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Bovine Viral Diarrhea Virus: Characterization of a Cytopathogenic Defective Interfering Particle with Two Internal Deletions

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Molecular characterization of bovine viral diarrhea virus pair 13 revealed that isolate CP13 is composed of a cytopathogenic (cp) defective interfering particle (DI13) and a noncytopathogenic (noncp) helper virus. The DI13 genome possesses two internal deletions of 1,611 and 3,102 nucleotides. Except for a small fragment of the gene coding for glycoprotein E1, all structural protein genes are deleted together with most of the N^{pro} gene, the region coding for nonstructural proteins p7 and NS2. While the amino terminus of NS3 seems to be strictly conserved for all other cp bovine viral diarrhea viruses, NS3 of DI13 is amino-terminally truncated and fused to 23 amino acids derived from N^{pro} and E1. Characterization of the DI-helper virus system revealed a striking discrepancy between RNA production and generation of infectious viruses.

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus* within the family *Flaviviridae*. Other members of the *Pestivirus* genus are border disease virus of sheep and classical swine fever virus (61). The pestivirus genome consists of a single-stranded nonpolyadenylated RNA molecule with a size of about 12.5 kb and positive polarity (10, 13, 33, 39). The first third of this RNA codes for an autoprotease and four structural proteins, while the 3′ part of the genome encodes the other nonstructural proteins (9, 11, 54, 58). Viral gene expression occurs via synthesis of a polyprotein and subsequent proteolytic processing by host cell and viral proteases (9, 11, 49, 54, 62, 63).

Cytopathogenic (cp) as well as noncytopathogenic (noncp) isolates of BVDV are found in cattle suffering from a variety of clinical syndromes such as diarrhea and abortion (1, 18, 57). Both cp and noncp BVDV are involved in the development of fatal mucosal disease (MD). As a first step in the unique pathogenesis of MD, congenital infection with a noncp BVDV occurs, which results in the birth of calves showing specific immunotolerance to the respective virus strain and lifelong persistence of the virus (1, 5, 6, 57). Such persistently infected animals may spontaneously develop MD, usually at an age of 6 to 18 months. Importantly, the cp biotype can always be isolated from such animals in addition to the noncp biotype (32). The cp and noncp virus derived from one animal are termed a "virus pair." Members of such a pair are antigenically very closely related (12, 42).

At the molecular level, one important difference between cp BVDV and noncp BVDV is the occurrence of NS3 (p80) in cells infected with a cp virus. NS3 is colinear with the carboxy-terminal part of NS2-3 (p125); the latter is present in cells infected with either cp BVDV or noncp BVDV (12, 19, 36, 37, 42, 44, 56). The genomes of several cp BVDV strains contain small host cell-derived insertions in the region coding for NS2-3 (34, 35, 55). For other cp BVDV strains, large duplications of viral sequences encompassing the NS3-coding region were identified together with cellular insertions or additional duplications of viral sequences (36, 37, 45). The available data indicate that the identified genome alterations are responsible for the expression of NS3 and the cytopathic effect (CPE) observed for these viruses. Accordingly, the generation of these cp BVDVs is a result of a recombination which leads to integration of additional sequences into the genome of noncp viruses. Since the generation of the cp viruses within persistently infected animals is regarded as crucial for the outbreak of MD, a direct linkage between RNA recombination and a lethal disease can be hypothesized.

In addition to insertion of sequences, deletions can result in cp BVDV. Investigation of BVDV CP9 led to identification of a cp defective interfering particle (DI) with an internal genomic deletion of 4,263 nucleotides (56). In the polyprotein encoded by the DI genome, the putative amino terminus of NS3 is fused to the carboxy terminus of the autoprotease N^{pro}, which is known to cleave at its own carboxy terminus (54, 62). In the case of DI9, cleavage by N^{pro} probably generates the amino terminus of NS3.

BVDV CP13 contains a cp DI. Characterization of virus pair 13 (12) was first performed by Northern (RNA) blot analysis. Total RNA from MDBK cells (American Type Culture Collection, Rockville, Md.) infected with either CP13 or NCP13 was isolated and hybridized with a BVDV-specific cDNA probe (36). In lanes with RNA from cells infected with NCP13 and CP13, a band migrated similar to the 12.5-kb genomic RNA of BVDV NADL. Analogous to BVDV CP9, a much stronger second BVDV-specific band with a size of about 7.7 kb was visible in the lane with RNA from CP13-infected cells (Fig. 1).

To assess the influence of the multiplicity of infection (MOI) on the amount of the 7.7-kb RNA, MDBK cells were infected with different dilutions of a CP13 stock. The cultures were analyzed 48 h postinfection by Northern blot hybridization. While at a high MOI, the 7.7-kb RNA band represented the only detectable signal on these blots, it decreased with higher dilutions, and the 12.5-kb genomic RNA became more and more prominent (Fig. 2). Thus the 7.7-kb RNA showed the typical features of a DI genome, namely dependence on and interference with helper virus replication. Interestingly, no

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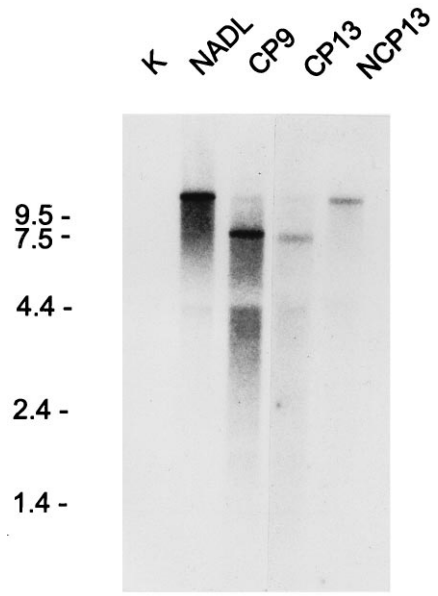


FIG. 1. Northern blot analysis of RNA from noninfected MDBK cells (K) or MDBK cells infected with BVDV isolates NADL, CP9, CP13, and NCP13. Gel electrophoresis, transfer, and hybridization with a BVDV NCP1-derived probe (36) labeled by nick translation (46) were done as described before (48). The exposure time was 24 h for all lanes. Numbers on the left indicate the sizes of an RNA ladder in kilobases. Additional bands in the range of 4.4 kb represent gel artifacts resulting from the 28S rRNA.

TABLE 1. Inoculation of MDBK cells with material isolated from individual BVDV CP13 plaques with and without BVDV NCP13 superinfection

Dish	CPE detection with[a]:	
	Superinfection	No superinfection
1	+	+
2	+	+
3	+	−
4	+	−
5	+	−
6	+	−
7	+	−
8	+	−
9	+	−
10	+	−
11	+	−
12	+	−
13	+	−
14	+	−
15	−	−
16	−	−
17	−	−
18	−	−
19	−	−
20	−	−
21	−	−
22	−	−
23	−	−
24	−	−

[a] +, detection of CPE; −, no CPE detectable. Isolation of plaque material and reinfection were performed as previously described (56).

CPE could be detected after infection of cells with CP13 at a dilution of 1:78,125. Thus, the CPE and the DI are both lost at higher dilutions. To verify that the presence of the DI correlates with the induction of CPE, material from individual plaques was isolated, diluted, and used for infection of MDBK cells. For each plaque, two equivalent infections were performed. One set of cultures was incubated without further treatment. CPE was detected in 2 of the 24 dishes. In a parallel experiment, the second set of cultures was superinfected 1 h after addition of the plaque material with noncp BVDV NCP13. In 14 of 24 cases, CPE was visible (Table 1). The result strongly suggests that the development of CPE is dependent on the presence of a helper virus.

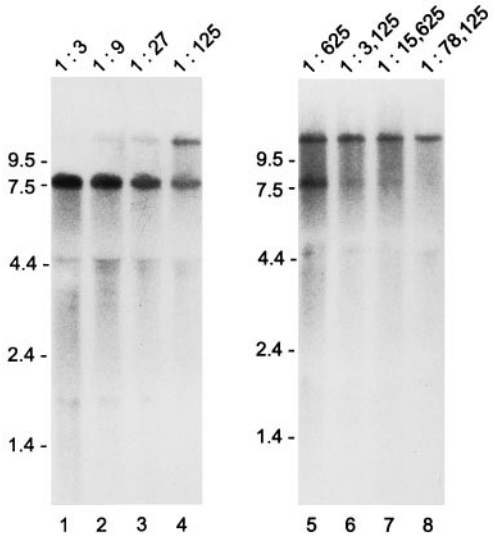


FIG. 2. Northern blot analysis of total RNA from MDBK cells infected with different dilutions of a CP13 virus stock. The dilution of the virus stock used for each infection is indicated above the lanes. Hybridization was done with a probe derived from cDNA clone D10 (this report). The sizes of an RNA ladder are indicated in kilobases on the left.

In addition to the plaque isolation experiment, RNA transfections according to the DEAE-dextran method (56, 59) were carried out. Total RNA from CP13-infected cells which contained no detectable amount of the 12.5-kb genomic RNA but a large amount of DI13 RNA was used for transfection (RNA comparable to Fig. 2, lane 1). Transfection of noninfected MDBK cells did not lead to CPE; in addition, BVDV-specific RNA could not be demonstrated after Northern blot analysis (Fig. 3, lane 3), and viral proteins were not detected by immunofluorescence (data not shown). However, when cells were infected with noncp BVDV NCP13 prior to transfection, CPE was observed. Northern blot analysis demonstrated the presence of DI RNA (Fig. 3, lane 4). These investigations again showed that the development of CPE strictly correlates with the presence of the DI RNA. Since the assistance of a noncp helper virus was obligatory for the induction of cell lysis, the presence of an autonomously replicating cp BVDV strain could be excluded.

Genome structure of the CP13 DI. To identify the location of the deletion in the DI13 genome, cDNA cloning and sequencing were performed (36, 51). Computer analysis (14) revealed that the determined sequence is colinear with the BVDV SD-1 genome up to nucleotide 424. The 30 nucleotides downstream of this residue correspond to positions 2036 to 2065. This fragment is followed by a sequence that can be aligned with the BVDV SD-1 sequence starting at nucleotide 5168. The resulting gaps in the genome of DI13 encompass nucleotides 425 to 2035 (1.611 kb) and 2066 to 5167 (3.102 kb).

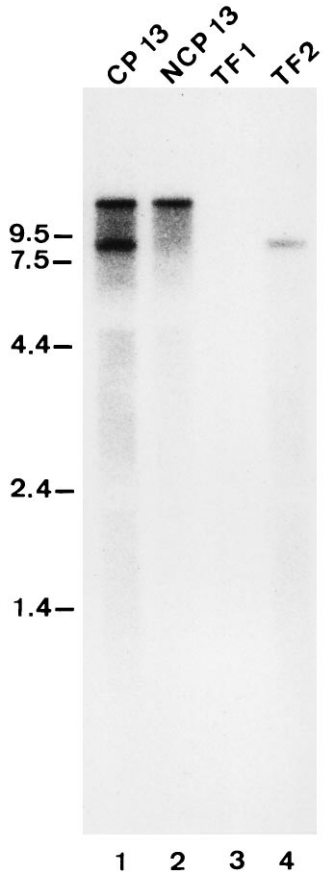


FIG. 3. Northern blot analysis of total RNA from MDBK cells infected with CP13 virus (lane 1) or NCP13 virus (lane 2). Lanes 3 and 4 show the results of a transfection experiment with RNA from CP13-infected cells (TF1 and TF2). CP13 DI-rich RNA was transfected in noninfected cells (TF1) and NCP13-infected cells (TF2). CPE was only detectable for TF2. The RNAs were isolated after one passage and hybridized against a homologous probe derived from D10 (this report). The sizes of an RNA ladder are indicated in kilobases on the left. After longer exposure, genomic BVDV RNA with a size of 12.5 kb could be detected in lane 4 but not in lane 3.

The sequences surrounding the deletions were confirmed by sequencing of four independent cDNA clones (D9, D10, D11, and D12). Thus, in contrast to DI9, the DI13 genome exhibits two deletions. The DI13 RNA has the capacity to code for a continuous protein, which starts with the original amino terminus of the BVDV polyprotein. In the DI13 polyprotein, only the first 13 amino acids of N^{pro} are present (Fig. 4). Because of the first deletion, this fragment of N^{pro} is fused to a stretch of 10 amino acids (aa) corresponding to aa 551 to 560, which is part of glycoprotein E1. The second deletion includes aa 561 to 1594. The analyzed sequence continues with aa 1595, which is part of NS2-3 (Fig. 4). Therefore, the deletion encompasses almost the complete autoprotease N^{pro} and the entire structural proteins, with exception of 10 aa of glycoprotein E1. In addition, protein p7 (15) and NS2 are missing (Fig. 4). For DI9, the deletion encompasses the genes for the core protein (C), the glycoproteins (E0, E1, and E2), p7, and NS2 (56).

The 3′ recombination site previously identified for DI9 is exactly the same as the one detected in three cp CSFV DI RNAs and cp BVDV genomes with cellular ubiquitin-coding insertions or duplication and rearrangement of viral sequences (34–38, 45, 55). Since in these cases the 3′ recombination position determines the amino terminus of NS3, all of these viruses express NS3 with the same amino-terminal sequence starting with the motif GPAVCKK or MGPAVCKK. The conservation of the 3′ recombination point was hypothesized to be of functional importance (36, 37). DI13 represents the first BVDV expressing an NS3 protein with an amino terminus which is clearly different from that of other cp isolates.

Internal deletions are known to occur in DI RNAs of both negative- and positive-strand viruses (8, 40, 41, 52). Deletions in RNA virus genomes result from recombinations which probably are a consequence of template switching by the viral RNA-dependent RNA polymerase (29, 31, 52). According to this mechanism, the DI13 genome was generated in a recombination including two template switches. Interestingly, most of the recombinations leading to cp BVDV require two template switches (36, 37, 45, 55). In contrast, BVDV DI9 and the CSFV DIs have been generated by one template switch (38, 56). The latter type of recombination should occur more often than the one requiring two switches. It is not known why DIs like DI9 or the classical swine fever virus DIs have been found only rarely, but it can be assumed that other types of cp pestiviruses have yet unknown selective advantages which overcome the improbability of their generation.

The helper virus. The dilution experiments demonstrated the presence of a noncp BVDV in the CP13 isolate. After infection of cells with high dilutions of the virus stock, only the 12.5-kb RNA band was visible in Northern blot analysis (Fig. 2, lane 8). With a similar RNA preparation as starting material, a cDNA library was established. The terminal sequences of two BVDV-specific clones were analyzed. In addition, a cDNA library with RNA of NCP13-infected cells was established. Several cDNA clones were obtained and analyzed by terminal sequencing. The data demonstrated the absence of the deletions identified in the DI13 genome. Moreover, comparison of the first 250 nucleotides of the NS3 gene revealed 100% identity between the sequences determined for DI13, NCP13, and the noncp helper virus derived from the CP13 virus stock. Apparently, the helper virus from the cp isolate is identical to NCP13, which was isolated from organ material of an infected animal and biologically cloned via end point dilution (12). In consequence, pair 13 consists of two viruses: a cp DI and the noncp helper virus NCP13. The sequence homology demonstrates that DI13 has developed from NCP13 by a process of RNA recombination.

Proteins derived from the DI13 genome. All cp BVDV isolates can be distinguished from noncp BVDV by their ability to express NS3, a protease which cleaves at its own C terminus and which is responsible for processing of the nonstructural protein region downstream of NS3 (63). In all strains analyzed so far, NS3 has the same size (19). This observation together with data from processing studies conducted for different BVDV strains led to the conclusion that proteolytic cleavage at the amino terminus of NS3 always occurs at amino acid position 1590 or very close to it (37, 55, 56). An obvious question resulting from the genome structure of DI13 was whether the fusion protein composed of the N^{pro} and E1 fragments and the amino-terminally truncated NS3 is further processed. In a first approach, protein expression in cells infected with different BVDV strains was analyzed by immunoprecipitation with an NS2-3/NS3 specific serum (antiserum A3 [58]). NS2-3 with a size of about 125 kDa was detected for CP13, NCP13, and the noncp helper virus derived from the CP13 stock. As expected, a second band (NS3) could be detected for CP13 but not for the two noncp viruses (Fig. 5). In a direct comparison with the corresponding protein from BVDV Osloss and NADL, NS3 of CP13 migrated more slowly, indicating a higher molecular weight of the protein (Fig. 6).

Processing of the DI13-encoded polyprotein was studied in a transient expression system with vaccinia virus vTF7-3, which expresses the bacteriophage T7 RNA polymerase (17). To analyze the expression of NS3, the DI13-derived cDNA insert of clone D10 was cloned according to standard procedures (50) into plasmid pCITE (Novagen, AGS, Heidelberg, Germany) after generation of an *Nco*I site at the translation start codon of the open reading frame. Site-directed mutagenesis was done according to the method of Kunkel et al. (30) with oligonucleotide BVDV-ATG (5′ CAA CTC CAT GGG CCA TGT ACA 3′). The resulting construct (D29) should be able to direct expression of the original amino-terminal region of the DI13 polyprotein encompassing the N^{pro} and E1 fragments and the NS3 protein. For a second construct (D34), an *Nco*I restriction site was introduced in the DI13 cDNA at the beginning of the NS3-coding region with oligonucleotide BVDV-NS3 (5′ TTT TCT TCA CCA GTG ACA ATA GGA 3′). D34 contains the NS3-coding region together with the translation initiation codon but not the 22 codons located between the AUG and the NS3 gene. Finally, two additional plasmids were constructed: D35 contains the N^{pro} but not the E1-coding fragment, while from D36 the N^{pro}-derived codons except for the initial AUG were removed. The 3′ end of the DI13-derived inserts was the same for all four expression clones and is located within the NS4B gene.

Transient expression was carried out as described before (56). The labeled cell extracts were subjected to immunoprecipitation with specific antisera. Expression of D29 gave rise to a protein comigrating with NS3 of CP13-infected cells (Fig. 6, lanes 3 and 5, respectively). In contrast, the corresponding protein of D34 comigrated with that from Osloss and NADL-infected cells (Fig. 6, lanes 4, 6, and 7, respectively). It therefore seems likely that the short fragments derived from N^{pro} and E1 are not removed from the truncated NS3 protein of DI13. Experiments with the NS3 protease of hepatitis C virus showed that amino-terminal truncations of 7 to 15 aa had dramatic effects on the cleavage efficiency of the enzyme (3, 16). These findings together with the conservation of the NS3 amino terminus in cp BVDV led to the idea that the presence of the 23 aa derived from N^{pro} and E1 is functionally important to compensate for the 5 residues deleted from NS3. To determine whether the foreign sequences at the amino terminus of NS3 from DI13 influence the proteolytic function of this protein, the generation of NS4A was studied after transient expression of D29, D34, D35, and D36. Immunoprecipitation with antiserum P1 (37) resulted in precipitation of NS4A with a size of about 8 kDa, NS4B*, a protein with a size of 15 kDa representing the carboxy-terminally truncated NS4B, and the precursor NS4A/4B* (Fig. 7). No difference in processing efficiencies could be detected. Thus, the additional 23 aa at the amino terminus of the DI13-encoded NS3 seem to be dispensable for processing at the NS3/NS4A and NS4A/NS4B sites. However, it has been reported for HCV that detection of the effects of truncations depends on the assay system and the processing site analyzed (4). Moreover, processing at the two sites analyzed here represents only one activity of this multifunctional protein. It is therefore still possible that the N^{pro}- and E1-derived parts of the NS3 fusion protein are required to obtain a viable cytopathogenic DI. Such questions can be directly addressed with genetically modified viruses recovered from infectious clones.

Titration experiments. DIs have been described for a large number of animal virus systems (for review, see references 2, 22, and 40). Different studies demonstrated that the ratio between DI and helper virus varies in the course of infection and is heavily influenced by the MOI used for the initial inoculation (22, 23, 47). Quantitative analysis of helper virus-DI systems usually is difficult, because the amount of DI cannot be easily determined. In other systems, measurement of DI titers was based on (i) determination of the rate of interference (24, 53),

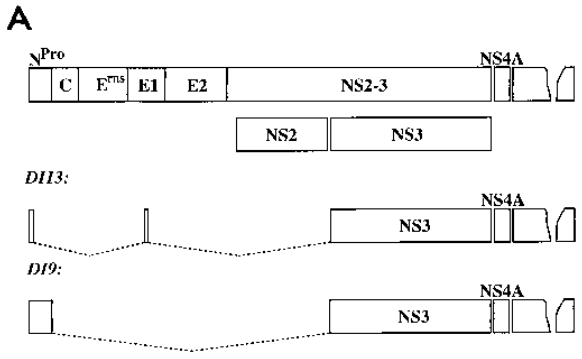
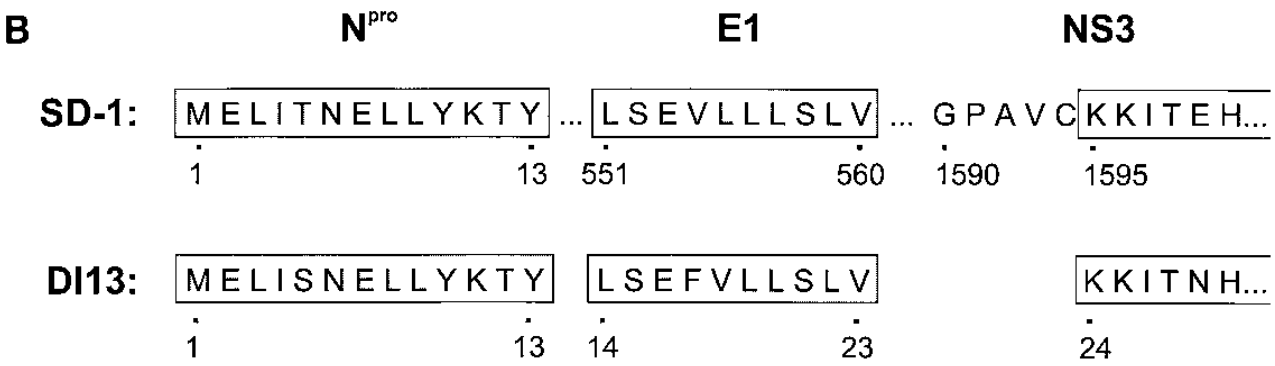


FIG. 4. (A) Schematic presentation of the DI13 genome in comparison with the organization of a standard BVDV genome and the genome of BVDV DI9 (56). The location of the genes coding for N^{pro} (viral autoprotease; nonstructural protein), the capsid protein (C), the three structural glycoproteins (E^{rns}, E1, and E2), and the nonstructural proteins NS2-3 (serine protease and helicase) and NS4A is indicated. The DI13 genome possesses two deletions. The first deletion spans almost the entire N^{pro} gene, the capsid protein, the E^{rns} gene, and part of the E1-coding region. The second deletion spans the 3′ part of the region coding for E1, and the genes for E2, p7, and NS2. The sequence of part of the CP13 DI genome was determined after establishment of exonuclease III/nuclease S1 deletion libraries (21) and was deposited at the GenBank EMBL sequence data library under accession number Z73248. (B) Alignment of part of the amino acid sequence deduced from the DI13 sequence with corresponding regions of the BVDV SD-1 polyprotein (marked by boxes). The positions of several amino acids in the respective polyproteins are indicated. Amino acid 1590 represents the hypothesized amino terminus of NS3.



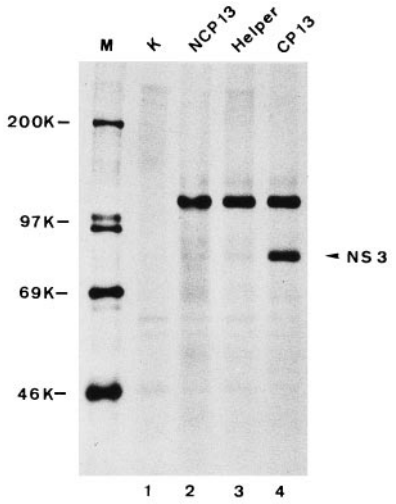


FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of immunoprecipitates after metabolic labeling of MDBK cells infected with the viruses indicated on top. The precipitation was carried out with antiserum A3, which is directed against NS2-3/NS3 (lanes 2 to 4) or as a control (K) with preimmune serum (lane 1). The cell extracts were prepared under denaturing conditions (20). Precipitates were formed with cross-linked *Staphylococcus aureus* (27).

(ii) counting, or (iii) physical separation of DI particles (28). Because of their cp phenotype, pestiviral DIs can easily be detected and distinguished from the noncp helper viruses. To learn more about the biological properties of the DI13-NCP13 system, the amount of infectious DI particles and helper viruses was analyzed. The induction of CPE served as a parameter for DI replication, while the helper virus was detected via indirect immunofluorescence. To circumvent the problem of wrong DI titers due to unsufficient amounts of helper virus at higher dilutions, titrations aiming at determination of plaques were carried out in two parallel experiments, namely with noninfected MDBK cells and with cells infected with NCP13 as exogenous helper virus. Titration of our virus stock revealed 4×10^3 PFU and, according to immunofluorescence, 1.5×10^5

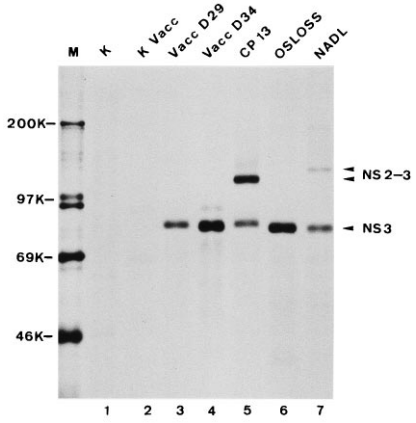


FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitates generated with antiserum A3 after metabolic labeling of MDBK cells infected with CP13 (lane 5), Osloss (lane 6), or NADL (lane 7) or BSR cells infected with vaccinia virus vTF7-3 (17) and transfected with constructs D29 (lane 3) and D34 (lane 4). The sizes (in kilodaltons) of marker proteins (M) are indicated. K, control of noninfected MDBK cells precipitated with antiserum A3 (lane 1); K Vacc, vaccinia virus control (BSR cells infected with vTF7-3 but not transfected) (lane 2).

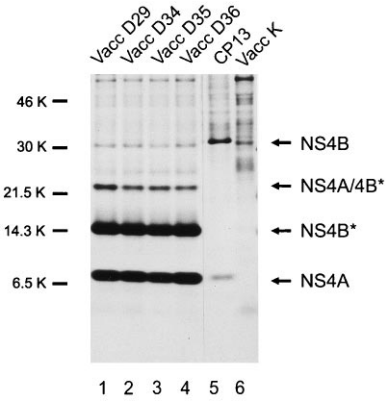


FIG. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of metabolically labeled proteins precipitated from BSR cells infected with vTF7-3 and transfected with the indicated plasmids (lanes 1 to 4) with antiserum P1, which reacts with NS4A and NS4B (37). In lane 5, the proteins precipitated from BVDV CP13-infected MDBK cells are shown as a control. Vacc K, vaccinia virus control; NS4B*, carboxy-terminally truncated NS4B; NS4A/4B*, precursor composed of NS4A and NS4B*. The locations and sizes (in kilodaltons [K]) of marker proteins are indicated.

focus-forming units per ml. The numbers of PFU determined with or without addition of exogenous helper virus were nearly identical. Thus, even at high dilutions, almost every DI is rescued by the internal helper viruses, which indicates that DI RNA apparently can persist within a cell until the helper viruses have replicated and spread to all cells present in the dish.

Infections of MDBK cells with different dilutions of the CP13 virus stock were carried out. After 48 h, part of the cells was harvested for titration, while RNA was prepared from the rest of the material. Production of the cp DI could be detected when infections were carried out with dilutions up to $1{:}5^{-5}$ (Table 2). Regarding the highest tested MOI, the DI yield was about 1.5×10^4/ml. Since the titer of the helper virus determined for the same samples was in a range of 10^5 to 10^6/ml (lowest to highest MOI, respectively) the number of infectious DIs was lower than that of the helper viruses for every dilution. However, the results of the Northern blot analysis carried out with the RNA derived from the same cultures were completely different. As shown in Fig. 2, infections with dilutions up to 1:625 resulted in detection of much more DI than helper virus RNA, which according to the behavior of marker bands is not due to insufficient transfer of the genomic RNA. Since similar

TABLE 2. Titration of cell extracts prepared from cultures infected with different dilutions of a BVDV CP13 stock

Inoculation[a]	CPE at dilution[b]:					
	5^{-1}	5^{-2}	5^{-3}	5^{-4}	5^{-5}	5^{-6}
1:3	+	+	+	+	+	−
1:9	+	+	+	+	−	−
1:27	+	+	+	+	−	−
$1{:}5^{-3}$	+	+	+	+	−	−
$1{:}5^{-4}$	+	+	+	+	−	−
$1{:}5^{-5}$	+	+	−	−	−	−
$1{:}5^{-6}$	−	−	−	−	−	−

[a] Dilutions of the CP13 stock used for the initial infections.

[b] Results of the titrations for the extracts prepared 48 h postinfection. +, detection of CPE; −, no CPE detectable. The titration was carried out according to the method of Spearman and Kaerber (25) with MDBK cells infected with NCP13 as exogenous helper virus 6 h before addition of 200 μl of the respective virus dilution.

results were obtained for the supernatant of the cultures, the discrepancy between the ratio of virus titers and the ratio of RNA yields observed for DI and helper virus is not due to a packaging defect of the DI RNA. One possible explanation for the finding is that not every infection with a DI leads to development of a CPE. Since, however, the titrations were all carried out in the presence of exogenous helper virus, this argument doesn't seem very strong. Another reason for our findings could be that DIs contain more RNA molecules than do the helper viruses. For alphaviruses, five to six DI genomes were found in one virion (7, 26). However, the respective DI genomes were small, and the authors hypothesized that the total RNA content was rather constant for the different particles, which in our case would allow an increase in molecule number to only 1.6 times compared with that of the helper virus. A third reason for our results could be that the ratio between particle number and infectious units is lower for the DI than for particles containing the full-length viral RNA. Further investigations are necessary to test these hypotheses and to analyze this issue for the other pestiviral DIs.

In our experiments, both the DI and helper virus titers were found to be about 1 order of magnitude higher in the supernatant than in cell extracts. This finding contradicts earlier reports that pestiviruses tend to be associated with the host cell and that therefore cell extracts contain more infectious viruses than the supernatant (43, 60). We cannot exclude that the difference between the published results and our observations is due to the virus strain and/or the cells used in this study.

The pathogenesis of MD is dependent of the development of a cp BVDV from a noncp ancestor within a persistently infected animal. Cytopathogenic DIs as described for BVDV CP9 and CP13 were isolated from animals that came down with MD. In both cases, the DIs are responsible for the development of a CPE in cell culture. It therefore seems likely that an outbreak of MD can be correlated with the development of cp DIs in the animal. Cell culture experiments have begun to unravel the complexity of the DI-helper virus systems. Elaborate investigations are necessary to elucidate how such a system behaves within a whole organism.

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