Pathogenesis of Mucosal Disease: a Cytopathogenic Pestivirus Generated by an Internal Deletion

NORBERT TAUTZ,¹ HEINZ-JÜRGEN THIEL,¹ EDWARD J. DUBOVI,² AND GREGOR MEYERS^{1*}

Federal Research Centre for Virus Diseases of Animals, 72001 Tübingen, Germany,¹ and New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853²

Received 19 October 1993/Accepted 4 February 1994

Cytopathogenic bovine viral diarrhea virus (BVDV) arises by RNA recombination in animals persistently infected with noncytopathogenic BVDV. Such animals develop fatal mucosal disease. In this report, the genome of a cytopathogenic BVDV isolate, termed CP9, is characterized. CP9-infected cells contained not only viral genomic RNA of 12.3 kb but also a BVDV-specific RNA of 8 kb. cDNA cloning and sequencing revealed that the 8-kb RNA is a BVDV genome with an internal deletion of 4.3 kb. The 8-kb RNA represents the genome of a typical defective interfering particle (DI), since its replication was strictly dependent on the presence of a helper virus and strongly interfered with the replication of the helper. Cell culture experiments demonstrated that the CP9 virus stock contains two viruses, namely, a helper virus and DI9. While the helper virus alone was noncytopathogenic, the presence of the DI conferred cytopathogenicity. Expression experiments demonstrated that p80, the marker protein of cytopathogenic BVDV, is translated from the defective genome. The occurrence of this cytopathogenic DI is linked to a fatal disease in cattle.

Bovine viral diarrhea virus (BVDV), a small enveloped RNA virus, belongs to the pestiviruses. This genus, together with the genus *Flavivirus* and the hepatitis C virus group, forms the family *Flaviviridae* (61a, 62). The pestiviral genome consists of a single-stranded RNA of positive polarity with a length of about 12.3 kb (12, 16, 35, 42, 50). However, certain BVDV strains have considerably larger RNA genomes, up to 16.5 kb (38, 39, 49). The viral RNA is translated into one polyprotein of about 4,000 amino acids (aa) which is cotranslationally and posttranslationally processed into the mature viral proteins. Proteolytic processing is mediated by cellular and viral proteases (10, 11, 55, 63, 64). The first protein of the open reading frame is a nonstructural autoprotease, N^{pro} (59, 61, 63). This protease releases itself from the polyprotein, thereby generating its own C terminus. In the viral polyprotein, N^{pro} is followed by the core protein (C), the viral glycoproteins (E0, E1, and E2), and the nonstructural proteins.

Cytopathogenic BVDV (cpBVDV) and noncytopathogenic BVDV (noncpBVDV) isolates can be differentiated by their effects on tissue culture cells (reviewed in references 2 and 41). Both biotypes play an essential role in the most severe manifestation of a BVDV infection, the so-called mucosal disease (MD), which shows a mortality of almost 100%. Several studies have demonstrated that infection of pregnant cows with noncpBVDV strains can result in the birth of calves persistently infected with the same noncpBVDV strains (7, 33). These animals are immunotolerant with respect to the particular BVDV strain. The tolerance is highly specific, since the immune system of persistently infected animals is able to clear superinfections with antigenically different BVDV strains without eliminating the persisting BVDV (6, 15, 40). Interestingly, a cpBVDV strain, in addition to the persisting noncpBVDV strain, can be isolated from animals which had developed MD (34). Both viruses isolated from one animal with MD are antigenically almost identical and are called a virus pair (14,

46). MD can also be induced experimentally by superinfection of persistently infected animals with cpBVDV strains. Importantly, only superinfection with a cpBVDV matching the antigenic pattern of the persisting noncpBVDV strain resulted in development of MD (5, 7, 40). Recently, viruses isolated from naturally occurring cases of MD have been characterized at the molecular level (38, 39, 49). These studies have shown that each of the analyzed cpBVDV strains developed from the respective persisting noncpBVDV virus by a process of RNA recombination.

One common feature of all the cpBVDV strains analyzed so far is the expression of a 80-kDa protein (p80), which is not found after infection of cells with noncpBVDV (11, 14, 46, 48). Therefore, p80 is regarded as a marker protein for cpBVDV. p80 is colinear to the carboxy-terminal two-thirds of p125, a nonstructural protein found in cells infected with either cpBVDV or noncpBVDV. Generation of p80 occurs in some cpBVDV strains by proteolytic cleavage of p125 (11, 13, 36, 60). Other cpBVDV strains express p80 from duplicated genomic regions and not by cleavage of p125 (38, 39). In all of these cases, proteolytic release of p80 is linked to the changes caused in the viral genomes by the RNA recombination process, e.g., the uptake of cellular ubiquitin-coding sequences. In this report, the viruses of pair 9, isolated from an animal with MD, were analyzed at the molecular level. The data obtained show that CP9 is a novel type of cytopathogenic pestivirus.

MATERIALS AND METHODS

Cells and viruses. MDBK cells were obtained from the American Type Culture Collection (Rockville, Md.). BHK-21 cells (BSR clone) were kindly provided by K. K. Conzelmann (Federal Research Centre for Virus Diseases of Animals), and BVDV strain NADL was provided by M. S. Collett (Medimmune, Inc., Gaithersburg, Md.). Isolation and serological characterization of BVDV strains NCP9 and CP9 have been described previously (14). Cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS).

* Corresponding author. Mailing address: Federal Research Centre for Virus Diseases of Animals, P.O. Box 1149, 72001 Tübingen, Germany.

Infection of culture cells. Since BVDV virions are mainly cell associated and appear only in very low titers in the supernatant of infected cells, lysates, prepared by freezing and thawing of the infected cells, were used for reinfection of culture cells. The cells were lysed 48 h postinfection by freezing and thawing and were stored at -70°C .

cDNA synthesis, cloning, and library screening. Synthesis and cloning of double-stranded cDNA in λ ZAPII (Stratagene, Heidelberg, Germany) as well as establishment and screening of phage libraries were done as previously described (38). The sequences of the oligonucleotide primers, Pes9 and BVD14, used for cDNA synthesis were also published previously (38).

Nucleotide sequencing. Dideoxy sequencing (57) of double-stranded DNA templates was carried out by using the T7 polymerase sequencing kit from Pharmacia (Freiburg, Germany). cDNA clones were truncated for sequence analysis with exonuclease III and S1 (25). Computer analyses of sequence data were performed on a Digital Microvax II, using the University of Wisconsin Genetics Computer Group software (17).

RNA preparation, gel electrophoresis, and Northern (RNA) hybridization. RNA was prepared as described previously (54). Five micrograms of glyoxylated RNA (56) was separated in phosphate-buffered 1% agarose gels containing 5.5% formaldehyde and transferred to Duralon-UV membranes (Stratagene). An RNA ladder (Bethesda Research Laboratories) served as a size standard. ^{32}P -labeled DNA probes were obtained by nick translation (nick translation kit; Amersham-Buchler, Braunschweig, Germany). The DNA probe used for nick translation and hybridization is a cDNA fragment derived from BVDV strain NCP1 and has been described elsewhere (38). Hybridizations were performed in 0.5 M sodium phosphate (pH 6.8)–1 mM EDTA–7% sodium dodecyl sulfate (SDS) at 55°C . For posthybridization washes, 40 mM sodium phosphate (pH 6.8)–1 mM EDTA–5% SDS and 40 mM sodium phosphate (pH 6.8)–1 mM EDTA–1% SDS were used two times each at hybridization temperature.

Radioimmunoprecipitation and SDS-PAGE. BVDV-infected MDBK cells ($1.5 \times 10^6/3.5\text{-cm-diameter dish}$) were labeled for 6 h with 0.5 mCi of [^{35}S]methionine–[^{35}S]cysteine per ml. Labeling medium contained no cysteine and 5% of the normal methionine content. Cell extracts were prepared under denaturing conditions (24). Extracts were incubated with 5 μl of undiluted serum. Precipitates were formed with cross-linked *Staphylococcus aureus* (31), analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and processed for fluorography using En^3Hance (New England Nuclear, Boston, Mass.).

Construction of the BVDV expression plasmids. Expression plasmids pCC9.1 and pCC9.2 are based on the pCITE vector system (Angewandte Gentechnologie Systeme GmbH, Heidelberg, Germany). pCITE contains the internal ribosome entry site of encephalomyocarditis virus downstream of a T7 RNA polymerase promoter. The translational start codon is located at the 3' end of the internal ribosome entry site sequence and followed by a polylinker sequence.

In a first step, expression vector pRN653A (60) was cleaved with *EcoRI*, and the ends were filled in with the Klenow fragment of DNA polymerase and treated with calf intestine phosphatase. Into this vector was ligated a *BclI*–*EcoRV* fragment of pSKC9.1, whose ends were filled in with Klenow enzyme. The *BclI* site corresponds to nucleotides 393 to 398 of BVDV strain SD-1 (16); the *EcoRV* site is located within the polylinker sequence of pSKC9.1. From a resulting plasmid, an *NcoI*–*BamHI* fragment was isolated and cloned into pCITE2A, restricted with the same enzymes. This cloning procedure resulted in construct pCC9, whose open reading frame starts

with the original translation start codon of defective interfering particle (DI) DI9. From this construct, a *NotI*–*XbaI* fragment, including exclusively polylinker sequences, was deleted from the 3' end of the cDNA insert; the ends were filled in with Klenow enzyme and religated. In the obtained expression construct, pCC9.1, a translational stop codon follows 2 amino acids (aa) downstream of the DI9 sequence. The open reading frame of pCC9.1 starts with aa 1 and ends with aa 2385 (numbers according to the strain SD-1 sequence). To generate pCC9.2, a *HincII*–*XbaI* fragment was deleted from pCC9, and the ends of the plasmid were filled in with Klenow enzyme and treated with calf intestine phosphatase. The *HincII* site corresponds to nucleotides 5434 to 5439 of the SD-1 sequence; the *XbaI* site is located in the polylinker sequence downstream of the cDNA insert. Into this pCC9-derived vector, a *HincII*–*DraI* cDNA fragment of pSKC9.1 was ligated. The *DraI* site corresponds to nucleotides 5627 to 5632. In the resulting plasmid pCC9.2, the last DI-derived amino acid is directly followed by a translational stop codon. The open reading frame of pCC9.2 starts with aa 1 and ends with aa 1748 (numbers according to the strain SD-1 sequence).

Plaque isolation. MDBK cells in 6.0-cm-diameter dishes were infected with BVDV CP9 and overlaid with 1.8% methylcellulose. After 48 h, plaque formation was observed in the cell layer. Material of 23 individual plaques was isolated with Pasteur pipettes. The material of each plaque was diluted in 500 μl of medium. These suspensions were used for infection of MDBK cells in 24-well plates. For the infection of one well, 250 μl of plaque suspension was used. Material from each plaque was used for the infection of cells in two wells. One of the wells was superinfected after 1 h with BVDV NCP1 (14); 72 h after the addition of the plaque suspension, the cells were passaged into 3.5-cm-diameter culture dishes and checked for the appearance of a cytopathic effect (CPE).

RNA transfection. A suspension of 3×10^6 MDBK cells was transfected with 10 μg of RNA by DEAE-dextran. Ten micrograms of RNA was mixed with 100 μl of HBSS and 100 μl of DEAE-dextran (1 mg/ml in HBSS) and incubated for 30 min on ice (61a). Pelleted cells were washed once with DMEM without FCS. The cells were then resuspended in the RNA/DEAE-dextran solution and incubated at 37°C . After 30 min, 20 μl of dimethyl sulfoxide was added, and the mixture was incubated at room temperature for 2 min. After addition of 2 ml of HBSS, cells were washed once with HeBSS and medium without FCS. The pelleted cells were then resuspended in medium with FCS and seeded in culture dishes.

Half of the transfected cells were grown in a 6.0-cm-diameter dish. These cells were passaged and split 1:2 after 48 to 72 h. Twenty percent of the transfected cells of each experiment were cultured in 3.5-cm-diameter culture dishes, treated like the corresponding cell culture in the 6.0-cm-diameter dish, and later used for the immunofluorescence assay. The RNA used for transfection was 10, 1, or 0.1 μg of total cellular RNA isolated from BVDV CP9-infected MDBK cells. For each transfection, 10 μg of RNA was used. If only 1 or 0.1 μg of cellular RNA was used, 9 or 9.9 μg of yeast tRNA was added. For each concentration of cellular RNA, three transfection experiments with noninfected MDBK cells and three transfections with BVDV NCP1-infected MDBK cells were carried out in parallel. Infection of MDBK cells with BVDV NCP1 was carried out 48 h prior to RNA transfection.

Immunofluorescence assay. Cells were fixed with methanol-acetone (1:1) at -20°C for 15 min and incubated with a BVDV-specific bovine hyperimmune serum at 37°C for 16 h. After addition of a fluorescein isothiocyanate-coupled mouse

FIG. 1. Northern blot analysis of total RNA of MDBK cells. Lanes: 1, noninfected; 2, infection with cpBVDV strain NADL; 3, infection with cpBVDV isolate CP9; 4, infection with BVDV strain NCP9. The probe used for hybridization is derived from BVDV strain NCP1 (38) and encompasses part of the p125-coding region. The BVDV-specific bands of 8 and 12.3 kb are marked with arrows.

anti-bovine antibody for 2 h at 37°C, fluorescence was evaluated.

Transient expression with the T7-vaccinia virus system. BSR cells (5×10^5 /3.5-cm-diameter culture dish) were infected with vaccinia virus vTF7-3 (21) in 1 ml of DMEM lacking FCS at a multiplicity of infection (MOI) of 10. After 1 h of incubation at 37°C, cells were washed two times with medium and transfected with 10 µg of plasmid DNA (mammalian transfection kit; Stratagene). After 5 h at 37°C, cells were washed twice with labeling medium. Labeling medium contained no cysteine and 5% of the normal methionine content. Cells were labeled overnight in 0.5 ml of labeling medium containing 0.5 mCi of [35 S]methionine-[35 S]cysteine per ml.

Nucleotide sequence accession number. The sequence data reported have been deposited with the EMBL/GenBank data libraries under accession number U03912.

RESULTS

Northern blot analysis. It has been shown recently that cpBVDV strains develop from noncpBVDV strains by a process of RNA recombination (38, 39, 49). The aim of the work presented here was the characterization of a cytopathogenic BVDV (CP9) and a noncytopathogenic strain (NCP9) isolated from an animal which died of MD. Both viruses, referred to as virus pair 9, were almost identical, as judged from their reaction patterns with a set of monoclonal antibodies (14).

To characterize the genomic RNAs of these isolates, total RNA from cells, infected with either CP9 or NCP9, was isolated and hybridized in a Northern blot to a 32 P-labeled BVDV-specific cDNA probe (Fig. 1); the probe encompasses part of the p125-coding region of a BVDV strain (38). The genomic BVDV RNA with a length of about 12.3 kb was

FIG. 2. Northern blot analysis of total RNA of MDBK cells infected with BVDV CP9. Infection of cells was carried out with a lysate of CP9-infected cells which was diluted prior to use. Lanes: 1, not infected; 2, 1:2 (MOI = 0.1, according to fluorescence assay); 3, 1:20; 4, 1:200; 5, 1:2,000; 6, 1:20,000. Cells were lysed for RNA preparation after 24 h (1:2, 1:20), 48 h (1:200), or 72 h (1:2,000, 1:20,000). The probe used for hybridization is derived from BVDV strain NCP1 (38) and encompasses part of the p125-coding region. The BVDV-specific bands of 8 and 12.3 kb are marked with arrows.

detected in cells after infection with NCP9 (Fig. 1, lane 4) and CP9 (Fig. 1, lane 3). A large duplication of viral sequences, as identified in the genomes of several cpBVDV strains (38, 39, 49), is thus not present in the genome of CP9. Probes specific for the cellular insertions found in the genomes of several cpBVDV strains, namely, ubiquitin and c-INS (36, 37), failed to detect the genome of CP9 or NCP9 (data not shown). Therefore, the modifications already described for the genomes of cpBVDV strains are not present in the genome of CP9.

Interestingly, in addition to the 12.3-kb genome, a second BVDV-specific RNA species was detected in the RNA of CP9-infected cells (Fig. 1, lane 3). This RNA with an estimated length of 8 kb was not found in cells after infection with NCP9. So far, pestivirus-specific RNAs with subgenomic length have not been described.

Characteristics of the 8-kb RNA. Viral genomes with deletions or truncations have been identified in a large number of animal and plant virus systems. They are termed DIs provided that (i) these RNAs are not able to replicate without the support of a helper virus and (ii) replication of the DI RNA interferes with the replication of the helper virus (28). To determine whether the BVDV-specific 8-kb RNA species found in CP9-infected cells represents the genome of a BVDV DI, cell culture experiments were carried out according to the criteria listed above.

A lysate of CP9-infected cells was diluted stepwise and used for infection of MDBK cells. Total RNA of the infected cells was analyzed in a Northern blot (Fig. 2). After infection with a low MOI, the genomic BVDV RNA of 12.3 kb was detectable (Fig. 2, lane 6). Infection at a higher MOI did not result in a higher level of genomic RNA. Instead, the amount of the

12.3-kb RNA species decreased significantly. Simultaneously, the 8-kb RNA species accumulated under these conditions of infection (Fig. 2, lanes 2 to 5). These results demonstrate interference of the 8-kb RNA with the replication of the complete viral genome. Infection at a high MOI favors coinfection of cells with helper virus and a DI which is essential for propagation of a DI. After infection with a low MOI, the amount of the 8-kb RNA decreased (Fig. 2, lanes 2 to 6). This result is consistent with a dependency of the 8-kb RNA, with regard to its propagation, on the presence of a helper virus in the same cell. The 8-kb BVDV-specific RNA therefore represents the genome of a DI. Hereafter, the 8-kb genome will be referred to as DI9.

The cytopathogenic agent. Repeated passages of BVDV CP9 at a low MOI led to elimination of the DI from the virus stock. Surprisingly, cells infected with such a DI-free virus stock exhibited no CPE. This striking difference between CP9-infected cells and cells infected with NCP9 or the DI-free virus stock led to the working hypothesis that the presence of DI9 and the occurrence of a CPE are directly linked.

To verify that DI9 is the cytopathogenic agent in the CP9 virus stock, the presence of an autonomous cpBVDV strain had to be excluded. The major difference between cpBVDV strains described so far and a cpBVDV DI concerns the dependence on a coinfecting helper virus, which applies only to the DI. To investigate the abilities of the viruses contained in the CP9 virus stock to cause a CPE, cell culture experiments were performed. After infection with CP9, the cytopathogenic agent should be present in high concentrations within the plaques. To isolate the cytopathogenic agent, 23 such plaques were picked. The material was diluted, and two aliquots obtained from each plaque were used in parallel for infection of MDBK cells. After incubation of noninfected cells with the material from 23 primary plaques, no CPE could be detected. In the second part of this experiment, cells were superinfected 1 h after addition of the plaque material with a noncpBVDV strain. In 12 of the 23 culture dishes, a CPE developed. Plaque picking experiments comparable to those described above were carried out with different cpBVDV strains. In none of these cases was a noncpBVDV infection a prerequisite for development of a CPE.

In another series of experiments, MDBK cells were transfected with total RNA of CP9-infected cells. The RNA used for transfection contained no 12.3-kb genomic RNA detectable in a Northern blot but a high amount of DI9 RNA (comparable to the RNA shown in Fig. 2, lane 2). Transfection of MDBK cells with 10, 1, or 0.1 μ g of this RNA did not lead to a CPE. For each concentration, three culture dishes were tested in parallel. In a BVDV-specific immunofluorescence assay, all cell cultures were found to be negative for BVDV replication, even when the cells were passaged up to three times (data not shown). A second subset of MDBK cells was infected prior to transfection with BVDV strain NCP1. Transfection of 10 or 1 μ g of RNA led to a CPE in all culture dishes. The CPE appeared more distinct after transfection of 10 μ g of RNA from CP9-infected cells. After one passage, a CPE could also be detected in one of three cell culture dishes transfected with 0.1 μ g of RNA from CP9-infected cells. Transfection of noninfected cells with total RNA isolated from noncpBVDV-infected cells did not result in a CPE, but the cells were shown to be positive for BVDV by the immunofluorescence assay. Development of a CPE after transfection of cells with RNA from cpBVDV (strain NADL)-infected cells was independent of a noncpBVDV infection (data not shown).

Both series of experiments demonstrate that in the case of BVDV CP9, there is a strict requirement of a helper virus for

the development of a CPE. Accordingly, the presence of an autonomously proliferating cpBVDV strain in the CP9 virus stock can be excluded. Therefore, the BVDV DI identified in this virus stock is the cytopathogenic agent. Taken together, the results indicate that BVDV isolate CP9 is a mixture of a cytopathogenic DI (DI9) with a noncytopathogenic helper virus, most likely strain NCP9.

Genome structure of DI9. For molecular characterization of DI9, cDNA of the respective genome was cloned. First-strand synthesis was carried out with DI-enriched RNA as the starting material (comparable with the RNA from Fig. 2, lane 2) and two BVDV-specific oligonucleotides complementary to the BVDV genome around positions 7000 and 9000, respectively, as primers (38). The cDNA inserts of several BVDV-specific lambda clones were analyzed by terminal sequencing of the cDNA inserts. Sequence alignment of the termini of the cDNA insert of clone pSKC9.2 (Fig. 3A) revealed that the BVDV-specific sequence starts at position 44 of the BVDV genome and ends with position 6818, covering a region of about 6.8 kb. However, the cDNA insert of pSKC9.2 has a length of only 2.5 kb, indicating a gap of about 4.3 kb in this cDNA fragment. Analogous results were obtained with cDNA clones pSKC9.1 to pSKC9.4 (Fig. 3A). It was thus expected that these clones contain cDNA fragments derived from the DI9 genome. cDNA clones without this large deletion, originating from the helper virus, were not isolated from the cDNA library by screening with the NCP1 probe, probably because of the low amount of helper virus genome present in the RNA preparation used for cDNA synthesis.

Part of the genomic sequence of DI9 was obtained by sequencing of both DNA strands of clone pSKC9.2 (Fig. 3A) and of the 3' *Eco*RI fragment of pSKC9.1 together encompassing nucleotides 44 to 7542 (numbers according to the BVDV SD-1 sequence [16]). The genomic sequence of DI9 is colinear with the BVDV genome up to nucleotide 889. The sequence downstream of this nucleotide also can be aligned with BVDV sequences, but the homology starts at position 5153. The resulting gap in the genome of DI9 encompasses nucleotides 890 to 5152 (4,263 bases). A deletion of about 4.3 kb is in good agreement with the observed length of the DI RNA (Fig. 2). The sequences surrounding the deletion site were confirmed by DNA sequencing of four independent cDNA clones (Fig. 3A). In the analyzed part of the DI9 genome, no further gaps or insertions are present 5' or 3' to this deletion. Analyses of the derived amino acid sequence of DI9 indicated one long open reading frame for this genome. The open reading frame of DI9 starts with the original amino terminus of the BVDV polyprotein (Fig. 3B and C). In pestiviruses, the first protein of the open reading frame, N^{pro}, is an autoprotease which releases itself from the nascent polyprotein by an autocatalytic cleavage (59, 61, 63). Surprisingly, the deletion identified in the polyprotein of the DI starts at aa 169, the first amino acid of the viral capsid protein (59). Accordingly, DI9 encodes the complete N^{pro} protein. The sequence downstream of the deletion starts with aa 1590 of the viral polyprotein located in the region of the nonstructural protein p125 (Fig. 3B). Therefore, the deletion encompasses the entire set of structural proteins and also the amino-terminal region of the nonstructural protein p125.

Generation of p80. BVDV p125/p80 is a protease responsible for generation of its own C terminus and processing of the downstream region of the viral polyprotein (64). p80 is colinear to the C-terminal two-thirds of p125. It has been shown that p80 can be either generated by cleavage of p125 or expressed from a duplicated genomic region (11, 38, 39, 60). Since expression of p80 is strictly limited to cells infected with

FIG. 3. (A) Scheme of cDNA clones representing parts of the BVDV DI9 genome. cDNA inserts are symbolized by lines; deleted genomic regions are symbolized by dashed lines. Nucleotides of the BVDV genome included in the cDNA clones: pSKC9.1, 204 to 7542; pSKC9.2, 44 to 6818; pSKC9.3, 285 to 7063; pSKC9.4, 48 to 6723 (numbers according to the BVDV strain SD-1 sequence [16]). Since the BVDV-specific oligonucleotides used for cDNA synthesis were complementary to the BVDV SD-1 genome at positions 6876 to 6895 (BVDV14) and positions 8933 to 8952 (Pes9), priming of none of the analyzed BVDV-specific cDNA clones has occurred with these oligonucleotides. (B) Deduced genome and polyprotein organization of DI9 in comparison with the genome and polyprotein organization of noncpBVDV (i.e., NCP9). N^{pro}, viral autoprotease, nonstructural protein; C, viral nucleocapsid protein; E0, E1, and E2, viral envelope proteins; ?, genomic region for which no corresponding protein has been identified; p125/p80 and p10, viral nonstructural proteins. (C) Alignment of the deduced amino acid sequences of DI9 and BVDV strain SD-1. 1, BVDV strain SD-1, aa 1 to 180; 2, BVDV strain DI9, aa 1 to 300; 3, BVDV strain SD-1, aa 1572 to 1721 (numbers according to the published SD-1 sequence [16]). Start and end points of homologous regions are boxed.

cpBVDV strains (19, 20, 46, 48), it can be regarded as the marker protein of cpBVDV. The absolute correlation between p80 expression and cytopathogenicity of BVDV isolates points toward a crucial role of this protein in induction of the CPE.

FIG. 4. Analysis of immunoprecipitates after metabolic labeling of MDCK cells by SDS-PAGE (12% gel). Shown are cells infected with BVDV NCP9 (lanes 1 and 3) or CP9 (lanes 2 and 4), preimmune serum (lanes 1 and 2), and anti-A3 serum directed against the p80 part of p125 (lanes 3 and 4). Numbers on the left indicate molecular masses of marker proteins in kilodaltons.

As expected, expression of p80 was detected only in CP9-infected cells (Fig. 4, lane 4), not in NCP9-infected cells (Fig. 4, lane 3). From the data presented above, it appeared likely that expression of p80 is linked to the presence of the DI. To determine whether p80 is actually expressed by the genome of DI9, polyprotein processing was studied in a transient expression system using vaccinia virus vTF7-3, which expresses the bacteriophage T7 RNA polymerase (21). Plasmid pCITE, an expression vector containing a bacteriophage T7 RNA polymerase promoter, was used to construct the expression plasmids. Part of the cDNA insert of pSKC9.1 was cloned into pCITE, resulting in clone pCC9.1 (Fig. 5A). The open reading frame contained in pCC9.1 starts with the original N-terminal methionine of the DI9 polyprotein and extends to aa 2385, close to the estimated C terminus of p10, the protein located downstream of p80. After transient expression, the labeled cell extracts were subjected to immunoprecipitation with BVDV-specific antisera (39, 61). Expression of pCC9.1 gave rise to an 80-kDa protein (Fig. 5B, lane 3) which comigrated with p80 from CP9-infected cells (Fig. 5B, lane 2). Thus, p80, the marker protein of cpBVDV, can be expressed from the genome of DI9.

The open reading frame of pCC9.1 encodes two proteases, the autoprotease N^{pro} and p80; the latter is located directly downstream of N^{pro}. To investigate whether cleavage between N^{pro} and p80 is due to the proteolytic activity of p80, a second plasmid was used. In construct pCC9.2, the 3' end of the cDNA insert of pCC9.1, together with part of the active center of the p80 protease domain, was removed (Fig. 5A). After transfection of this construct, antiserum G1 precipitated a protein (Fig. 5C, lane 7) which comigrated with N^{pro} obtained either from CP9-infected cells (lane 5) or by expression of pCC9.1 (lane 6). Accordingly, the autoprotease N^{pro} generates its own C termi-

nus also in the polyprotein encoded by DI9. The proteolytic activity of p80 is not involved in this processing step.

Antiserum P1 precipitated p10 and p30 from cells infected with BVDV CP9 (Fig. 5C, lane 2). After transient expression of pCC9.1, we detected a protein (Fig. 5C, lane 3) which comigrates with p10 from CP9-infected cells (lane 2). This result shows that cleavage at the C terminus of p80 takes place. In BVDV-infected cells, generation of p10 is mediated by the p80 protease (64). Accordingly, the proteolytic activity of p80 encoded by DI9 is not affected. In addition, these experiments show that a p80 protein, starting with glycine 1590, is still proteolytically active.

DISCUSSION

Analyses of BVDV pairs isolated from cattle after outbreaks of MD revealed that cpBVDV strains evolve from persisting noncpBVDV. In the genomes of cpBVDV strains, either insertions of cellular sequences (36), sometimes together with duplications of viral sequences (38, 49), or mere rearrangements and duplications of viral sequences (39) have been identified. According to these data, the genomes of all cpBVDV strains analyzed so far are generated by complex RNA recombination processes. In spite of these genomic variations, all of these viruses are replicating autonomously. The genome analyzed in this report also represents a product of RNA recombination. In the case of pair 9, the resulting cytopathogenic agent is, however, an internal-deletion-type DI.

A common property of all cpBVDV strains analyzed so far is the expression of p80, the marker protein of cpBVDV. The N terminus of p80 can be generated by different proteases (39, 60). In all cases, this processing event is strictly linked to the changes in the polyprotein caused by the RNA recombination. The genomes of cpBVDV strains CP14 and Osloss contain ubiquitin-coding sequences, and it was demonstrated that a cellular ubiquitin-specific protease generates the N terminus of p80 (60). cpBVDV strains which contain a duplicated p80 region downstream to a ubiquitin-coding sequence, i.e., CP1 (38), apparently express p80 in the same way. In the polypro-

FIG. 5. (A) Scheme of the polyprotein structure of DI9 and structures of pCITE expression constructs pCC9.1 and pCC9.2. Numbers under the bars indicate positions of the first and last amino acids of the encoded viral proteins (numbers according to the BVDV strain SD-1 sequence [16]). S(1752) indicates the position of the catalytic serine residue in the active center of the p125/p80 protease. p80* is a C-terminally truncated p80; truncation includes S(1752). (B) Analysis of immunoprecipitates after metabolic labeling of MDBK or BSR cells by SDS-PAGE (12% gel). Lanes: 1 and 2, proteins precipitated from metabolically labeled MDBK cells after infection with BVDV CP9; 1, preimmune serum; 2, anti-A3 serum; 3 and 4, proteins precipitated by anti-A3 serum from metabolically labeled BSR cells; 3, BSR cells infected with T7-vaccinia virus (vTF7-3) and transfected with pCC9.1; 4, BSR cells after infection with T7-vaccinia virus only. Numbers on the left indicate molecular masses of marker proteins in kilodaltons. (C) Analysis of immunoprecipitates after metabolic labeling of MDBK or BSR cells by SDS-PAGE (12% gel). Lanes: 1, 2, and 5, proteins precipitated from metabolically labeled MDBK cells infected with BVDV CP9; 1, preimmune serum (NS); 2, anti-P1 serum, directed against p10 and p30; 5, anti-G1 serum directed against N^{pro}; 3, 4, 6, 7, and 8, proteins precipitated from metabolically labeled BSR cells with anti-P1 (lanes 3 and 4) or anti-G1 (lanes 6, 7 and 8) serum; 3, 6, and 7, BSR cells infected with T7-vaccinia virus (vTF7-3) and transfected with pCC9.1 (lanes 3 and 6) or pCC9.2 (lane 7); 4 and 8, BSR cells infected only with T7-vaccinia virus. From CP9-infected cells, both p10 and p30 are precipitated (lane 2). p30 is encoded downstream of p10. Numbers on the left indicate molecular masses of marker proteins in kilodaltons.

FIG. 6. Scheme of the polyproteins of DI9 and cpBVDV Pe515CP (39), each symbolized by bars. D and B mark the amino acids flanking the recombination site (D, aa 168; B, aa 1590 [numbers according to BVDV strain SD-1 sequence [16]]. N^{pro} , an N-terminally truncated N^{pro} molecule encoded by the short duplicated region in Pe515CP; see also Fig. 3.

teins of two additional cpBVDV strains (Pe515CP and CP6) a duplicated autoprotease N^{pro} (N^{pro*}) is located N terminally to a duplicated p80 region (Fig. 6). In these cases, the autoproteolytic activity of N^{pro*} generates the N terminus of p80 (39). The genome of DI9 exhibits a large deletion instead of duplications or rearrangements, but the amino acid sequence at the N^{pro} /p80 border in the polyprotein of DI9 is identical to the one identified for Pe515CP and CP6. As shown in this report, the generation of the N terminus of p80 in the polyprotein of DI9 is also mediated by N^{pro} . The proteolytic activity of p80 is not involved in this cleavage.

DIs have been identified in most virus systems (reviewed in references 4, 26, 27, 44, 53 and 58). Interference of DIs with the replication of the helper virus leads to a strong reduction of virus yield and in some virus systems also to the suppression of the virus-induced CPE (30, 47). These tissue culture experiments led to the proposal that DIs also may change the course of viral diseases. Ameliorating effects on disease symptoms and attenuation of viruses by DIs were shown for influenza virus (43), vesicular stomatitis virus (22), Semliki Forest virus (18), and other virus systems (reviewed in references 4, 27, and 53). It was proposed that in the vesicular stomatitis virus system, DIs are involved in changes from acute to persistent infections (23). For a Semliki Forest DI, immunosuppression was observed in mice (3).

Different plant viruses are associated with so-called satellite RNAs which sometimes exacerbate viral symptoms (1, 29). Recently, DI RNAs were identified in two plant viruses, a carmovirus (turnip crinkle virus) and a bromovirus (broad bean mottle virus); both augmented the symptoms caused by the parental virus (32, 52). In animal virus systems, defective measles virus isolated from patients with subacute sclerosing panencephalitis (8) and defective retroviruses with oncogenic potential are involved in development of diseases (45; reviewed in reference 9). However, in the latter two cases, the respective defective viruses do not represent DIs (53). To our knowledge, a DI causing a natural disease of humans or animals has not been described.

Fatal MD can be induced experimentally in cattle persistently infected with a noncpBVDV strain by superinfection with an antigenically closely related cpBVDV strain (5, 7, 40). Therefore, the cpBVDV strain found in each of the animals with MD is regarded as causative for development of MD. Since BVDV DI9 is the cytopathogenic agent isolated from an animal with MD, DI9 is most probably responsible for the outbreak of this case of fatal disease. Thus, cpBVDV DI9 is the first DI linked to a lethal disease in animals.

cpBVDV and noncpBVDV strains isolated from one animal with MD show in each case a very high degree of similarity which has been shown serologically (14, 46, 51) as well as by

molecular analyses (38, 39, 49). This finding is not surprising, since persistently infected calves are immunotolerant with respect to the persisting noncpBVDV strain but are competent to clear superinfections with serologically different BVD viruses (6, 15, 40). Thus, only a cpBVDV strain which is covered by the immunotolerance of the persistently infected animal can induce MD (40). BVDV field isolates show a very high degree of antigenic variation. Horizontal spread of newly developing cpBVDV strains therefore can lead to MD only in a strictly limited subset of persistently infected animals.

The situation is quite different for cpBVDV DI9. The genome of DI9 does not encode any of the viral structural proteins. After infection of MDBK cells, the genomic RNA of DI9 is packaged into virus particles. Accordingly, DI9 virions always contain the structural proteins of the respective BVDV helper. Inoculation of a persistently infected calf with a cpBVDV stock composed of cytopathogenic DI9 and a noncpBVDV helper should result in a specific immune response and clearance of the helper virus. The DI genome, however, will probably be replicated and packaged by the persisting BVDV and is therefore expected to evade the immune response. Accordingly, cpBVDV DI9 may be able to induce MD in all animals, each persistently infected with a serologically different noncpBVDV. The actual proportion of naturally occurring MD cases caused by cpBVDV DI particles remains to be investigated.

The BVDV-MD system is an attractive model for studying the development of virus mutants with altered pathogenic potential. Results obtained in this system may be of general interest for understanding the pathogenesis of virus-induced diseases, especially with regard to persistent infections.

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