Molecular Cloning of Complementary DNA from a Pneumopathic Strain of Bovine Viral Diarrhea Virus and its Diagnostic Application

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ABSTRACT

A pneumopathic strain of bovine viral diarrhea virus was grown in cell culture and purified. Genomic ribonucleic acid was extracted, polyadenylated at the 3′ end, and copied into complementary DNA after oligo-dT priming. Complementary DNA was made double stranded and cloned into the pUC9 plasmid. Approximately 200 complementary DNA clones varying in length from 0.5 to 2.5 kilobases were obtained. Hybridization assays indicated that the sequences isolated were specific for bovine viral diarrhea virus and that at least 5.5 kilobases of bovine viral diarrhea virus genome was represented in the library of complementary DNA clones, the majority of which may have originated from the 3′ end of the virus genome. One cloned complementary DNA sequence was used as a ^{32}P-labelled hybridization probe for bovine viral diarrhea virus detection. The probe hybridized with all cytopathic and noncytopathic strains of bovine viral diarrhea virus tested and was 100 times more sensitive than infectivity assays for the detection of bovine viral diarrhea virus. Hybridization did not occur with nucleic acids from bovine coronavirus, bluetongue virus, bovine adenovirus or uninfected cell cultures. Native plasmid DNA sequences, labelled with ^{32}P, did not hybridize with bovine viral diarrhea virus ribonucleic acid.

RÉSUMÉ

Cette expérience consistait à faire croître sur culture cellulaire et à purifier une souche du virus de la diarrhée à virus bovine, responsable de pneumopathie. On procéda à cette fin à l'extraction de l'ARN de son génome, on lui ajouta plusieurs molécules d'adénine, à l'extrémite 3′, et on le copia en ADN complémentaire, après une amorce avec de l'oligo-dT. On rendit cet ADN complémentaire, bicaténaire; on en fit ensuite des clones dans le plasmide pUC9. Ce procédé donna environ 200 clones d'ADN complémentaire dont la longueur variait de 0,5 à 2,5 kilobases. Des tentatives d'hybridation démontrèrent la spécificité des séquences isolées, à l'endroit du virus de la diarrhée à virus bovine, et qu'au moins 5,5 kilobases du génome du dit virus étaient représentées dans l'ensemble des clones de l'ADN complémentaire, dont la majorité pourrait avoir originé de l'extrémité 3′ du génome du virus. On utilisa un clone d'une séquence d'ADN complémentaire, comme sonde d'hybridation marquée au P^{32}, pour la détection non virus expérimental. Cette sonde réalisa une hybridation avec toutes les souches cytopathogènes ou non du virus de la diarrhée à virus bovine éprouvées à cette fin et elle se révéla 100 fois plus sensible que les tests d'infectivité utilisés pour les détecter. Aucune hybridation ne se produisit avec les acides nucléiques du coronavirus bovin, du virus de la fièvre catarrhale du mouton, de l'adénovirus bovin ou des cultures cellulaires non infectées. Les clones des séquences d'ADN bicaténaire, marqués au P^{32}, ne réalisèrent pas d'hybridation avec l'acide ribonucléique du virus de la diarrhée à virus bovine.

INTRODUCTION

Three defined syndromes of cattle caused by bovine viral diarrhea virus (BVDV) are recognized: bovine viral diarrhea, mucosal disease (MD) and fetal disease. Most primary postnatal BVDV infections are subclinical but explosive outbreaks of BVD may occur (1,2). Bovine viral diarrhea usually is a trivial disease characterized by fever, salivation, leukopenia, and erosion of the oral mucosa (1,2). However, many primary postnatal infections, whether subclinical or not, may render animals transiently immunosuppressed (1,2). The exact clinical consequence of BVDV-induced immunosuppression depends on superimposed environmental (or managemental) stress and intercurrent infections (1,3). Evidence indicates that BVDV may be a pivotal respiratory pathogen in cattle acting as a primary infectious agent resulting in severe disease by opportunistic organisms such as *Pasteurella haemolytica* (3,4). However, BVDV strains vary in

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their capacity to impair pulmonary immunity (4,5).

Transplacental infection is a very common sequel in persistently-infected cattle or after BVDV infections in susceptible, pregnant heifers and cows (1,2). The virus is fetopathic, primarily early in pregnancy, but fetal infections may result in persistent immunotolerant infections in postnatal life. Such animals may appear to be healthy but some die prematurely, often after chronic illness, and all have the potential of developing MD (1,2). Evidence indicates that MD is precipitated by superinfection of persistently-infected, seronegative animals with a different strain of the virus and thus represents the final outcome of *in utero* infection (6-8). Another significant repercussion of *in utero* infection is that persistently-infected cattle may be the primary source of BVDV in nature (2).

The economic impact of BVDV infections is enormous and is primarily the consequence of immunological dysfunction and efficient transplacental infections (with all the diverse consequences of fetal infection) in cattle (9,10). Although much still has to be learned about the natural history of this virus, considerable progress in recent years has been made in understanding its epidemiology and pathogenesis so that rational guidelines for control of the disease are possible (9).

Since the virus is endemic in the USA, attempts to maintain a herd free of infection to the virus likely would invite disastrous economic losses (9). The aim of rational control measures should be to break the cycle of transmission by identifying (and removing) persistently-infected animals and by preventing transplacental infections (9,10). Another important objective must be prevention of BVDV infection of stressed animals (1). Effective BVDV control, therefore, would require screening of herds for persistently-infected cattle and use of effective vaccines (9).

Genetic engineering techniques hold the promise of identifying the mechanism of cross immunity to BVDV strains and to develop more effective vaccines for BVD control such as synthetic peptide vaccines and vaccines produced by the expression of cloned complementary DNA (cDNA) of various BVDV strains in suitable vectors (9). In addition, novel approaches for identifying persistently-infected animals are required since methods currently employed are inefficient, expensive and of questionable accuracy (11,12). Genetic engineering of BVDV is predicated on the cloning and sequencing of the ribonucleic acid (RNA) genomes of several strains of BVDV which will allow genetic comparison and expression of various sequences to establish functional parameters of gene products. Presently, BVDV is classified in the Togaviridae family in the pestivirus genus (13). The BVDV genome consists of positive sense, single-stranded RNA approximately 12.5 kilobases (kb) in length (13,14). Unfortunately, BVDV is among the least characterized of the animal viruses (13,15) which is primarily due to its relatively poor growth in cell cultures and susceptibility to manipulations (14,15). This report describes the development of a partial cDNA library from the genome of a field isolate of BVDV which experimentally impairs pulmonary resistance, and the use of some of the cloned sequences as hybridization probes for the sensitive and specific detection of BVDV.

MATERIALS AND METHODS

CELL CULTURES AND VIRUS

Bovine viral diarrhea virus strains used in this study included cytopathic strains (72, NADL, Singer, Oregon C24V) and noncytopathic strains (2724, 7443, New York-1). The origin of strains 72 and 2724 has been described (16); the other BVDV strains were obtained from Dr. S.R. Bolin, National Animal Disease Center, Ames, Iowa. Other viruses included were a field isolate of bluetongue virus (17), the Mebus strain of bovine coronavirus (18), and serotype III of bovine adenovirus obtained from Dr. D.E. Reed, Veterinary Medical Research Institute, Ames, Iowa. Dulbecco's MEM supplemented with 10% fetal bovine serum that had been treated with β-propiolactone (0.05% final concentration) to remove adventitious viruses was used for cell growth (unpublished data). Primary bovine turbinate cell cultures, passaged 5 to 12 times and negative for BVDV by indirect immunofluorescence, were used for propagating BVDV. All buffers and water were treated with diethyl pyrocarbonate and autoclaved.

PREPARATION OF BVDV GENOME RNA

Confluent bovine turbinate cell cultures were inoculated with BVDV strain 72 at a multiplicity of infection of 0.05 median cell culture infectious doses ($CCID_{50}$) per cell and incubated at 37°C until cytopathic effect reached 80 to 100%, usually for two to three days. The titer of progeny virus was 10^7 $CCID_{50}$ per mL. Cell culture supernatant fluids were clarified at 15,000 x *g* for 20 min at 4°C and virus was pelleted from clarified supernate by centrifugation at 100,000 x *g* for 2 h at 4°C. The viral pellet was resuspended in 0.5 mL of NTE buffer [0.5 M tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.6] containing 2.0% SDS and 0.5 mg of proteinase K and the mixture was incubated at 37°C for 30 min. Ribonucleic acid was extracted using phenol:chloroform:isoamyl alcohol [24:24:2] and precipitated by adding 0.1 volumes of 3.0 M sodium acetate and two volumes of ethanol. The concentration of RNA (redissolved in water) was determined by spotting 1 μL quantities adjacent to known amounts of transfer RNA on DE-81 fiber filters and staining with ethidium bromide (19). Some preparations of BVDV RNA were obtained from virions purified by isopycnic centrifugation. Virus from clarified cell culture supernatant fluids was first concentrated by sedimentation at 100,000 x *g* for 3 h at 4°C through 20% sucrose (wt/wt in NTE) onto a cushion of 60% sucrose (wt/wt). The virus band was collected, diluted 1:2 with NTE, layered onto an 18 mL continuous gradient of 20 to 60% [wt/wt] sucrose, and virions were sedimented to their buoyant density by centifugation at 100,000 x *g* for 14 h at 4°C. Fractions (0.5 mL) were collected and the absorbance at A260 was determined. Peak absorbance was detected in a band with a buoyant density of 1.13 g/cm^3 which was presumed to be virus on the basis of

previous studies (20-22) and infectivity (data not shown). Virus then was pelleted at 100,000 x *g* for 5 h at 4°C and RNA was extracted as previously described.

PREPARATION OF cDNA CLONES FROM BVDV GENOME RNA

Bovine viral diarrhea virus genomic RNA was polyadenylated at the 3′ end before using it for cDNA cloning. Tailing was done in a reaction volume of 100 μL which consisted of 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 0.25 mM ATP, 50 μg BSA, 10 μg BVDV RNA, and 6 units poly-A polymerase [Bethesda Research Laboratories (BRL), Bethesda, Maryland]. The reaction was carried out for 12 min at 37°C, conditions that add approximately 100 adenosine monophosphate residues to the same number of pmoles of control RNA (7.5 kb yeast transfer RNA) (BRL) (data not shown). The reaction was stopped by the addition of 2 μL of 0.5 M EDTA and heating at 60°C for 5 min. The RNA was precipitated by the addition of 0.1 volumes of 3.0 M sodium acetate and three volumes of 100% ethanol.

Complementary DNA cloning was done essentially by the method of Gubler and Hoffman using commercial reagents (Amersham Corporation, Arlington Heights, Illinois) for first and second strand DNA synthesis (23). Briefly, first strand cDNA was synthesized with reverse transcriptase using 10 μg poly-A-tailed BVDV RNA as template and oligo-$dT_{12\text{-}18}$ as primer in a reaction volume of 20 μL. Second strand cDNA synthesis was done in a reaction volume of 100 μL using all the product from first strand synthesis. No radiolabel was included in the reactions. Double-stranded cDNA (dscDNA) was homopolymer tailed at the 3′ end by the method of Roychoudhury and Wu (24). Conditions used were those that yielded an addition of 15-20 dCMP's at each end of control DNA. For tailing, DNA was incubated for 1.5 min at 37°C in a reaction volume of 50 μL that consisted of 1 X tailing buffer (0.1 M potassium cacodylate, 0.025 M Tris base, 1.0 mM $CoCl_2$, and 0.2 mM DTT), 10 mM dCTP, and 30 units of terminal deoxytransferase (Pharmacia Inc., Piscataway, New Jersey). The reaction was stopped by adding 5 μL of 0.5 M EDTA and heating at 68°C for 5 min, and the product was precipitated with ethanol.

C-tailed dscDNA was annealed to *Pst* I-digested, G-tailed pUC9 plasmid (Amersham Corp., Arlington Heights, Illinois) in a reaction volume of 30 μL consisting of annealing buffer (10 mM Tris-HCl, 1 mM EDTA, and 0.15 M NaCl), 1-5 ng C-tailed BVDV dscDNA, and 15 ng *Pst* I-digested, G-tailed pUC9 plasmid. The mixture was incubated at 58°C for 3 h and then gradually cooled to 4°C over 1 h. *Escherichia coli* cells, strain JM-83, were transformed by the method of Hanahan (25) and white colonies obtained on agar containing ampicillin, X-gal, and IPTG, were selected and screened by colony hybridization using the method of Maniatis *et al* (19,25). The bacterial colonies were transferred to nitrocellulose, lysed and probed with a single-stranded BVDV cDNA (produced by first-strand cDNA synthesis using purified genomic BVDV RNA as a template and random primers). Primer was used at five times the molar concentration of BVDV RNA (26) and probe was synthesized in the presence of alpha $dCT^{32}P$, $dAT^{32}P$ and $dGT^{32}P$. The final wash following colony hybridization was done with 0.1 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M trisodium citrate [pH 7.0]) and 0.5% SDS for 30 min at 68°C. The approximate size of the cloned insert was initially determined by agarose gel electrophoresis of supercoiled plasmids. Recombinant plasmids migrating slower than parental supercoiled pUC9 were digested with *Pst* I (or *Hin*D III and *Bam*H I) restriction endonuclease and the released inserts were isolated by electrophoresis and electroelution (19). Size of cloned cDNA sequences was determined by comparison after electrophoresis with a 123-base pair ladder standard (BRL). Cross hybridization of Southern blots was done with selected purified BVDV cDNA sequences to establish relationships among the cDNA clones isolated.

PREPARATION OF cDNA PROBES

Purified cloned cDNA sequences were labelled with ^{32}P to a specific activity of 1-4 x 10^8 cpm/μg by nick translation as described by Rigby *et al* (27). Labelled DNA was separated from unincorporated nucleotides by gel filtration through Sephadex G-50 (19). Native pUC9 plasmid DNA was labelled also by nick translation to serve as a control in hybridization assays. Prior to hybridization, probe was heated for 5 min at 100°C and chilled on ice.

HYBRIDIZATION ASSAY

Blots for DNA hybridization were prepared, hybridized and washed following hybridization as described by Maniatis *et al* (19). Southern blots were prepared from agarose electrophoresis gels containing restriction endonuclease digests of recombinant plasmids by osmosis onto nitrocellulose sheets as described by Maniatis *et al* for cross hybridization mapping of cloned cDNA (19). After the transfer of the DNA and before hybridization the nitrocellulose filters were baked for 2-3 h at 80°C.

Hybridization of cDNA probes with BVDV RNA was done by the dot blot method as described by Shockley *et al* (28). Samples tested included various cell culture-grown viruses, uninfected cell culture supernatant fluids, phenol-extracted whole cells, known quantities of BVDV RNA and yeast transfer RNA. Specimens were clarified by centrifugation, treated with nonidet P-40 and formaldehyde and applied to nitrocellulose in a dot-blot apparatus. Air-dried nitrocellulose sheets then were baked at 80°C for 90 min before they were placed into plastic bags with prehybridization solution at 42°C for 4-6 h with gentle agitation. The cDNA probes (10^7 cpm/mL) were denatured by boiling for 5 min followed by rapid cooling in ice and added to the hybridization solution (50% formamide). Hybridization was allowed to proceed at 42°C for 16-24 h. The hybridized filters were washed (2 X SSC and 0.1% SDS, four times for 5 min at 22°C and then 0.1 X SSC and 0.1% SDS, twice for 15 min at 50°C) and dried at room temperature on filter paper before their placement into radiographic film cassettes containing radiographic film and an intensifying screen for 24-72 h.

INFECTIVITY ASSAYS

Serial tenfold dilutions of virus were made in chilled cell culture medium and

TABLE I. Characterization[a] of 19 BVDV cDNA Clones Identified by Gel Electrophoresis after Digestion of Recombinant Plasmid DNA with Restriction Enzyme *Pst* I

Clone	Size of Inserts (kb)[b]			Total kb[c]
A	0.4	0.5		0.9
B	0.6	0.6		1.2
C	0.5	0.6		1.1
D	0.5			0.5
E	2.0			2.0
F	0.7			0.7
G	0.3	0.5	1.8	2.6
H	1.3			1.3
I	0.5	0.6	0.8	1.9
J	0.2	0.9		1.1
K	1.4			1.4
L	1.3			1.3
M	0.3	0.5	1.8	2.6
N	1.3			1.3
O	0.7	1.6		2.3
P	0.6	1.3		1.9
Q	0.6			0.6
R	0.2	0.6	1.0	1.8
S	0.1	0.5		0.6

[a]Size of fragments was estimated by comparison with a 123-base pair ladder, size standard
[b]Fragments produced by *Pst* I digestion
[c]Total size of insert in kilobases

0.1 mL aliquots of the dilutions were adsorbed onto secondary bovine turbinate cells in 96-well cell culture plates for 1 h at 37°C. The virus dilutions were removed and replaced with medium. The cells were incubated at 37°C in a 5% CO_2 atmosphere and examined daily with an inverted microscope. The highest dilution of virus which resulted in cytopathic effects in two of four cell cultures was considered to be the median infectious dose ($CCID_{50}$). Infectivity endpoints of noncytopathic viruses were determined by immunofluorescence (5).

RESULTS

Strain 72 of BVDV virus grew to titers of approximately 10^7 $CCID_{50}$ per mL. Purified virions were obtained by isopycnic density gradient centrifugation from fractions corresponding to a specific gravity of 1.128 to 1.138. Approximately 250 μg BVDV RNA was extracted from BVDV purified from 500 mL of infected cell culture fluids.

Approximately 5,000 colonies of ampicillin-resistant *E. coli* with disrupted lac-Z gene, indicating probable transfection with recombinant plasmids, were obtained. The transformation efficiency of the bacteria with plasmid DNA was 5.0 x 10^7 cells per μg of plasmid DNA. Approximately 200 bacterial colonies had strong hybridization signals with a random-primed, first-strand cDNA probe and were subcultured for further analysis. Forty-seven of the bacterial isolates yielded recombinant plasmids with cDNA inserts longer than 0.5 kilobases (kb) whereas 67 had plasmids with sequences from 100 basepairs (bp) to 500 bp. *Pst* I restriction endonuclease digestion of 19 selected recombinant plasmids yielded inserts that ranged from 0.5 to 2.6 kb in length (Table I). Eleven cloned cDNA sequences contained one or two internal *Pst* I restriction endonuclease cleavage sites since digestion generated two to three distinct fragments. Intact sequences were obtained after *Hin*D III and *Bam*H I digestion.

Cross hybridization of Southern blots under conditions of high stringency with probes prepared from three cDNA clones (C, O and I), identified three separate regions of the genome (Table II). The library of clones represented at least 5.3 kb or 42.4% of the total viral genome (12.5 kb) since the three probes represented areas that did not overlap and their combined size was 5.3 kb. As indicated in Table II, several cDNA clones were identified which represented connecting regions between those represented by the three probes and areas also not represented by the three probes. Complementary DNA clone C hybridized with seven, clone O hybridized with nine and clone I hybridized with four of 19 other sequences isolated (Table II).

Cloned cDNA sequences, identified as clones B, C, E and I, were obtained from recombinant plasmids which had strong signals by colony hybridization with random-primed, single-strand cDNA probe. Probe made from clones B, C, E and I hybridized with all cytopathic and noncytopathic BVDV strains tested (Fig. 1) but not with bluetongue virus, bovine coronavirus or bovine adenovirus (even when samples contained more than 50 ng of RNA or DNA). Probe from clone C hybridized with purified BVDV RNA but not with 100 ng samples of cellular nucleic acids (uninfected cells) or yeast transfer RNA (Fig. 2). Probes prepared from native pUC9 plasmid DNA did not hybridize with any of the samples tested.

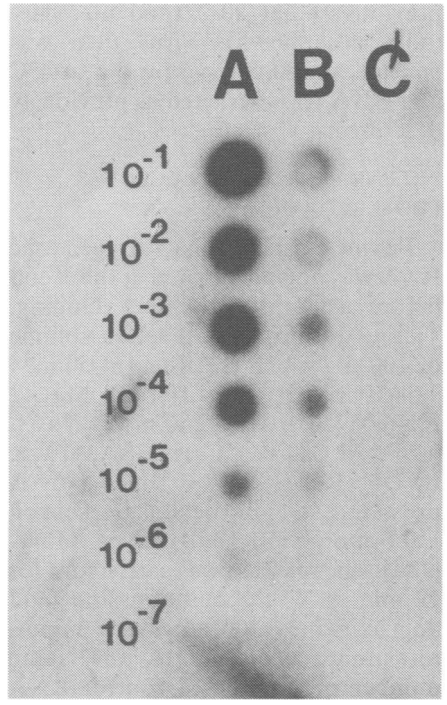

Fig. 1. Dot blots of tenfold dilutions of infected cell culture supernates of cytopathic BVDV strain 72, 1.0 x 10^6 $CCID_{50}$ (column A); noncytopathic BVDV strain 2724, 1.0 x 10^5 $CCID_{50}$ (column B); and uninfected cell culture (column C). Dot blots were hybridized with clone C. The BVDV strains were detected to a dilution of 1.0 x 10^{-7} and 1.0 x 10^{-6}, respectively. Hybridization signals were not detected with uninfected cell culture material.

The detection limit of hybridization assay of 0.1 mL samples with the probe of clone C was at the 1.0 x 10^7 dilution of cell culture fluid infected with either BVDV 72 or BVDV 2724 whereas the infectivity titers ($CCID_{50}$) of these materials were 10^5 and 10^4 per 0.1 mL, respectively (Fig. 1). As little as 10-20 pg of purified BVDV RNA resulted in detectable hybridization signals with probe C (Fig. 2).

DISCUSSION

Sufficient purified virions of BVDV strain 72 were obtained to extract genomic RNA for polyadenylation and cDNA synthesis. This allowed the cloning of a large BVDV-specific genomic cDNA library which represented at least 45% of the entire genome (12.5 kb) (14). Several probes were made from some of the cloned

TABLE II. Results of Cross Hybridization Mapping of 19 BVDV cDNA Clones with Probes Prepared from Three BVDV cDNA Clones Labelled with dCT^{32}P by Nick Translation

Clones	Size (kb)	Reaction with Hybridization Probe		
		C	O	I
A	1.0	+		
B	1.2	+		
C	1.1	+		
J	1.2	+	+	
P	1.6	+	+	
Q	0.6	+	+	
G	2.6		+	
H	1.3		+	
O	2.3		+	
F	0.7		+	+
K	1.4		+	+
M	2.65		+	+
I	1.9			+
D	1.4			
E	2.0			
L	0.8			
N	1.4			
R	1.9			
S	0.5			

DNA from clones D, E, L, N, R and S did not hybridize with any of the probes (C, O and I)

sequences which hybridized with all the BVDV strains tested but not with other virus or cellular nucleic acids and these allowed a limited cross-hybridization analysis.

The major problems encountered in the cloning of BVDV were difficulties in obtaining sufficient amounts of purified BVDV RNA to use as a template for first-strand cDNA synthesis and the absence of 3′ polyadenylation of the genome (14,22). Several methods of BVDV purification have been reported in the literature but difficulties were encountered in obtaining adequately purified virus (14,20,29-31). Bovine viral diarrhea virions may be highly cell associated (32) and tend to copurify with cell vesicles and membranes (14,31). Successful purification of a BVDV strain may be dependent on the capacity of the virus to replicate to high titer in cell culture (14).

In this study, gradient-purified virus had a density of 1.128 to 1.139 g/cm^3) which corresponded to some previously-reported values (20,21,33). However, various values for the density of BVD virions, ranging from 1.12 to 1.174 g/cm^3, have been reported (20,21,33,34). Infectivity, density, and absorbance of fractions, collected after centrifugation in sucrose gradients, were determined and compared to identify the location of purified virions and to obtain the accurate density value of BVDV.

Initially random primer-initiated cDNA synthesis was attempted but inadequate amounts of dscDNA were obtained for cloning (data not shown) and therefore genomic RNA was polyadenylated prior to first-strand synthesis to allow the use of oligo-dT primers which resulted in adequate amounts of dscDNA to proceed further with cDNA cloning. The cloned BVDV sequences obtained, which ranged from less than 0.1 kb to 2.6 kb in length, were consistent with cDNA cloning results of others using the methods of Gubler and Hoffman which usually have resulted in clones averaging approximately 2.0 kb in length (23,35).

It was necessary to characterize the recombinant plasmids by several parameters to ensure that the cDNA clones obtained represented BVDV genomic sequences and not contaminating cellular RNA or DNA. Colony hybridization with first-strand cDNA synthesized from purified BVDV RNA indicated that the identified bacteria contained BVDV cDNA sequences but the presence of traces of cellular DNA contaminating the purified virus preparation still could have hybridized with plasmids containing cellular DNA. Hybridization of probes, prepared by nick translation of cDNA from several recombinant plasmids, with BVDV-infected cell cultures and with RNA extracted from isopycnic density gradient-purified BVDV and the lack of hybridization with uninfected cell culture extracts (which included dot blots of cell culture supernates and of phenol-extracted, formalin-denatured whole cells) confirmed that the DNA sequences identified by colony screening were indeed BVDV cDNA sequences. It was important to establish that the cloned cDNA sequences were virus-specific since some putative viral cDNA probes have hybridized with cellular nucleic acids (36). Additional proof that the cloned cDNA sequences were BVD-specific was that probe prepared from native pUC9 plasmid did not hybridize with uninfected cell cultures or BVDV-infected cell cultures. Further evidence of the specificity of the recombinant BVDV cDNA sequences was that cross hybridization of these sequences with other bovine RNA and DNA viruses tested did not occur.

Theoretically, by the addition of a 3′ poly-A tail to the BVDV RNA template and use of an oligo-dT primer for first-strand cDNA synthesis, all transcripts should have had common sequences representing the 3′end of the genome. Mapping by cross hybridization with Southern blots of a limited number of the largest clones identified at least three separate regions represented by three clones (C, I and O) and with a total size of 5.3 kb. The locations of these areas were relatively close to one another since relatively short overlapping sequences (0.6 to 0.7 kb) were identified (Table II). Although only a limited number of clones have been examined in this manner, since only the largest clones were selected, hybridization results indicated that most belonged to one of two groups (clone C and O) and a few to one small group (clone I). Thus many cDNA sequences cloned may have had a common point of origin in the BVDV genome.

Because BVDV does not contain a polyadenylated genome, the 3′end-specific polyadenylated tail cannot be used as a marker to establish orientation of the cloned cDNA sequences for a physical map of the genome. Cross hybridization indicated that 47% of the 19 cDNA sequences cloned originated within a 2.3 kb genomic segment represented by clone O

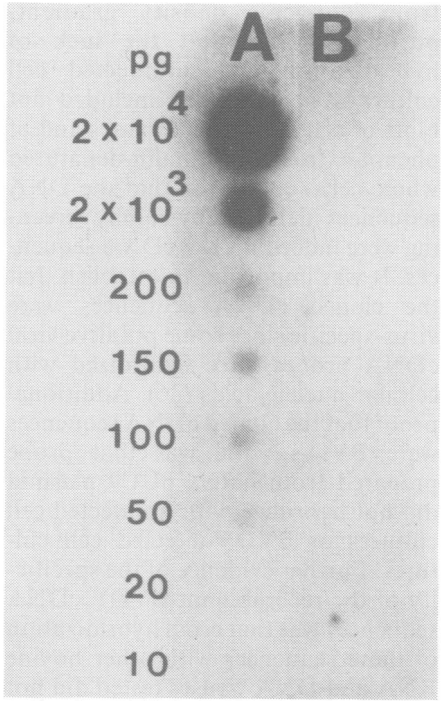

Fig. 2. Dot blots of known concentrations of BVDV (strain 72) genomic RNA (column A) and transfer RNA (column B). The BVDV genomic RNA was detected to a level of 20 pg. Blots were hybridized with cDNA from clone C.

whereas 32% originated from the area of clone C and 21% from the area of clone I. Therefore the largest number of clones may have originated from the same general area of the genome. Since an oligo-dT primer was used with the polyadenylated genomic template, and assuming that a majority of genome molecules were intact prior to polyadenylation during cDNA cloning, it may be concluded that the related clones of the largest groups originated at or near the 3′ end of the BVDV genome.

Cross hybridization indicated that cDNA clone O was located between that of C and I and because fewer sequences hybridized with the latter it was concluded tentatively that clone C originated closer to the 3′ end of the BVDV genome. Obviously not all the cDNA sequences cloned originated from the 3′ end of the genome since some cDNA probes did not cross hybridize with the majority of the cloned sequences. If the BVDV RNA was degraded by contaminating nucleases into smaller segments prior to polyadenylation and cDNA synthesis, other regions of the genome also could be represented in the cDNA library. In addition, newly synthesized cDNA sequences could have rehybridized with available regions of BVDV RNA template during first-strand cDNA synthesis and served as primers at sites closer to the 5′ end of the genome (37). It is known also that reverse transcriptase can initiate first-strand synthesis at random sites on the template RNA in the absence of a primer. These arguments support the premise that the majority of transcripts originated from the 3′ end but that some transcripts may have originated at random sites somewhat further away from this point.

The potential usefulness of cloned BVDV cDNA sequences as hybridization probes evaluated in the present study may rival that reported for other viruses (28,38,39). The sensitivity threshold for detection of hybridization signals was 10 to 20 pg of BVDV genomic RNA. This was determined by dot blot hybridization with known concentrations of RNA extracted from density gradient-purified BVD virions. This level of sensitivity was obtained with a hybridization time of 16 h and after stringent washing (low salt concentrations, 0.1 X SSC) of the hybridized dot blots before they were exposed to radiographic film for 24 to 48 h. Standard methods of measuring hybridization by autoradiography have had a detection limit of 10 to 25 pg of genomic RNA or DNA in other systems (28,39). Morace *et al* reported the detection of as little as 10 pg of hepatitis B viral DNA under similar conditions to those employed in the present study (40). In the present study, detection of BVDV RNA in cell culture fluids by hybridization with cDNA was at least as sensitive as infectivity assays. In fact, in most assays hybridization was approximately 100 times more sensitive than infectivity assays when serial dilutions of BVDV-infected cell culture fluids were examined.

Few markers which characterize BVDV strains exist with the exception of *in vitro* cytopathogenicity. Therefore hybridization studies with specific sequences from different strains may be of great value in genetic classification of BVDV strains. Hybridization probes prepared from cloned cDNA sequences were specific for BVDV strain 72. In the present study, cDNA sequences from clones B, C, E and I used as hybridization probes reacted with all the cytopathic and noncytopathic BVDV strains tested.

Use of small specific sequences as probes could result in greater specificity which may allow discrimination among closely-related viruses (41). The ideal probe would consist of sequences that are not highly conserved among strains of BVDV thereby allowing strain differentiation. Optimal specificity has been obtained by decreasing probe length to the minimal number of base pairs needed for stable hydrogen bonding (15-20 bp) but, unfortunately, probes of this size are 100-fold less sensitive than larger probes (41).

Persistently-infected animals are the major source of virus in the environment but many of these animals appear healthy and are difficult to identify. Therefore the detection of the immunotolerant, persistently-infected animals is important. The most common method of herd screening for BVDV infection is by seroconversion, but seronegative, persistently-infected animals cannot be identified in this manner. Serum virus neutralization is commonly used for identification and assaying BVDV antibodies in bovine sera. It is expensive, relatively insensitive, and laborious. Neutralizing antibody titers to homologous BVDV strains often are much higher than to heterologous strains which complicates diagnostic serology when serum virus neutralization is done (42). Many of these disadvantages exist also when virus isolation is done to identify infected animals (1).

It was anticipated that the cloning of BVDV would provide tools for the study of the pathogenesis and diagnosis of BVDV infections. One particular rewarding avenue may be that by comparison of base sequences of several strains of the virus, insight may be gained into the genetic basis which determines the biological activity of different strains of BVDV. Establishing the genetic determinants that influence cytopathogenicity, viral persistence, cross immunity, and strain interaction in disease production is necessary to adequately determine pathogenesis, prophylaxis, and control of the disease.

The cloned cDNA segments provide a mechanism to completely sequence the BVDV genome. By examining sequence data, the exact coding regions can be determined and gene expression examined.

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