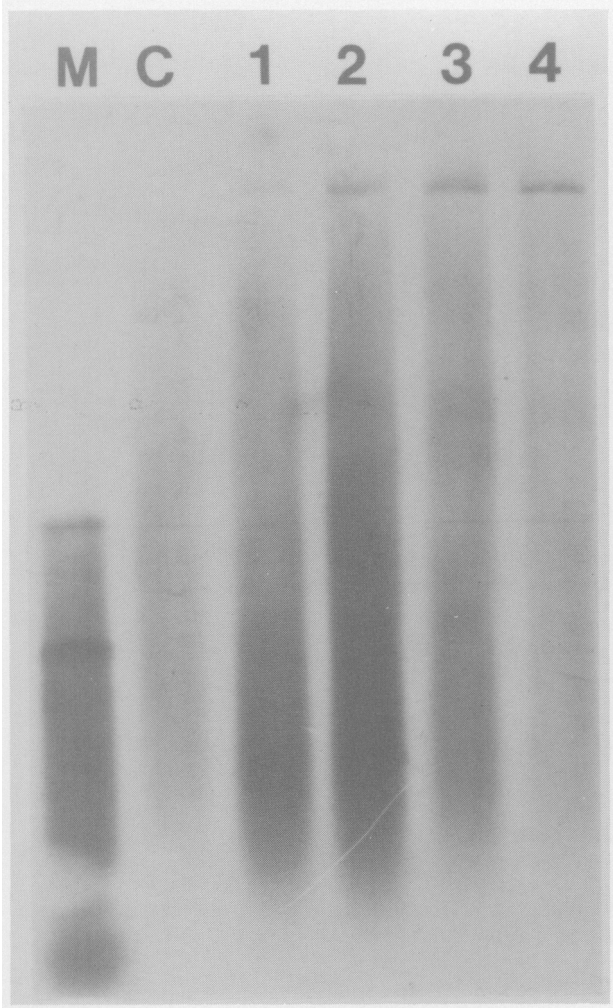

FIG. 2. Intracellular BVDV RNA synthesis. Newly synthesized viral RNA, pulse-labeled with [^{3}H]uridine, was analyzed on 0.9% agarose gels in the presence of 2.2 *M* formaldehyde. Lane M, *E. coli* 16S and 23S ribosomal RNA. Lanes 1–4, RNA from infected cells at 12, 15, 18, and 21 hr after infection, respectively. Lane C, Uninfected cell RNA at 18 hr.

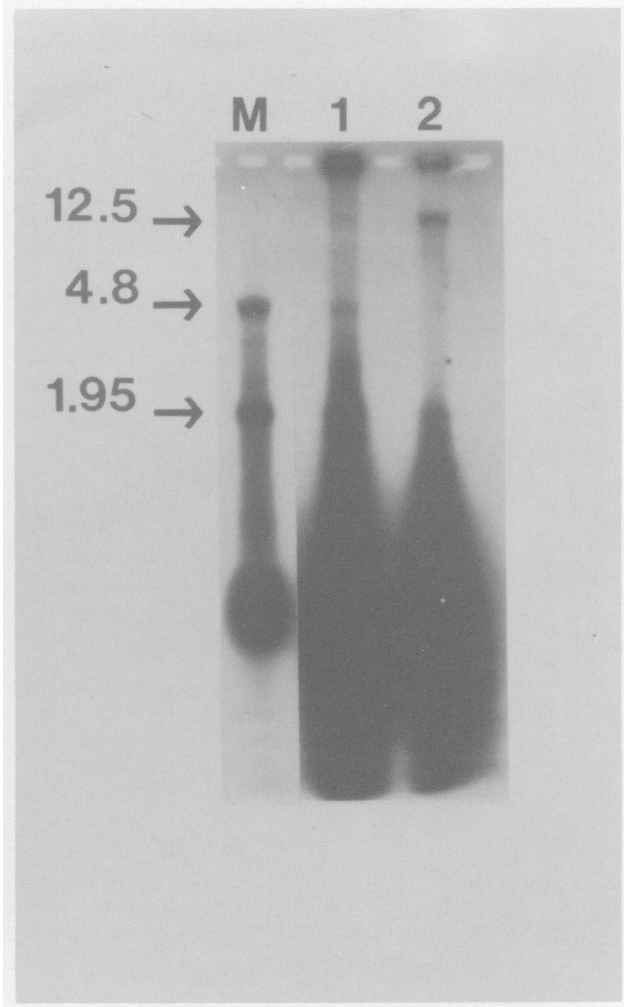

FIG. 3. Agarose gel electrophoresis of viral RNA preparations. RNA isolated from a virus pellet was labeled with pCp using RNA ligase and electrophoresed on a 0.9% agarose gel in the presence of 2.2 *M* formaldehyde. Lane M contains labeled 18S and 28S ribosomal RNAs. Lanes 1 and 2 contain RNA from a low (10^7 pfu/ml) and high-titer virus preparation (10^8 pfu/ml), respectively.

RNA samples were substantially contaminated with cellular RNA, presumably random cellular sequences, *i.e.*, ribosomal RNA, pelleted during the virus concentration step. To prepare representative probes which would preferentially hybridize to BVDV sequences (BVDV-specific cDNA), random cDNA transcripts of sucrose gradient-purified BVDV genomic RNA were synthesized. This cDNA was first prehybridized to an excess of uninfected cellular RNA and the resultant unfractionated RNA: cDNA mixture then hybridized to a nitrocellulose filter on which uninfected BEK-1 cell RNA, infected cell RNA, control yeast tRNA, and template BVDV genomic RNA had been spotted.

The results of this experiment are presented in Fig. 5. Some residual hybridization to uninfected RNA (dot 1) could be detected but a visibly stronger signal was obtained with infected cell RNA (dot 2) and template BVDV RNA (dot 4). No hybridization was observed with the yeast tRNA control (dot 3). The difference in signal obtained in the hybridization to infected *versus* uninfected cells provided evidence that our BVDV-specific probe was adequate for screening of a recombinant cDNA library. Virus-negative cDNA probes (representative of total cellular RNA sequences) were prepared by reverse transcription of total uninfected BEK-1 cell RNA.

Attempts to polyadenylate BVDV RNA

A specific priming site at the 3′ terminus of the BVDV RNA was desired for use during complementary DNA synthesis and DNA cloning experiments. Our preliminary characterization of BVDV RNA using oligo(dT) binding suggested that the virus genome lacked a detectable poly(A) tract. While

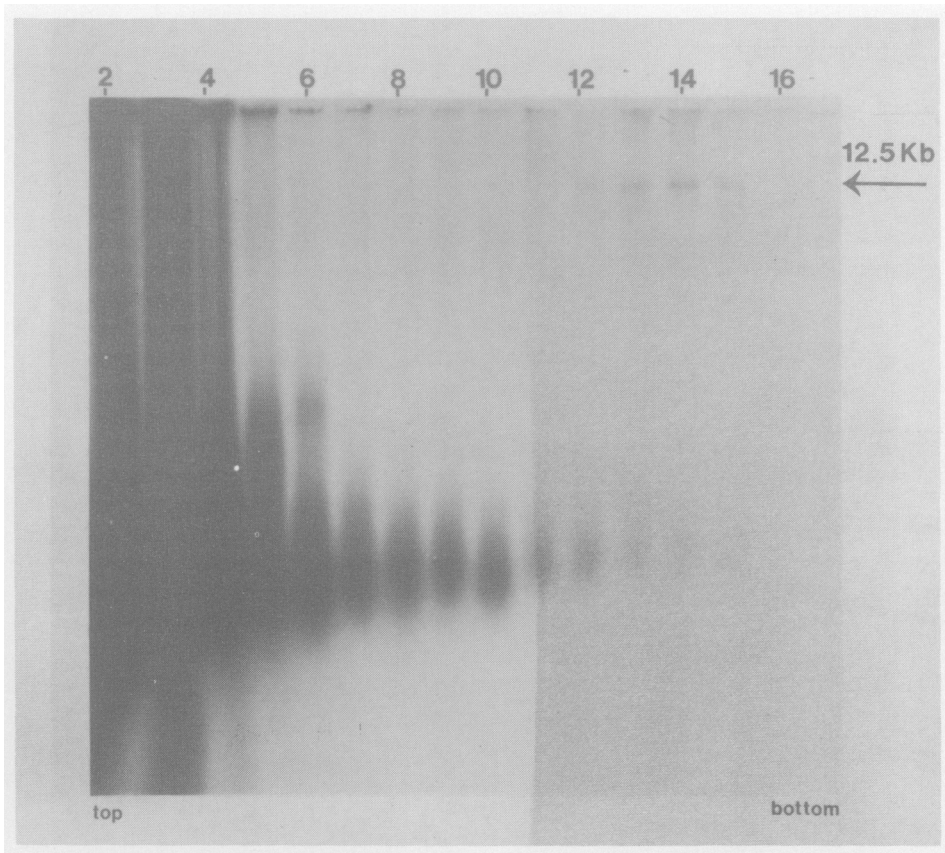

FIG. 4. Purification of BVDV RNA by centrifugation on sucrose gradients. The purification was carried out as described in Materials and Methods. Lanes 2–16, Fraction number, top to bottom; the arrow indicates the position of 12.5-kb material.

these studies did not exclude the presence of a very short oligo(A) tail, it seemed prudent to attempt the addition of new A residues.

Polyadenylation reactions were carried out in the presence of *E. coli* poly(A) polymerase in molar excess, using both poliovirus RNA and ribosomal RNA as positive controls. Polyadenylation reactions proceeded poorly under the standard conditions described by Sippel (1973); preincubation of the RNA with 10 m*M* methylmercury hydroxide was found to be essential for extensive polyadenylation with all of our RNA samples. Under these conditions both control samples and total BVDV RNA were efficiently labeled *in vitro* with [^{3}H]ATP; however, after displaying the RNAs on denaturing agarose gels, no polyadenylation of the full-length 12.5-kb BVDV RNA was detected by fluorography/autoradiography (data not shown). Probably the 12.5-kb BVDV RNA is susceptible to trace nucleases which do not affect poliovirus RNA. Alternately, the 3′ end of BVDV RNA may be inaccessible to the poly(A) polymerase (but available to RNA ligase). Further experiments are necessary to resolve this issue.

Cloning of BVDV-specific cDNA sequences

Although we were unable to resolve some uncertainties generated by the polyadenylation experiments we proceeded to prepare cDNA libraries using oligo(dT) primers in the synthetic step with *in vitro* polyadenylated BVDV RNA as substrate (previously partially purified on sucrose gradients, see Fig. 4).

Approximately 0.7 μg of genomic BVDV RNA was used in a single reaction under conditions selected to obtain optimal cDNA elongation (see Materials and Methods). The resulting cDNA:RNA hybrids were treated with RNase A to remove free RNA and purified on a Sepharose 4BCL column to eliminate relatively short hybrid molecules (<800 bp). Cloning in *E. coli* was performed after tailing of the RNA:cDNA hybrids with dC and annealing to plasmid pBR322 tailed with dG at the *Pst* I site.

Screening of the clones was performed on replicates with either virus-negative or BVDV-specific (prehybridized as above) ^{32}P-labeled cDNAs as probe. Colonies which gave a clear signal with the BVDV-specific probe but no response with the virus-negative probe were selected. By this method, 95 positive clones from a library of 2900 clones were obtained. The length of the inserts in these clones as determined by electrophoresis after *Pst* I digestion varied from approximately 500 to 1500 bp. No full-length virus-specific cDNA clones were recovered.

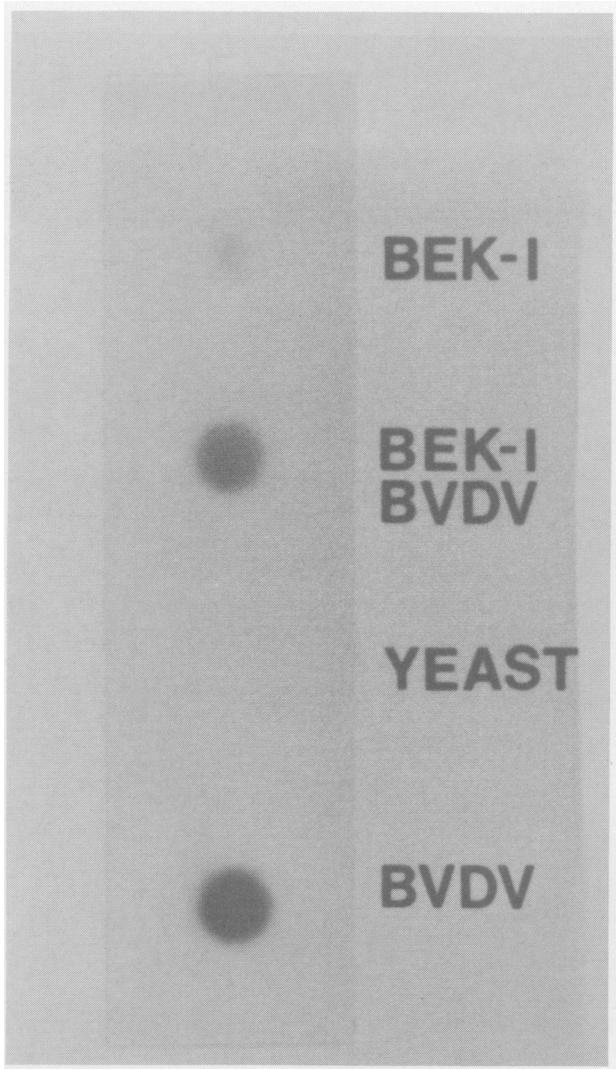

FIG. 5. RNA dot blot hybridizations with a BVDV-specific cDNA probe. One microgram of each indicated RNA sample was spotted on a nitrocellulose strip which was then hybridized with the BVDV-specific cDNA probe as described in the text.

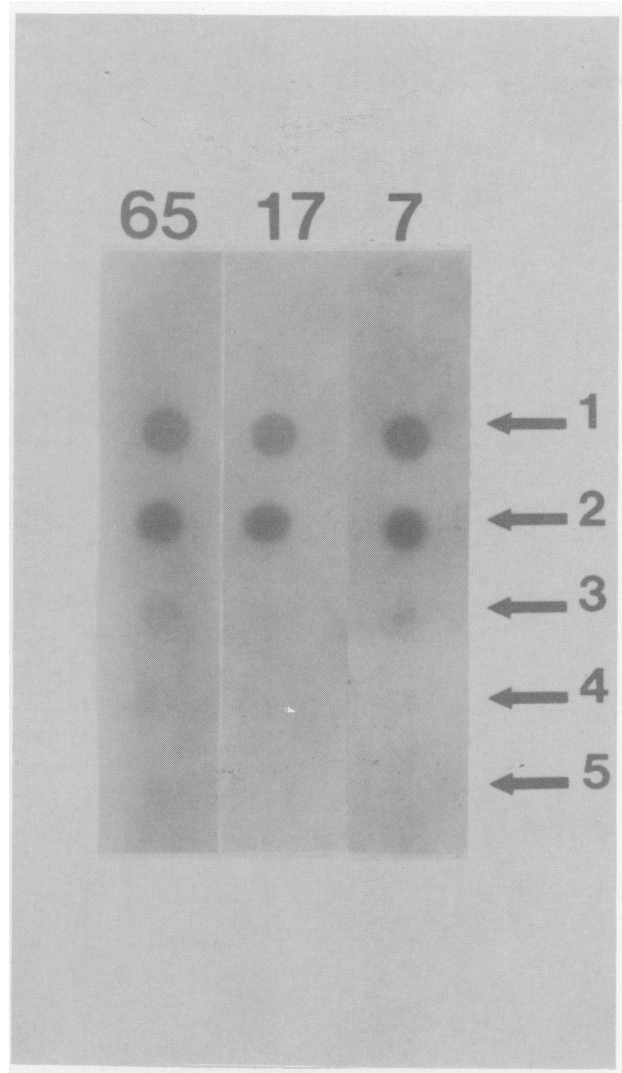

FIG. 6. RNA dot blot hybridizations with BVDV-specific clones. Twenty nanograms of partially purified BVDV genomic RNA (1), 4 μg each of RNA isolated from BVDV-infected BEK-1 cells (2), uninfected BEK-1 cells (3), BHK21 cells infected with Sindbis virus (4), and 4 μg of yeast tRNA (5) were spotted on nitrocellulose strips which were then hybridized with probes prepared from the inserts to plasmids pDT65, pDTH17 and pDT7, as described in Materials and Methods.

Characterization of BVDV-Specific cDNA clones

Our principal criteria for the initial identification of BVDV genomic RNA were its size (12.5 kb), presence in infected cells, and absence from uninfected cells. Thus, clones derived from genomic BVDV RNA should (i) hybridize back to infected cell RNA (but not uninfected cell RNA), and (ii) similarly react with the 12.5-kb genomic RNA band present exclusively in and isolated from infected cells.

For this second step in the identification of recombinant cDNA clones representing BVDV genomic sequences, we subscreened a set of clones, designated pDT7, 17, 28, 32, 39, 40, 65, and 87. Each of these clones had given distinct positive signals in the initial screening and were subsequently shown to contain different size inserts by restriction endonuclease analysis. Individual plasmid inserts were labeled *in vitro* as described in Materials and Methods, and hybridized to nitrocellulose strips spotted with control yeast tRNA, Sindbis virus-infected BHK21 cell RNA, uninfected BEK-1 cell RNA, BVDV-infected BEK-1 cell RNA, and template BVDV RNA (isolated genome). The hybridization observed was limited to BVDV-containing RNA samples only, thus confirming the specificity of our screening procedure; Fig. 6 shows three representative strips with inserts from plasmids pDT7, 17, and 65 as probes.

To identify the RNA species in infected cells which hybridized to labeled, cloned cDNAs, RNA gel hybridizations

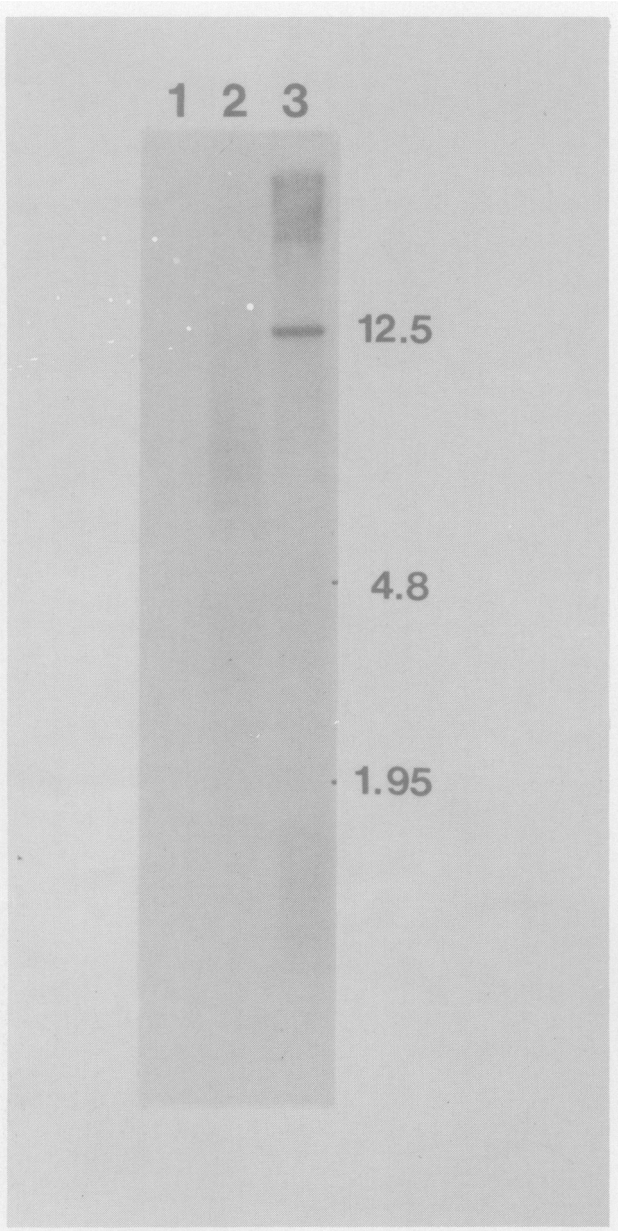

FIG. 7. RNA gel hybridization with BVDV-specific clones. RNA gel hybridizations were performed according to Smiley *et al.* (1983) with a probe prepared from plasmid pDT28 insert DNA. Lanes 1 and 2 contained 10 μg of RNA isolated from uninfected and BVDV-infected BEK-1 cells, respectively, and lane 3, 20 ng of partially purified BVDV genomic RNA.

FIG. 8. Southern blot of cellular genomic DNA with a BVDV-specific clone. DNA (10 μg) isolated from (uninfected) BEK-1 cells was digested with *Eco* RI and electrophoresed on 1% agarose. The DNA was blotted onto nitrocellulose and the blot hybridized with a probe prepared from a plasmid containing bovine growth hormone cDNA (lane bgh) or the insert from pCT185 plasmid DNA (lane 185).

were performed. Fig.7 shows a typical result obtained with the labeled insert from clone pDT28. Lane 1, containing uninfected cell RNA, gave a completely blank pattern; lane 2, containing infected cell RNA, exhibits a faint band at 12.5 kb, while in lane 3, containing partially purified BVDV genomic RNA, a distinct 12.5-kb RNA band is observed.

As a final control for the viral origin of the clones, we hybridized pCT185, a clone from a separate cDNA library overlapping pDT 40 (see Fig. 9, group 2, below) to bovine genomic DNA. Since BVDV sequences are believed to be unrelated to host cell DNA, no hybridization should be observed with clones of viral origin; a bovine growth hormone cDNA clone (Miller *et al.*, 1980) was used as a positive control. As shown in Fig. 8, lane B, the viral cDNA clone insert did not hybridize to bovine DNA, while the growth hormone cDNA probe (lane A) identified a band of ~4 kb as expected (Keshet *et al.*, 1981).

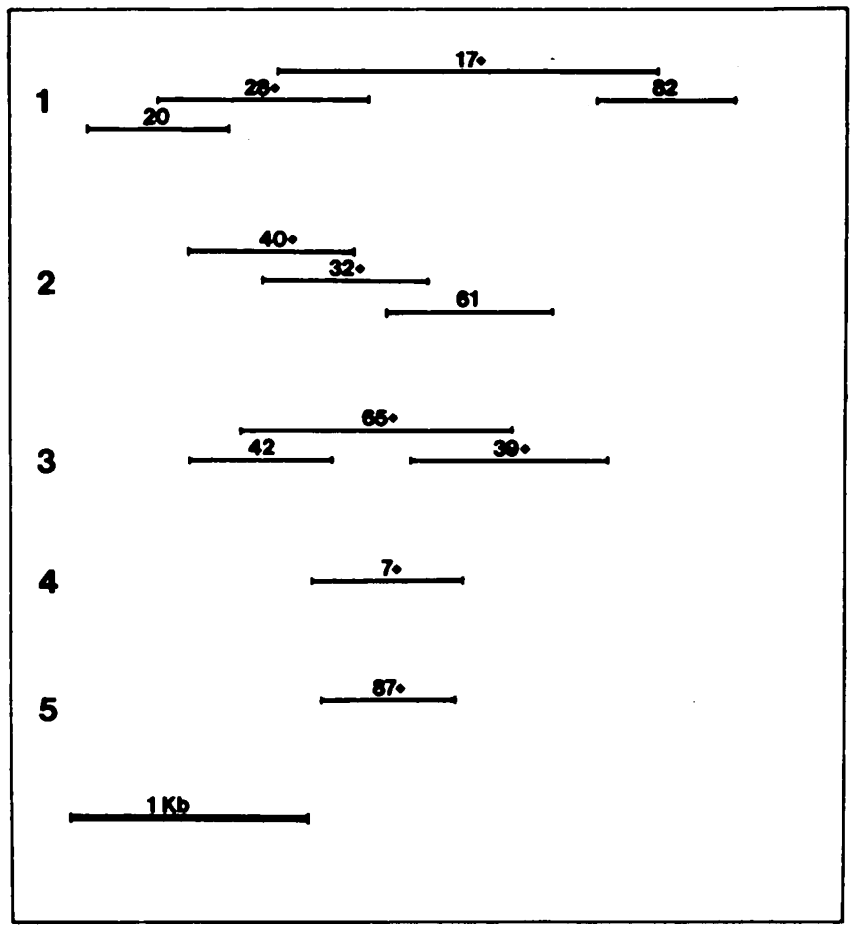

FIG. 9. Mapping of BVDV-specific clones. The five, nonoverlapping (designated 1–5) families were deduced from the hybridization experiments described in the text. The star indicates clones used as probes. Overlaps within families are approximate and based on restriction mapping and hybridization intensities.

Mapping of BVDV-specific clones

To map the 95 pDT clones relative to one another, we used Southern blots of Pst I-digested plasmid DNAs fractionated on agarose gel. These blots were hybridized with the eight probes described previously (labeled inserts from plasmids pDT7, 17, 28, 32, 39, 40, 65, and 87). These hybridizations allowed us to classify the 95 clones into five nonoverlapping families (Fig. 9) which account for at least 6 kb of the 12.5-kb BVDV genome. Some clones of the pDT series did not react with any of the eight probes employed and thus comprise additional portion(s) of the BVDV genome outside the region represented by the five family groups.

DISCUSSION

The molecular structure of BVDV has been difficult to analyze due to the lack of a good *in vitro* virus production system which yields adequate material for further study. In this paper we describe a new cell-virus strain pair, Osloss strain of BVDV and BEK-1 cells, which allowed us to produce consistently virus at high titers (10^7 to 10^8 pfu/ml) not previously reported (Horzinek, 1981). We have excluded the possibility that another contaminant virus has been isolated because the virus described here was biologically cloned and is completely neutralized by sera from BVDV infected calves. In addition this virus exhibits all the characteristics previously reported for BVDV: a small enveloped virion diameter 45–55 nm) of density of 1.12 g/cm^3 containing a single-stranded RNA genome of ~12.5 kb (Horzinek, 1981).

Partial purification of sucrose gradients of the BVDV RNA isolated from a virus pellet permitted an initial molecular characterization of the BVDV genome. Based on its nonretention on an oligo(dT) column, the viral RNA appears not to be polyadenylated. Also, no subgenomic-length viral RNAs were detected in infected cells. It should be noted that these properties (absence of polyadenylation and of subgenomic RNA species) have been reported previously for other togaviruses, in particular flaviviruses, (Schlesinger, 1980; Wengler and Wengler, 1981). Our results agree in general with the literature on BVDV but are at variance with

the data of Purchio *et al.* (1983) who have reported an 8.2-kb genomic RNA. This difference in the molecular weight of the BVDV genome might be related to strain differences or to the fact that these authors may have isolated a noncytopathogenic strain of BVDV, which has been reported to be present in the MDBK cells used for their study (Nuttall, 1978).

We have attempted to polyadenylate the purified genomic RNA *in vitro* with ATP and *E. coli* poly(A) polymerase. Whereas this technique worked well with control poliovirus RNA and ribosomal RNAs, it did not proceed with intact BVDV RNA as judged by (i) the absence of labeled BVDV RNA band after the enzymatic reaction with labeled ATP, (ii) the appearance of lower-molecular-weight products which may indicate a degradation of the viral RNA during the reaction, and (iii) the fact that the clones we obtained after oligo(dT) priming of this putatively polyadenylated RNA can be classified in different families covering dispersed parts of the genome indicative of random initiation. In contrast, RNA ligase was successfully employed to 3′ label BVDV genomic RNA, but we have not investigated the reasons for this difference in behavior between the enzyme-catalyzed polyadenylation and pCp ligation reactions; a possible explanation is the presence of a 3′-phosphate which blocks poly(A) polymerase activity but not RNA ligase (Krug and Uhlenbeck, 1982).

Finally and most importantly, we describe in this paper the first successful molecular cloning of BVDV sequences. Ninety-five virus-specific clones, containing BVDV sequences clustered in five, nonoverlapping families accounting for at least half the viral genome, were obtained. Identification of virus-specific clones relies on hybridization to the genomic viral RNA present in infected cells and on the absence of hybridization to either uninfected cellular RNA or with *Eco* RI-digested bovine genomic DNA. This successful initial cloning should provide the route to a complete molecular characterization of the BVDV genome and the identification and expression of the virus-coded proteins.

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