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Recovery of Cytopathogenic and Noncytopathogenic Bovine Viral Diarrhea Viruses from cDNA Constructs

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After cDNA cloning of the genome of bovine viral diarrhea virus (BVDV) isolate CP7, a full-length cDNA clone was constructed. RNA transcribed in vitro from this construct was shown to direct the generation of infectious BVDV upon transfection into bovine cells. To confirm the de novo generation of infectious BVDV from cloned cDNA a genetically tagged virus was constructed. In comparison with parental BVDV, the recombinant virus was slightly retarded in growth. The NS2 coding region of the CP7 genome contains a duplication of 27 nucleotides which is not present in the genome of its noncytopathogenic counterpart, NCP7. Exchange of a small fragment harboring this insertion against the corresponding part of the NCP7 sequence led to recovery of noncytopathogenic BVDV. Alteration of the construct by introduction of a fragment derived from a cytopathogenic BVDV defective interfering particle resulted in a chimeric defective interfering particle which exhibits a cytopathogenic phenotype. These findings confirm the hypothesis that the recombination-induced alterations in the genomes of cytopathogenic BVDV are responsible for the induction of cell lysis.

Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus*, which also includes classical swine fever virus (CSFV) and border disease virus of sheep. The genus *Pestivirus* is part of the family *Flaviviridae*, which also includes the genera *Flavivirus* and hepatitis C virus. The enveloped virions of the family members harbor positive-strand RNA genomes of about 9.5 to 12.3 kb (30). The genomic RNAs contain continuous long open reading frames, which are translated into polyproteins that are co- and posttranslationally processed by cellular and viral proteases to give rise to the mature viral proteins.

For all members of the genus *Pestivirus*, both cytopathogenic (cp) and noncytopathogenic (noncp) isolates have been described (2, 6, 10, 15, 23, 41). Genome analyses revealed insertions of cellular sequences, sometimes accompanied by duplication of viral sequences, and different rearrangements of viral sequences including deletions in the genomes of cp pestiviruses but not in the RNAs of the corresponding noncp viruses (2, 14, 19–23, 28, 37, 38). Thus, cp pestiviruses represent mutants of noncp viruses which at least in most cases have been generated by recombination. For BVDV, the cp phenotype is strictly correlated with the expression of the nonstructural protein NS3, since this protein is found only in cells infected with cp viruses whereas both cp and noncp BVDV generate NS2-3 (6, 11, 21, 22, 27, 38). Expression of NS3 is obviously a result of the genome rearrangements observed for cp BVDV.

BVDV represents a widely distributed pathogen of cattle which often induces only mild symptoms or causes subclinical infections (39). However, serious clinical conditions characterized by thrombocytopenia and hemorrhages have been observed in calves infected with a bovine pestivirus which belongs to the so-called type 4 pestiviruses (1, 7, 26, 29, 31). Sporadically, infection with BVDV results in a severe syndrome which is called mucosal disease and leads to death in almost 100% of cases. noncp and cp BVDV can always be isolated from such animals (16). During the last few years, evidence has been provided that mucosal disease develops when a recombination process leads to generation of a cp virus within an animal persistently infected with a noncp BVDV.

Even though the complex issue of BVDV cytopathogenicity and development of mucosal disease has been intensely studied, many questions are still open and substantial parts of the hypotheses await direct proof. However, since BVDV was not amenable to genetic manipulation, some crucial experiments could not be performed. Recently, the establishment of infectious clones has been described for three different strains of CSFV (24, 25, 32). We report here for the first time the recovery of a CP BVDV strain entirely from cloned sequences. By exchanging fragments of this construct, noncp BVDV and a cp BVDV defective interfering particle (DI) could be generated.

MATERIALS AND METHODS

Cells and viruses. MDBK cells were obtained from the American Type Culture Collection (Rockville, Md.). BVDV isolates CP7 and NCP7 were kindly provided by E. J. Dubovi, Cornell University, Ithaca, N.Y., and have been described previously (6, 36). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids.

Infection of cells. Since pestiviruses tend to be associated with the host cells, suspensions composed of supernatant and lysed infected cells were used for reinfection of culture cells. Material for infection was prepared by freezing and thawing cultures 48 h postinfection and was stored at −70°C. If not indicated differently in the text, a multiplicity of infection of about 0.1 was used. Infection with NCP BVDV was detected by immunofluorescence with a bovine hyperimmune serum.

cDNA synthesis, cloning, and nucleotide sequencing. Establishment of cDNA libraries in lambda ZAPII (Stratagene, Heidelberg, Germany) was done as described previously (21). For the library covering the 5′ one-third of the genome, cDNA synthesis was primed with oligonucleotide B18R. The probe for the first screening was a *Pst*I fragment derived from CSFV cDNA clone 4.0 (corresponding to genomic positions 1507 to 2580) (18); the probe for the second screening was the CP7-derived cDNA fragment corresponding to nucleotides 845 to 2738 (5′-terminal *Eco*RI fragment from clone pCP7.10 described in this report). The library for the 3′ region of the genome was established with randomly primed cDNA (18). The first screening was done with a *Kpn*I-*Hin*dIII

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fragment from CSFV cDNA clone 1.9 which corresponds to nucleotides 11209 to 11727 (18); the second screening was performed with a *Cla*I-*Eco*RI fragment from clone pCP7.21 (this report). Subcloning of cDNA fragments into pBluescript plasmids by in vivo excision was performed as recommended by the supplier (Stratagene). Exonuclease III and nuclease S1 were used to establish deletion libraries of cDNA clones (12). Dideoxy sequencing (35) of double-stranded DNA templates was carried out with the T7 sequencing kit (Pharmacia, Freiburg, Germany). Computer analysis of sequence data was performed with the Genetics Computer Group software (9). Oligonucleotide B18R has the sequence AARTARTCNGTNACRTARCT, with R representing G or A.

Construction of full-length BVDV cDNA clones. Restriction, subcloning, and other standard procedures were done essentially as described elsewhere. (34). Restriction and modifying enzymes were purchased from New England BioLabs (Schwalbach, Germany), Pharmacia (Freiburg, Germany), and Boehringer (Mannheim, Germany). Plasmid pACYC177 was obtained from New England BioLabs.

The 5′ part of the genome was assembled in two steps with the *Sal*I-*Bam*HI fragment from clone pCP7.17 (the *Sal*I site is located in the polylinker of the vector), the *Bam*HI-*Nsi*I fragment from clone pCP7.10, and the *Nsi*I-*Eag*I fragment from clone pC7.1. pBR322 cut with *Sal*I and *Eag*I served as a vector for the resulting clone, 146II. The 3′ part was constructed by insertion of the *Sal*I-*Eco*RV fragment from clone pCP7.21 together with the *Eco*RV-*Eco*RI fragment from clone pCP7.31 into pBR322 *Eco*RI-*Sal*I. Into this plasmid, cut with *Aat*II, treated with Klenow polymerase, and cut with *Cla*I, a *Sac*I (blunt-end)-*Cla*I fragment was inserted, which was derived from a construct obtained via insertion of oligonucleotides ol3′-plus and ol3′-minus together with the *Aat*II-*Cla*I fragment from clone pCP7.18 into pBluescript *Cla*I-*Sma*I. This construct was cut with *Nru*I and *Sal*I and ligated with the *Eco*RV-*Sal*I fragment from expression construct pC7.1 (36), leading to clone 145. The inserts from clones 146II, cut with *Xho*I and *Nco*I, and 145, cut with *Nco*I and *Sma*I, were assembled in pBluescript SK−, cut with *Xho*I and *Sma*I, resulting in clone 148. To obtain the 5′-most end, oligonucleotides ol5′-plus and ol5′-minus were combined with the *Bst*1107I *Xho*I fragment from clone pCP7.17 in pBluescript SK− *Eco*RI-*Xho*I. The insert of this clone was released with *Xba*I and *Xho*I and introduced into pACYC177 *Nhe*I-*Xho*I. Construct pA/BVDV was obtained by digesting this plasmid with *Ssp*I and *Xho*I and inserting the insert from clone 148, partially cut with *Not*I, treated with Klenow polymerase, and cut with *Xho*I. Construct pA/BVDV/N was obtained after subcloning of an *Xho*I-*Bgl*III fragment of pA/BVDV into pCITE2a, mutagenesis with oligonucleotide ol-Nco/AUG, and insertion of the mutagenized fragment into pA/BVDV.

Clone pA/BVDV/Ins− was generated by digesting pA/BVDV with *Nsi*I and *Aat*II and inserting the *Nsi*I-*Sac*I fragment from expression construct pC7.1Ins− (36) together with the *Sac*I/*Aat*II fragment from pA/BVDV. Clone pA/BVDV/D9 was generated by introduction of an *Nco*I-*Hpa*I fragment from construct pCC9.1 (38) together with an *Hpa*I-*Sal*I fragment from pC7.1 (36) into pA/BVDV/Nco restricted with *Nco*I and *Sal*I. The oligonucleotides have the following sequences: ol5′-plus, AATTCTAATACGACTCACTATAGTATACGAGA ATTAGAAAAGGCACTCGTA; ol5′-minus, TACGAGTGCCTTTTCTAATT CTCGTATACTATAGTGAGTCGTATTAG; ol3′-plus, CCATAGTTGGACT AGGGAAGACCTTTAACAGCCCCC; ol3′-minus, GGGGGCTGTTAAAGG TCTTCCCTAGTCCAACTATGGACGT; and ol-Nco/AUG, TGTACATGGC CCATGGAGTTG.

Site-directed mutagenesis. Mutagenesis by the method of Kunkel et al. (13) was done with the MutaGene Phagemid in vitro mutagenesis kit (Bio-Rad, Munich, Germany) essentially as recommended by the manufacturer, except that single strands were produced with the filamentous phage VCSM13 (Stratagene). The presence of the desired mutations was verified by nucleotide sequencing.

The sequence of oligonucleotide ol-Nco/AUG used for mutagenesis is given above.

In vitro transcription. A 2-μg portion of the respective cDNA construct was linearized with the appropriate restriction enzyme and purified by phenol extraction and ethanol precipitation. Transcription with T7 RNA polymerase (New England BioLabs) was carried out in a total volume of 50 μl of transcription mix (40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM each ATP, GTP, CTP, and UTP; 10 mM dithiothreitol; 100 μg of bovine serum albumin per ml) with 50 U of T7 RNA polymerase in the presence of 15 U RNAguard (Pharmacia). After incubation at 37°C for 1 h, the reaction mixture was passed through a Sephadex G-50 spun column (34) and further purified by phenol extraction and ethanol precipitation.

RNA transfection. Transfection was done with a suspension of 3×10^6 MDBK cells and about 2.5 ng of in vitro-transcribed RNA bound to DEAE-dextran (Pharmacia). For positive controls, usually 5 μg of total RNA from MDBK cells infected with the respective BVDV isolate was used for transfection. The RNA/DEAE-dextran complex was established by mixing RNA dissolved in 100 μl of HBSS buffer (5 g of HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 8 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO_4 \cdot 2H_2O$, 1 g of dextrose per liter; pH 7.05) (40) with 100 μl of DEAE-dextran (1 mg/ml in HBSS) and incubating the mixture for 30 min on ice (40). Pelleted cells were washed once with Dulbecco's modified Eagle's medium without fetal calf serum, centrifuged, and then resuspended in the RNA–DEAE-dextran mixture. After 30 min of incubation at 37°C, 20 μl of dimethyl sulfoxide was added, and the mixture was incubated for 2 min at room temperature. After addition of 2 ml of HBSS, cells were pelleted and washed once with HBSS and once with medium without fetal calf serum. The cells were resuspended in Dulbecco's modified Eagle's medium with fetal calf serum and seeded in a 10.0-cm-diameter dish. At 48 to 72 h posttransfection, the cells were split and seeded as appropriate for subsequent analyses. For determination of transfection efficiency, the cells were seeded after transfection in two tissue culture dishes (10 cm in diameter), and plaques were counted 3 to 4 days posttransfection.

Northern (RNA) hybridization. RNA preparation, gel electrophoresis, radioactive labelling of the probe, hybridization, and posthybridization washes were done as described previously (33). The insert of a BVDV cDNA clone NCII.1 (21) was used as a probe.

RT-PCR. RNA for reverse transcription-PCR (RT-PCR) was isolated from cells infected with material from a stock of the recovered viruses that was established after one passage of the viruses obtained by transfection. Reverse transcription of 2 μg of heat-denatured RNA (2 min at 92°C followed by 5 min on ice in 11.5 μl of water in the presence of 30 pmol of reverse primer) was done after addition of 8 μl of RT mix (125 mM Tris-HCl [pH 8.3], 182.5 mM KCl, 7.5 mM $MgCl_2$, 25 mM dithiothreitol, 1.25 mM each dATP, dTTP, dCTP, dGTP), 15 U of RNAguard (Pharmacia), and 50 U of Superscript (Life Technologies/Bethesda Research Laboratories, Eggenstein, Germany) for 45 min at 37°C. After addition of paraffin (Paraplast; melting point, 55°C) and 2 min at 80°C, the tubes were placed on ice and 30 μl of PCR mix (8.3 mM Tris-HCl [pH 8.3], 33.3 mM KCl, 2.2 mM $MgCl_2$, 0.42 mM each dATP, dTTP, dCTP, dGTP, 0.17% Triton X-100, 0.03% bovine serum albumin, 5 U of *Taq* polymerase [Appligene, Heidelberg, Germany]) was added. Amplification was carried out in 30 cycles (30 s at 94°C, 30 s at 54°C, and 60 s at 72°C). The oligonucleotides have the following sequences: ol-B1, CTCGTATACATATTGGAC; ol-B2R, CTCCTCTTTCTCC AAACA.

Nucleotide sequence accession number. The sequence data for BVDV CP7 were deposited at the GenBank/EMBL data library (accession number U63479).

RESULTS

cDNA cloning and sequencing. In a previous paper, we reported cloning and sequencing of the central region of the BVDV CP7 genome. Clones which correspond to the genomic RNA from about 3.3 to 8.5 kb were isolated (36). To obtain clones from the 5′ one-third of the genome, a library was established with cDNA specifically primed with an oligonucleotide complementary to positions 3999 to 4022 of the genome. Screening of the library with a CSFV-derived probe and, in a second round, with a CP7-specific probe resulted in isolation of clones pCP7.10 and pCP7.17, which cover the region from residues 24 to 4025 of the BVDV genome (numbers refer to the sequence of BVDV SD-1 (8) unless otherwise indicated).

An additional library was established with randomly primed cDNA to obtain clones from the 3′ part of the genome. Initially, screening was again performed with a CSFV cDNA fragment; then, part of cDNA clone pCP7.21 was used to identify further clones. Clones pCP7.21, pCP7.31, and pCP7.18 cover the genomic region from residues 6727 to 12276.

The analysis of the BVDV CP7 genome was completed by sequencing appropriate parts of clones pCP7.10, pCP7.17, pCP7.18, pCP7.21, and pCP7.31. Comparison of the resulting sequences with the BVDV SD-1 genome (8) did not reveal the presence of insertions, duplications, or deletions in the 5′ and 3′ regions of the BVDV CP7 RNA. Thus, the insertion of 27 duplicated nucleotides identified previously within the NS2-coding region represents the only major difference with respect to the genome of a noncp BVDV isolate (36).

Construction of a full-length cDNA. The strategy of choice for recovery of recombinant positive-strand RNA viruses is transfection of target cells with genome-like RNA which is generated in vitro from full-length cDNA constructs by runoff transcription with a bacteriophage RNA polymerase (for a review, see reference 3). This approach was successfully used for three CSFV isolates (24, 25, 32) and was therefore also used for the BVDV infectious clone.

The 5′-most sequence of the BVDV CP7 genome has not been determined. With regard to published sequences, the first 23 nucleotides are missing. Comparison of the respective region from different pestivirus cDNA sequences revealed the presence of two conserved complementary 9-mer motifs with

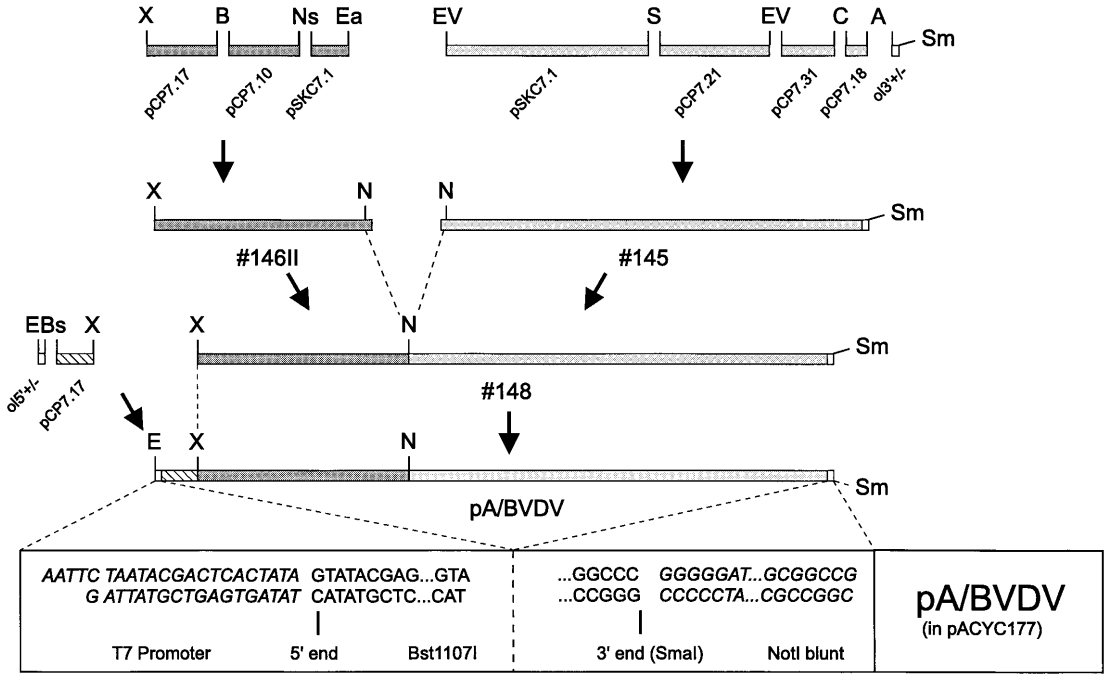

FIG. 1. Construction of BVDV full-length cDNA clone pA/BVDV. The upper region shows the fragments of the different cDNA clones present in the full-length construct. Below, the strategy for generation of pA/BVDV via intermediates 145, 146II and 148 is indicated. The box at the bottom shows the sequences surrounding the 5′ and 3′ ends of the viral sequence, which include a T7 RNA polymerase promoter and restriction sites for enzymes necessary for cloning or linearization of the plasmids. Restriction endonuclease sites: A, *Aat*II; B, *Bam*HI; Bs, *Bst*1107I; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; Ea, *Eag*I; N, *Nco*I; Ns, *Nsi*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

the consensus sequences GTATACGAG and CTCGTATAC. These two motifs are separated by a stretch of 11 to 15 less conserved nucleotides (17, 25). We decided to complete the full-length clone with the sequence of BVDV strain NADL (5). Thus, oligonucleotides composed of an *Eco*RI site, T7 RNA polymerase promoter, and the 5′ end of the BVDV sequence down to the third base of a *Bst*1107I site at position 29 were inserted together with a *Bst*1107I-*Xho*I fragment of cDNA pCP7.17 into pBluescript SK minus. In analogy to the 5′ end, the 3′-terminal sequences of the full-length clone downstream of an *Aat*II site were produced as oligonucleotides. According to published data, the pestivirus genome terminates with a conserved element which as a consensus ends with 3 to 5 C residues (4, 8, 24, 25, 31). For CSFV, it was shown that 3 terminal C residues are sufficient for infectivity of the RNA (24, 25, 32). Since, however, 5 C residues were reported for three different isolates of BVDV (4, 8, 31), this sequence was chosen as the 3′ end of the full-length CP7 clone. In addition to the synthetic oligonucleotides, cDNA fragments derived from the published clone pC7.1 (36) and from clones pCP7.17, pCP7.10, pCP7.21, pCP7.31, and pCP7.18 were used for the full-length construct (Fig. 1). The different components were fused at appropriate restriction sites by standard molecular cloning procedures (34).

The final version of the full-length cDNA clone, termed pA/BVDV, was constructed in the low-copy-number plasmid pACYC177 (Fig. 1). A variant of this clone (pA/BVDV/N) which contains an *Nco*I site overlapping the translational start codon of the long open reading frame was generated. Restriction with *Sma*I can be used for linearization prior to in vitro transcription.

Recovery of infectious virus from RNA transcripts. RNA was transcribed in vitro from *Sma*I-linearized pA/BVDV DNA. All available data indicate that pestivirus RNAs contain no 5′ cap. Moreover, recovery of CSFV was reported after transfection of RNA without 5′ cap (24, 25, 32). Therefore, no cap was introduced during transcription.

BVDV CP7 represents a cytopathogenic virus. Thus, generation of infectious virus after RNA transfection should result in the detection of plaques. Indeed, transfection of total RNA from cells infected with BVDV CP7 leads to cell lysis (Fig. 2A). The same effect was observed when RNA transcribed in vitro from pA/BVDV or pA/BVDV/N was transfected. In contrast, transfection of a 3′ terminally truncated RNA generated by transcription from *Aat*II-linearized pA/BVDV did not result in detection of a cytopathic effect (CPE) (Fig. 2A). In a Northern blot analysis, BVDV genomic RNA could be detected in samples obtained after transfection of BVDV CP7 RNA and RNA transcribed from the *Sma*I-linearized full-length clones but not in cells transfected with the 3′ terminally truncated RNA (Fig. 2B). Infection of cells with lysates prepared from cells transfected with full-length RNA again resulted in CPE (data not shown).

To provide formal proof for the recovery of BVDV entirely from cloned sequences, the RNA of cells infected with the viruses obtained from pA/BVDV and pA/BVDV/N was analyzed by RT-PCR with oligonucleotides ol-B1 and ol-B2R. DNA fragments of the expected size (0.48 kb) were detected for the samples containing viral RNA but not for the control (Fig. 3). The amplification products could not result from carryover of plasmid DNA, since a control reaction without addition of reverse transcriptase did not yield a PCR product (data not shown). To verify the presence of the mutated sequence, aliquots of the amplified products were incubated with *Nco*I. No difference was observed for the fragments amplified from RNA of V(pA/BVDV), the virus recovered from pA/BVDV (Fig. 3). However, the product amplified from V(pA/BVDV/N) was cut to completion into two fragments of the

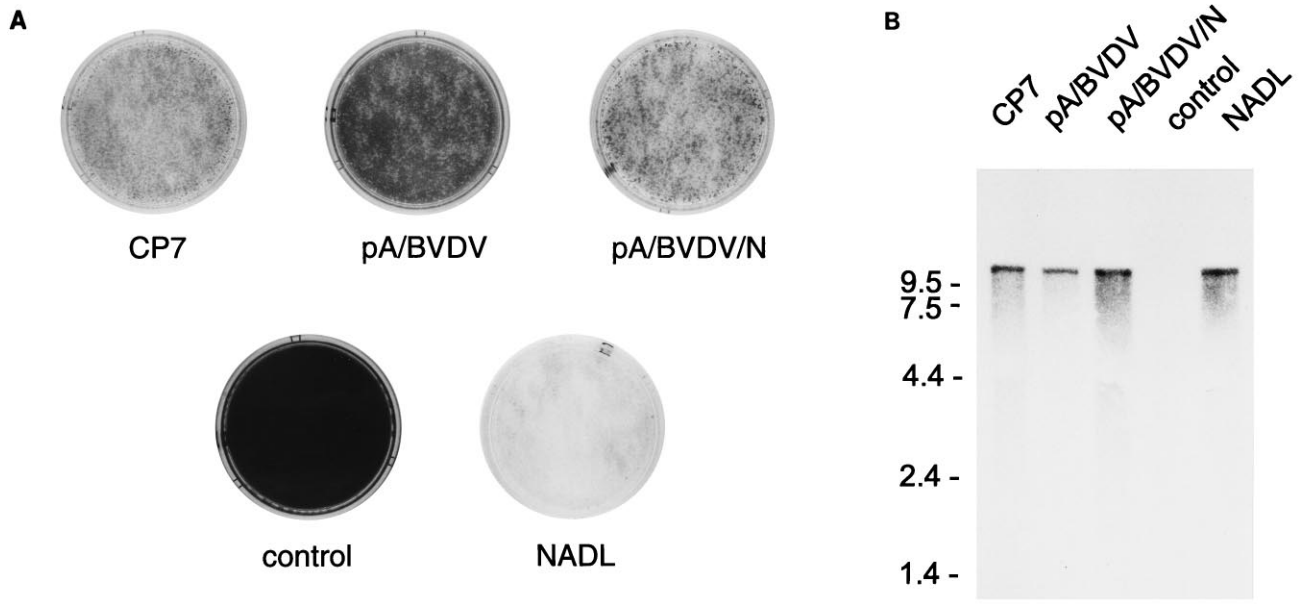

FIG. 2. Analysis of transfection experiments carried out with RNA from BVDV-infected MDBK cells (CP7 or NADL) or with RNA transcribed from plasmid pA/BVDV or pA/BVDV/N, respectively. The control panel contains 3′ terminally truncated RNA transcribed from pA/BVDV linearized with *Aat*II. (A) Crystal violet staining of tissue culture cells which had been seeded after RNA transfection. Cells were washed once with phosphate-buffered saline, fixed for 10 min with 5% formaldehyde, washed with water, and stained for 5 min with 1% (wt/vol) crystal violet in 50% ethanol. (B) Northern blot with RNA derived from the cells 48 h posttransfection hybridized to a BVDV-specific probe.

expected size. Nucleotide sequencing of the amplified fragments confirmed the absence of the mutation in the wild-type genome and its presence in the V(pA/BVDV/N) RNA (data not shown). Thus, a genetically tagged virus had been generated from the cDNA construct. The presented data clearly show that in vitro-transcribed full-length RNA is able to initiate an infectious cycle resulting in production of infectious virus.

Properties of the transcribed RNA and the recovered virus. Attempts aiming at purification of RNA from BVDV CP7 virions failed. The infectivity of BVDV CP7 RNA was therefore determined in control experiments with total RNA from cells infected with BVDV CP7. Transfection of 5 μg of total RNA led to recovery of 8 to 13 virus plaques. On the basis of comparison with defined amounts of in vitro-transcribed RNA, the respective samples contained approximately 1 ng of the viral genome. About 90% of the viral RNA that was detected in infected cells or after in vitro transcription was estimated to be full length (data not shown). Accordingly, the amount of viral RNA sufficient to produce 1 PFU in these assays was about 100 pg. Transfection of RNA transcribed in vitro from pA/BVDV/N yielded 4.7×10^4 PFU/μg. Thus, the specific infectivity of the in vitro-transcribed RNA is in the same range as that of BVDV virion RNA.

To analyze the growth characteristics of the recovered viruses, stocks were generated for both V(pA/BVDV) and V(pA/BVDV/N) directly after transfection. Cells were infected with the recombinant viruses or BVDV CP7 at a multiplicity of infection of 0.1, and the number of infectious viruses present in the culture was determined at different time points (Fig. 4). At about 30 h postinfection, the first indications of CPE were detected, which proceeded to pronounced cell lysis between 48 and 72 h. While the parental virus reached a level of 5×10^4 PFU/ml after 48 h, the titer of the recovered viruses was in a range of about 1×10^4 to 2×10^4 PFU/ml at the same time point. This slight difference in the growth characteristics of the recovered viruses and BVDV CP7 was constantly observed in different independent experiments.

In vitro generation of noncytopathogenic BVDV. Analysis of the genome of BVDV CP7 resulted in the identification of a small insertion of 27 nucleotides which is not present in the RNA of the corresponding noncp BVDV isolate NCP7. This insertion represents the only major difference between the CP7 sequence and the published sequence of the noncp BVDV isolate SD-1. Protein analyses revealed that this insertion is responsible for cleavage of NS2-3 and thus leads to generation of the cp BVDV marker protein NS3 (36). It therefore seems likely that the insertion is responsible for the cp phenotype of CP7. To test this hypothesis, pA/BVDV was reconstructed in such a way that a fragment containing the insertion was exchanged against a corresponding fragment from the NCP7 genome leading to pA/BVDV/Ins−. The only difference between pA/BVDV and pA/BVDV/Ins− is the absence of the 27-nucleotide insertion in the latter construct. After transfection of RNA transcribed from the clone without insertion, CPE could not be detected (Fig. 5A). However, immunofluorescence analysis with BVDV-specific antibodies clearly showed foci of positive cells (data not shown). Passaging the recovered virus again did not result in detection of CPE. The viral RNA was demonstrated in a Northern blot (Fig. 5B). Thus, removal of the 27-nucleotide insertion changes BVDV CP7 into a noncp virus.

Construction of a chimeric cp BVDV DI. Molecular characterization of several cp BVDV isolates resulted in identification of a variety of cp-specific genome alterations. One possible way leading to a cp virus is the generation of a cytopathogenic DI as found for isolate CP9. DI9 is able to induce CPE in cell culture when grown together with a noncp virus, whereas no lysis is observed when the helper virus is missing (38). To test whether a chimeric DI composed of sequences from DI9 and CP7 was viable and able to induce CPE, a 5.1-kb *Nco*I-*Hpa*I fragment was deleted from pA/

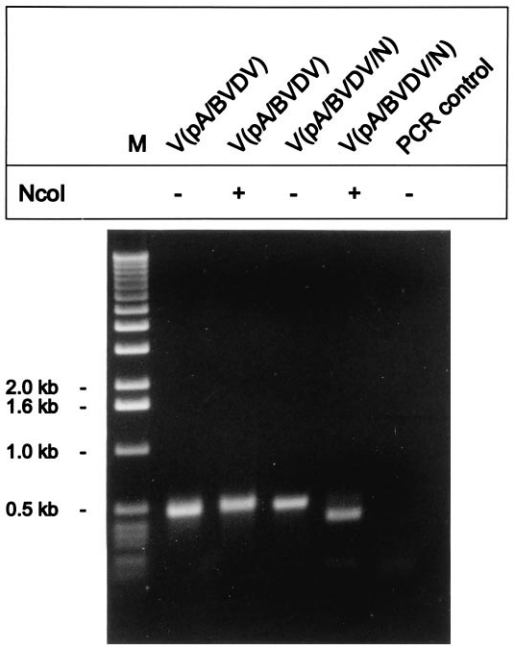

FIG. 3. Ethidium bromide-stained agarose gel with products of RT-PCR derived from RNA of cells infected with the indicated viruses which were obtained after RNA transfection and one passage in tissue culture. The amplified fragments were loaded either without further treatment (−) or after restriction with *Nco*I (+). The 3′-terminal fragment resulting from *Nco*I cleavage of the V(pA/BVDV/N)-derived fragment is only 120 nucleotides and is barely visible on the photograph.

BVDV/N and substituted by a 0.79-kb fragment harboring the genomic region with the deletion of DI9. The resulting construct was termed pA/BVDV/D9. As expected, transfection of noninfected cells with pA/BVDV/D9-derived RNA did not result in CPE (Fig. 6A). The same was true for the control dishes transfected with total RNA from cells infected with BVDV CP9 or a noninfectious in vitro-transcribed RNA. However, when cells were infected with the noncp BVDV isolate NCP1 prior to transfection, lysis was detectable for cells transfected with the CP9 RNA and the RNA transcribed from

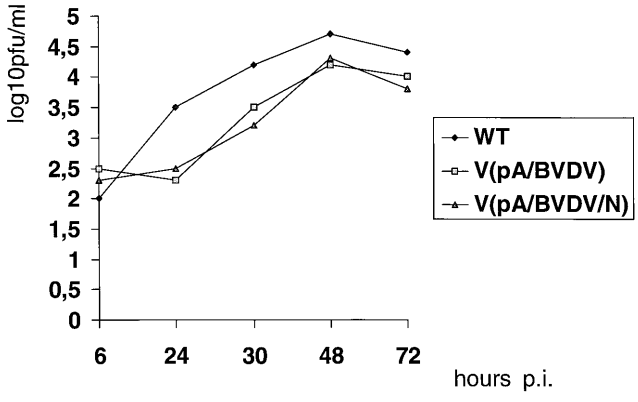

FIG. 4. Growth curve of BVDV CP7 (WT) and the viruses derived from constructs pA/BVDV [V(pA/BVDV)] and pA/BVDV/N [V(pA/BVDV/N)]. Monolayers of MDBK cells were infected with the different viruses at a multiplicity of infection of about 0.1 and harvested at the indicated time points, and titers were determined by counting the number of plaques. The results are given as $\log_{10}$ PFU per milliliter. p.i., postinfection.

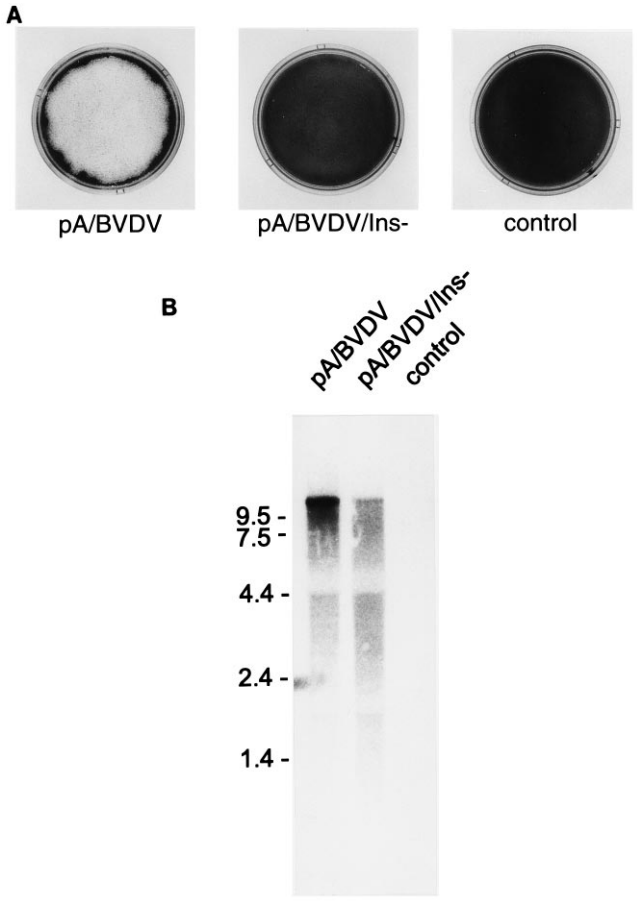

FIG. 5. Results of transfection experiments with RNA transcribed from plasmids pA/BVDV or pA/BVDV/Ins−, respectively. The control panel shows transfection of noninfectious RNA. (A) Crystal violet staining of transfected tissue culture cells. (B) Northern blot.

pA/BVDV/D9 whereas the negative control showed no CPE (Fig. 6A). Equivalent results were obtained when the RNA isolated from the transfected cells was analyzed in a Northern blot. No virus-specific RNA could be detected after transfection of noninfected cells (results not shown), whereas both DI and helper virus genome were visible after transfection of the CP9 RNA or the chimeric DI RNA in the presence of noncp helper virus (Fig. 6B). Interestingly, the RNA of the DI recovered from the pA/BVDV/D9 appears to be slightly larger than that of DI9. The reason for this difference is unclear. Since only part of the DI9 genome has been sequenced, it might be that this RNA contains another, as yet unidentified deletion.

The specific infectivity of the synthetic RNA was determined to be 1.9×10^4 PFU/μg and thus was similar to that of the pA/BVDV/N-derived RNA.

DISCUSSION

Until now, recovery of infectious pestiviruses from cloned sequences has been described for three isolates of CSFV (24, 25, 32). In all these cases, full-length clones were assembled from cDNA fragments and combined with a bacteriophage T7 RNA polymerase promoter and a unique restriction site for linearization at the 3′ end of the viral sequence. RNA produced in vitro by runoff transcription was introduced into appropriate target cells, resulting in the production of infectious

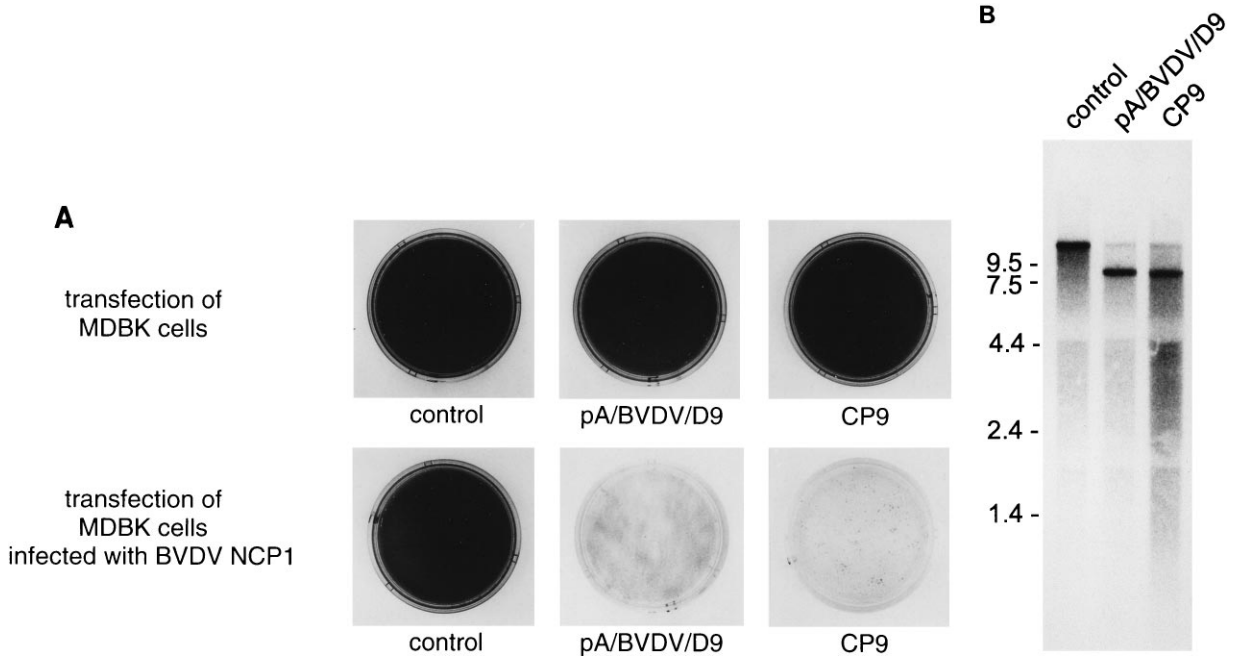

FIG. 6. (A) Crystal violet staining of MDBK cells or BVDV NCP1-infected MDBK cells after transfection with RNA transcribed from pA/BVDV/D9 or RNA from cells infected with BVDV CP9. The control panels show transfection of noninfectious RNA. (B) Northern blot with RNA derived from the BVDV NCP1-infected cells transfected with the indicated RNAs.

viruses. We applied the same strategy to another pestivirus species, namely, BVDV CP7. In contrast to the approaches used for CSFV, the 5′-most and 3′-most sequences of the viral genome were not determined for BVDV CP7. Thus, to obtain a full-length construct, the CP7-derived cDNA fragments had to be combined with oligonucleotides deduced from the sequences of other BVDV isolates. This approach seemed reasonable since the respective regions of the viral genomic RNA show a high degree of conservation for different strains (17, 25); however, a certain risk of incompatibility of the fused sequences could not be excluded.

To obtain information about intrusive mutations of an infectious clone, two criteria, namely, specific infectivity of the in vitro-transcribed RNA and the growth characteristics of the recovered viruses, have to be evaluated. The specific infectivity of the synthetic BVDV RNAs (1.9×10^4 or 4.7×10^4 PFU/μg) was found to be in the same range as that of viral RNA (10^4 PFU/μg). Similar values were found for one infectious CSFV clone (5.0×10^4 PFU/μg for wild-type CSFV Alfort/187 RNA and 6.3×10^4 PFU/μg for in vitro-transcribed RNA [32]). In contrast, the specific infectivity of transcripts derived from the other two CSFV full-length constructs were found to be 2 to 3 orders of magnitude lower than those estimated for the respective viral RNAs (24, 25). This finding points toward the presence of mutations in the last two cDNA clones. It has not been finally determined whether pestivirus genomic RNAs contain three, four, or five C residues at the 3′ terminus or simply are variable in this respect (17, 24, 25). Differences between the viral and synthetic RNAs with regard to the termini could result in a reduction of specific infectivity. Since, however, synthetic RNAs with both three and five 3′-terminal C residues show wild-type activity (32; also see above), the decreased specific infectivity of the two CSFV genome analogs is not because they end with only three C residues but has to result from as yet unidentified mutations.

The recovered viruses V(pA/BVDV) and V(pA/BVDV/N) exhibit a slight growth retardation in comparison with wild-type BVDV CP7. Even though the observed difference is small, it was reproducible in different experiments. This finding can be explained by the presence of one or more mutations in the respective cDNA constructs. The utmost terminal sequences have not been determined for the BVDV CP7 genome. Thus, the full-length cDNA constructs contain terminal sequences analogous to those displayed by another BVDV isolate. Since it is possible that the respective sequences are involved in secondary-structure formation important for RNA replication, nucleotide differences could have a significant influence on virus growth. Alternatively, exchanges in other parts of the genome, which could have been acquired during plasmid propagation in *Escherichia coli* could be responsible for the growth retardation. Sequencing of the cDNA construct will be necessary to identify such mutations.

During the past 5 years, the search for the molecular basis of cytopathogenicity of pestiviruses has led to highly interesting findings. Most cp pestiviruses analyzed so far arose from noncp ancestors by recombination. Integration of cellular sequences, sometimes accompanied by duplication of viral sequences, and different rearrangements of viral sequences, including the generation of cp DIs, have been found for cp pestiviruses (2, 14, 19–23, 28, 37, 38). Analysis of the genome of BVDV CP7 resulted in the identification of a small insertion of 27 nucleotides which is not present in the RNA of the corresponding noncp BVDV isolate NCP7 (36). This insertion was found to be the only major difference between the CP7 sequence and the published sequence of the noncp BVDV isolate SD-1. The 27 additional nucleotides represent a duplication of bases 4064 to 4090 of the viral genome integrated between the formerly neighboring nucleotides 4353 and 4354. Since the insertion of this sequence occurred in another reading frame, it encodes an amino acid sequence which is not present elsewhere in the polyprotein. Analyses based on transient expression of a variety of cDNA constructs revealed that this insertion is respon-

sible for cleavage of NS2-3 and thus leads to generation of the cp BVDV marker protein NS3 (36). The experiments described in this report show that the presence of the 27 nucleotides in the genome of BVDV CP7 is responsible for the cytopathogenicity of this virus. This conclusion is based on the fact that the only difference between constructs pA/BVDV and pA/BVDV/Ins− is the presence or absence of the 27-nucleotide insertion and the observation that transfection of pA/BVDV-derived RNA leads to cell lysis while introduction of RNA transcribed from pA/BVDV/Ins− directs the generation of a noncp virus.

The cytopathogenicity of CSFV is dependent on the presence of cp DIs in all three cases analyzed so far (23). After establishment of an infectious CSFV clone, it was demonstrated that CP DIs could be generated by introduction of the deletion (24). For BVDV, two isolates containing cp DIs have been described (14, 38). The data reported above for pA/BVDV/D9 strongly support the hypothesis that the presence of the appropriate deletion is the only prerequisite for a cp DI. This is interesting because only one mutation at the genome level is responsible for three different phenotypic changes, namely, defectiveness, the ability to interfere with the helper virus replication, and the induction of CPE. It is an open question at the moment whether the reduction of genome size alone is sufficient to create a pestivirus DI. Future analyses with defective RNAs derived from appropriately mutated versions of the infectious pestivirus clones will help to answer this question.

The cytopathogenicity of pestiviruses is correlated with the expression of the nonstructural protein NS3. For BVDV and some border disease virus strains, NS3 is expressed only by cp viruses while noncp isolates express solely NS2-3 (2, 6, 11, 21, 22, 27, 36, 38). In the case of CSFV, noncp isolates also express a protein which is identical or at least very similar to NS3. However, the amount of NS3 is highly increased in cells infected with the cp isolates (23). Transient-expression studies and in vitro translation experiments revealed that the (enhanced) expression of NS3 is due to the genomic changes found in cp pestiviruses (14, 22, 23, 36–38). Protein analyses conducted for the BVDV strains recovered from in vitro-transcribed RNA showed the expected results. In cells infected with V(pA/BVDV), both NS2-3 and NS3 were detected, while V(pA/BVDV/Ins−) was able to express NS2-3 but not NS2 or NS3. For pA/BVDV/D9, expression of NS3 was demonstrated after transient expression (data not shown). Thus, the data resulting from the analyses with the infectious CSFV and BVDV clones clearly show that the mutations leading to the expression of NS3 represent the only prerequisite for a cp phenotype and therefore prove that (enhanced) expression of NS3 results in CPE. Future work will be directed toward elucidation of the mechanism by which NS3 is able to induce cell lysis.

Application of so-called reverse genetics represents one powerful tool of modern virology. The fact that both CSFV and BVDV are now amenable to this approach will certainly have great influence on pestivirus research and on the work on related human viruses.

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