Noncytopathogenic Pestivirus Strains Generated by Nonhomologous RNA Recombination: Alterations in the NS4A/NS4B Coding Region

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Several studies have demonstrated that cytopathogenic (cp) pestivirus strains evolve from noncytopathogenic (noncp) viruses by nonhomologous RNA recombination. In addition, two recent reports showed the rapid emergence of noncp *Bovine viral diarrhea virus* (BVDV) after a few cell culture passages of cp BVDV strains by homologous recombination between identical duplicated viral sequences. To allow the identification of recombination sites from noncp BVDV strains that evolve from cp viruses, we constructed the cp BVDV strains CP442 and CP552. Both harbor duplicated viral sequences of different origin flanking the cellular insertion Nedd8*; the latter is a prerequisite for their cytopathogenicity. In contrast to the previous studies, isolation of noncp strains was possible only after extensive cell culture passages of CP442 and CP552. Sequence analysis of 15 isolated noncp BVDVs confirmed that all recombinant strains lack at least most of Nedd8*. Interestingly, only one strain resulted from homologous recombination while the other 14 strains were generated by nonhomologous recombination. Accordingly, our data suggest that the extent of sequence identity between participating sequences influences both frequency and mode (homologous versus nonhomologous) of RNA recombination in pestiviruses. Further analyses of the noncp recombinant strains revealed that a duplication of 14 codons in the BVDV nonstructural protein 4B (NS4B) gene does not interfere with efficient viral replication. Moreover, an insertion of viral sequences between the NS4A and NS4B genes was well tolerated. These findings thus led to the identification of two genomic loci which appear to be suited for the insertion of heterologous sequences into the genomes of pestiviruses and related viruses.

Together with *Classical swine fever virus* and *Border disease virus*, the genus *Pestivirus* includes the species *Bovine viral diarrhea virus 1* (BVDV-1) and BVDV-2; the latter are causative agents of economically important diseases in cattle (9). Pestiviruses belong to the family *Flaviviridae*, which also comprises the closely related human *Hepatitis C virus* as well as flaviviruses like *Yellow fever virus* and *West Nile virus* (23).

Pestivirions are small, enveloped particles with a single-stranded RNA genome of positive polarity and a length of about 12.3 kb. The viral genomic RNA is neither capped nor polyadenylated and contains one large open reading frame (ORF) that is flanked by 5' and 3' nontranslated regions (NTRs). The NTRs harbor *cis*-active elements that are essential for viral replication. The ORF encodes a polyprotein of approximately 3,900 amino acids that is co- and posttranslationally processed to the mature viral proteins by cellular and viral proteases. The N-terminal third of the ORF encodes the viral autoprotease N^{pro}, a nonstructural protein (NS), as well as the structural proteins, namely, the capsid protein C and the glycoproteins E^{rn}s (RNase soluble), E1, and E2. The remaining part of the polyprotein is processed to the nonstructural proteins p7, NS2-3 (NS2, NS3), NS4A, NS4B, NS5A, and NS5B. Together with putative cellular cofactors, NS3 to NS5B are part of the viral replication complex (for reviews, see references 34, 38, and 48).

According to their effects on susceptible tissue culture cells, cytopathogenic (cp) and noncytopathogenic (noncp) pestiviruses are distinguished (22, 32). Molecular analyses of cp pestivirus strains revealed that they originate from noncp viruses mostly by nonhomologous RNA recombination. As a result, cp pestiviral genomes harbor genomic alterations that frequently include insertions of cellular mRNA or viral sequences as well as deletions or duplications of viral sequences (7, 10, 20, 21, 37, 38, 44). The generation of cp BVDV strains in animals persistently infected with noncp BVDV was frequently reported (for examples, see references 5 and 8) and is crucial for the induction of lethal mucosal disease in cattle (11, 12). Interestingly, two recent studies demonstrated that cell culture passages of recombinant cp BVDV strains led to reversion to the noncp phenotype (4, 8). Sequence analyses of the respective noncp strains revealed that homologous recombination between identical duplicated viral sequences resulted in the removal of both one copy of the duplicated viral sequences and the inserted host-derived sequences. In consequence, a precise determination of the recombination sites was not possible.

To investigate the reversion to noncp viruses in more detail, we describe here a different approach using recombinant cp BVDV strains CP442 and CP552; both harbor sequences encoding part of cellular Nedd8 (neural precursor cell expressed developmentally down-regulated). This Nedd8*-termed sequence was previously shown to be responsible for viral cytopathogenicity when inserted in the 5'-terminal region of a duplicated NS3 gene (4). For both CP442 and CP552, Nedd8* is flanked by duplicated viral sequences of different origin. This modification allowed us to precisely determine the recombination sites of emerging noncp

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recombinant strains that had lost the Nedd8* insertion after cell culture passages. Moreover, the significance of sequence identity between participating sequences was investigated with regard to frequency and mode (homologous versus nonhomologous) of RNA recombination. Finally, characterization of the isolated recombinant noncp viruses provided interesting insights into changes tolerated within the genomic region encoding the BVDV replicase.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum. BVDV field isolate CP821 has been described previously (4). All other BVDV strains were obtained after transfection of synthetic RNAs derived from infectious cDNA clones pCP442, pCP552, pNCP7-5A, pNCP7/552-R3, pNCP7/552-R4, and pNCP7/552-R5, respectively (see below).

Oligonucleotides. The following primers (MWG Biotech, Ebersberg, Germany) were used for PCR: OI 821-5333-AgeI (5'-ATCACCGTTGGGCTTACACA CATCAAG-3', nucleotides (nt) 5333 through 5360); OI 821-7756-R-SalI (5'-A TCGTCTGACTGCTGCCTGCTG-3', nt 7756 through 7735); OI 821/7-NS4-R-NsiI (5'-GCCATGTCATAGTCCGAGATGGAACCCATGATTTTGTTCA AGTCAACCACTGCCAGTTCTTCAGTTTCG-3', nt 7469 through 7402); OI CP7-B24 (5'-CCGGCCTTCTTCGACCT-3', nt 5627 through 5643); OI CP7-7863-R-ApaI (5'-AGTGGGCCCAAGCGGAGATGAACAG-3', nt 7863 through 7838); OI BVD-7209 (5'-AGCAAGTGGT[A/C]GGAGTGTG-3', nt 7209 through 7227). Letters in brackets represent wobbled nucleotides. The underlined sequences indicate restriction sites of endonucleases included in the names of the oligonucleotides.

Reverse transcription (RT)-PCR, molecular cloning, and nucleotide sequencing. Respective methods have been described previously (8).

Construction of cDNA clones. Nucleotide numbering throughout this study refers to the sequence of infectious full-length BVDV cDNA clone pCP7-5A (6). For the construction of plasmid pCP442, first, an RT-PCR fragment was generated encompassing part of the genomic region encoding NS3, NS4A, and NS4B of BVDV strain CP821. Thereby, an AgeI (5336) site and a SalI (7748) site, both representing silent mutations, were introduced by the sense and antisense primers used for PCR (OI 821-5333-AgeI; OI 821-7756-R-SalI). The obtained CP821-derived fragment was cloned into the vector pCR 2.1 (Invitrogen, Carlsbad, Calif.). Finally, the AgeI (5336)-SalI (7748) fragment of the resulting plasmid p821-NS3/NS4B was introduced into the previously described infectious cDNA clone p7/821 (4) precut with AgeI-SalI (see Fig. 1). For the construction of pCP552, a PCR was performed with primers OI 821-5333-AgeI and OI 821/7-NS4-R-NsiI, using p821-NS3/NS4B as template. The obtained PCR product was cloned into pCR 2.1, resulting in plasmid p821-NS3/CP7-NS4B. The latter harbors sequences corresponding to the 3' part of the NS3^{4-end} gene and the NS4A gene of CP821, which are directly followed by the genomic region encoding the N-terminal part of NS4B of CP7-5A. Eventually, the AgeI (5336)-SalI (7748) fragment of plasmid p821-NS3/CP7-NS4B was introduced into p7/821 precut with AgeI-SalI. The infectious noncp BVDV cDNA clone pNCP7-5A has been described previously (4). To generate cDNA clones pNCP7/552-R3, pNCP7/552-R4, and pNCP7/552-R5, RT-PCRs were performed using ProofStart polymerase (QIAGEN, Venlo, The Netherlands) and oligonucleotides OI CP7-B24 (nt 5627 through 5643) and OI CP7-7863-R-ApaI; total RNA from cells infected with the recombinant noncp BVDVs NCP552-R3, -R4, and -R5 served as templates. Respective products were treated with the A-Addition kit (QIAGEN) and cloned into vector pDrive (QIAGEN). Finally, respective plasmids were cut with SacI (5883)-SalI (7748) and the resulting fragments with lengths of about 1.47 to 1.65 kb were cloned into pNCP7-5A precut with SacI-SalI (see Fig. 4A). All cDNA clones were controlled by nucleotide sequencing. Further information on cloning procedures and construction of cDNA clones is available on request.

Indirect immunofluorescence and determination of viral growth kinetics. Infection with noncp BVDV was detected by indirect immunofluorescence (IF) with monoclonal antibody 8.12.7 directed against NS3 (17). Indirect IF analysis and determination of viral growth kinetics have been described elsewhere (6).

In vitro synthesis of transcripts and transfection of RNA. After complete digestion of plasmids pCP442, pCP552, pNCP7-5A, pNCP7/552-R3, pNCP7/552-R4, and pNCP7/552-R5 with SmaI, linearized DNA was extracted with phenol-chloroform and precipitated with ethanol. RNA then was transcribed by use of SP6 RNA polymerase (Takara, Shiga, Japan) using standard conditions. After

transcription, a DNase I (Ambion, Austin, Tex.) digestion was performed for 15 min. The quality and amount of RNA were controlled by ethidium bromide staining after agarose gel electrophoresis. The RNA transcripts used for transfection contained >80% intact RNA. For transfection, the confluent MDBK cells from a dish 10 cm in diameter were trypsinized, resuspended in 0.4 ml of phosphate-buffered saline without Ca²⁺ and Mg²⁺, and mixed with in vitro-transcribed RNA immediately before the pulse (950 μ F and 180 V). For electroporation, Gene Pulser II (Bio-Rad, Munich, Germany) was used. The electroporated cells were resuspended in 8 ml of medium, and 7.8 ml of this cell suspension was distributed to three wells of a six-well plate immediately post-transfection (p.t.). For control, cells were electroporated without adding synthetic RNA. Cells were checked 1 and 3 days p.t. by light microscopy for the appearance of a cytopathic effect (CPE) and by IF analysis for the expression of viral protein NS3.

Cell culture passages of infectious material derived from single plaques. To obtain infectious material derived from single plaques, 200 μ l from an 8-ml suspension of electroporated cells (see above) was serially diluted directly after transfection with CP442 RNA and CP552 RNA and used for plaque assays. Five days p.t., respective plates were examined by light microscopy, and for wells containing 1 to 10 individual plaques, the areas of cells exhibiting a CPE were marked with a pen. Thereafter, material of such plaques was picked, resuspended in 1 ml of medium, and used for further cell culture passages. After each cell culture passage, material for subsequent infections was prepared by freezing and thawing of cells 48 to 72 h postinfection (p.i.). If necessary, infectious material was stored at -70°C.

Isolation of noncp recombinant strains. After the first and every fifth passage, supernatants derived from infections with CP442 and CP552 were titrated and used for infections of cells in 24-well plates. Three days p.i., these plates were subbed for IF analysis. When foci of infected cells not showing signs of a CPE were detected in addition to cp plaques (for an example, see Fig. 2B), respective supernatants were diluted and used for plaque assays on 24-well plates. For wells that showed 5 to 10 plaques 3 days p.i., the plaques were removed by using a new pipet tip for each plaque. Finally, the agarose overlay was completely removed by washing with phosphate-buffered saline and the remaining cells were trypsinized, resuspended in medium, and seeded onto new six-well plates. After this procedure had been repeated several times and the ratio between noncp and cp BVDV estimated by IF analyses had become >1, respective cell culture supernatants were titrated on MDBK cells. Three days p.i., supernatants from cells showing no CPE were collected and the cells were examined by IF. Of these samples, only supernatants from wells whose cells did not show a CPE but were positive for BVDV in IF analysis were used for further infections of MDBK cells. Finally, total RNA of infected cells was harvested, thus representing samples from isolated noncp BVDVs.

RNA preparation, gel electrophoresis, and Northern (RNA) hybridization. Preparation of RNA, gel electrophoresis, radioactive labeling of the probes, hybridization, and posthybridization washes were performed as described previously (6). As BVDV-specific probe, the ClaI (11076)-AatII (12259) fragment of pCP7-5A was used. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific cDNA was a kind gift obtained from C. Grassmann (Institut für Virologie, Gießen, Germany) and has been described previously (29).

RESULTS

Genome organization of BVDV CP821. In a previous study, cp BVDV field isolate CP821 was characterized. In contrast to the common genome organization of noncp BVDV (for an example, see Fig. 1A, NCP7-5A), CP821 harbors a large duplication of viral sequences together with an insertion encoding part of cellular Nedd8 (Nedd8*); the latter is located in the 5' terminus of the 3'-terminal NS3 gene (Fig. 1B). After introducing Nedd8* together with flanking viral sequences into the genome of noncp BVDV strain NCP7-5A, it could be demonstrated that the CP821-derived sequences are responsible for the viral cytopathogenicity of the resulting chimeric BVDV strain 7/821 (Fig. 1C) (4). In the respective study, the genomic region downstream of the 3'-terminal NS4A gene of CP821 was not investigated. In the meantime, RT-PCR and sequence analyses of the respective genomic region revealed that the 5'-terminal NS4A gene is not directly followed by a

FIG. 1. Schematic representation of BVDV strains. (A) RNA genome of noncp BVDV strain NCP7-5A containing one large ORF (box) flanked by 5' and 3' NTRs (4). The positions of the genes encoding the viral structural proteins (capsid protein C and envelope proteins E^{ns}, E1, and E2) and NS proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B are indicated. Positions of restriction sites SalI, AgeI, and MluI present in the corresponding infectious cDNA clone pNCP7-5A are marked above and below the genome. The genomic region between the AgeI and MluI sites is diagonally hatched. (B) Genome organization of cp BVDV field isolate CP821. Sequences derived from noncp BVD strain ncp821 are shaded in light gray; sequences originating from BVDV strain H are displayed in dark gray (4). Sequences encoding part of cellular Nedd8 (Nedd8*), which are inserted in the 5' region of the 3'-terminal NS3 gene, are highlighted by a black box (4). On top, a detailed section of the CP821 genome is displayed for the region between the 5'-terminal NS4A gene and the NS4B gene. The length of each segment is given in nucleotides below the diagram. (C) Genome organization of cp BVDV strains 7/821 (4), CP552, and CP442. The gray scales refer to panels A and B. The introduction of CP821-derived sequences into the respective genomes is indicated by arrows. The positions of MluI, AgeI, and SalI sites that were used for construction of the corresponding cDNA clones are marked below each genome. A mutated AgeI site (Δ AgeI) served as genetic marker. For 7/821, the hatched NCP7-5A-derived duplicated sequences (see panel A) which flank the CP821-derived insertion are connected by brackets. The lengths of the bars are not drawn to scale. The genomic sizes of NCP7-5A, 7/821, CP552, and CP442 are indicated in kilobases (kb) on the right.

complete NS4B gene. Instead, the NS4A and NS4B genes are separated from each other by a chimeric duplicated viral sequence of 171 nt (Fig. 1B). The latter encodes an N-terminal fragment of NS4B which is fused to a 3'-terminal fragment of the NS4A gene (Fig. 1B, NS4B-N² and NS4A-C^{*}).

Construction of BVDV strains CP442 and CP552. After cell culture passages of 7/821, noncp BVDV rapidly emerged (4). Further analyses revealed that respective noncp BVDVs resulted from homologous RNA recombination between the identical duplicated sequences that flank Nedd8*. In consequence, a precise determination of recombination sites was not possible. The study presented here was designed to study RNA

recombination between duplicated viral sequences of different origin; this novel approach should allow us to precisely determine the crossover sites of emerging noncp BVDV recombinant strains. For that purpose, we first constructed BVDV strain CP442 (Fig. 1C, bottom), whose genome organization is mainly based on 7/821. In contrast to 7/821, where the duplicated sequences downstream of Nedd8* mostly originate from NCP7-5A (Fig. 1C, top), the genome of CP442 contains more than 2.6 kb of duplicated sequences derived from CP821. Accordingly, for CP442, most of the duplicated viral sequences are derived from two different BVDV strains (Fig. 1C, bottom). For CP442, the impact of the CP821-derived 171-nt

FIG. 2. Origin of isolated noncp BVDVs, immunofluorescence analysis, and Northern blot analyses. (A) After transfection of MDBK cells with synthetic transcripts derived from infectious cDNA clones pCP442 or pCP552, cells were either divided into separate cell pools (CP442 I-1 and CP442 I-2) or serially diluted and used for plaque assays (CP442 II + III, CP552 I + II). Two to five days posttransfection, supernatants CP442 I-1 and CP442 I-2 as well as material from single picked plaques (CP442 II + III, CP552 I + II) were used to perform subsequent cell culture passages. Passages were regularly checked by immunofluorescence analysis. Emergence of noncp BVDV strains was detected by IF analysis (for an example, see panel B); noncp BVDV strains NCP442-R1 to NCP442-R8 and NCP552-R1 to NCP552-R6 were isolated as described in Materials and Methods. (B) Cells were infected with CP442 (CP442 I-1, 11th cell culture passage) and fixed 48 h postinfection. Cells were subsequently examined by light microscopy (left) and IF using a monoclonal antibody directed against NS3 (right). Both images depict the same area of cells. Note that, in addition to plaques (left, indicated by arrows), a large focus of cells infected with noncp BVDV was detected only by IF (right). Magnification, $\times 100$. (C) Northern blot analyses of RNAs derived from different cell culture passages (p.) of CP442 and from infection with the isolated noncp BVDV strains NCP442-R1, NCP442-R2, and NCP442-R3. Note that for the latter three, the sizes of viral genomic RNAs were considerably shorter than the one of CP442 RNA obtained from 1st, 5th, and 10th cell culture passages. n.i., noninfected cells. The genomic RNA of BVDV NCP7-5A served as a marker whose size is depicted in kilobases on the right.

insertion between the 3'-terminal NS4A and NS4B genes (Fig. 1C, bottom) on viral viability and replication was unclear. Accordingly, an additional BVDV strain (CP552) was constructed; apart from the absence of the described alteration within the NS4A/NS4B coding region, the genome of CP552 is identical to that of CP442 (Fig. 1C, middle).

Emergence and isolation of noncp BVDV strains after cell culture passages of CP442 and CP552. After the transfection of synthetic CP442 RNA into MDBK cells, positive staining by IF assays as well as the appearance of a CPE indicated efficient viral replication. Directly after the first transfection experiment, cells were divided into two cell pools (CP442 I-1 and CP442 I-2) and supernatants derived from both attempts were passaged separately on MDBK cells (Fig. 2A, left). While IF analysis of cells infected with limiting dilu-

tions of supernatants derived from the first cell culture passages did not indicate the emergence of noncp BVDV strains, the presence of noncp strains was observed after the fifth passages (Fig. 2B; data shown only for CP442 I-1, 11th cell culture passage). Subsequently, to favor the release of virions from cells infected with noncp BVDV, two freeze-thaw cycles were performed after each cell culture passage. To facilitate the isolation of noncp BVDV in the background of cp BVDV infection, cells were overlaid with semi-solid medium. Four to five days p.i., areas of emerging plaques were removed from the cell monolayer; only the remaining cells were further cultivated (see also Materials and Methods). After a total of 15 to 25 cell culture passages, the repeated removal of cp plaques led to the successful isolation of noncp BVDV for both CP442 I-1 and CP442 I-2;

respective noncp strains were termed NCP442-R1 and NCP442-R2 (Fig. 2A, bottom).

In the following experiment, it was investigated whether noncp BVDVs also emerge when single picked cp plaques are used as starting material for subsequent passages of CP442 and CP552. For that purpose, MDBK cells were first transfected with synthetic CP442 RNA or CP552 RNA in two separate attempts each (CP442 II, CP442 III, CP552 I, and CP552 II) (Fig. 2A). Directly p.t., the resuspended cell pools were diluted in a suspension of noninfected MDBK cells and used to perform plaque assays. For each experiment, wells with 1 to 10 plaques each were selected 5 days p.t. and material from 3 to 18 single plaques was picked, resuspended in cell culture medium, and used for subsequent cell culture passages (Fig. 2A, right). IF analyses performed after the first and every fifth subsequent cell culture passage indicated in most cases the emergence of noncp BVDV after the 5th to 15th passage. However, noncp BVDV could never be detected already after the first cell culture passage (data not shown). After 2 to 15 additional cell culture passages, in total, 12 noncp BVDVs could be isolated that were termed NCP442-R3 to NCP442-R8 and NCP552-R1 to NCP552-R6 (Fig. 2A, bottom); isolation of noncp BVDV was performed as described above. It should be noted that some of the noncp BVDVs that were initially detected by IF analyses were lost during the isolation procedure.

Northern blot analysis of recombinant noncp BVDV strains.

To characterize the noncp BVDV strains obtained after cell culture passages of cp CP442 and CP552, Northern blot hybridizations were performed using total cellular RNA from the first cell culture passage of CP442 or CP552 and from the first passage of the isolated noncp BVDV strains. In addition, for some recombinant strains, viral RNAs derived from different passages of the noncp virus isolation procedure were also investigated (for an example, see Fig. 2C, CP442 II-1, 10th and 15th passage; also data not shown). The results of these analyses revealed that the genomes of the isolated noncp BVDV strains were significantly smaller than the genomes of the parental cp viruses CP442 and CP552 (genomic sizes of approximately 15 kb) (Fig. 1) but in most cases were larger than the genome of NCP7-5A with a size of about 12.3 kb (Fig. 2C; data not shown). Interestingly, the genomes of the individual noncp BVDV strains slightly differed in size (Fig. 2C; data not shown). Moreover, viral RNAs corresponding to noncp BVDV genomes could not be detected after the first and fifth passages of the cp viruses (for an example, see Fig. 2C, CP442 I-1 and -2; also data not shown) while analysis of some RNAs derived from later passages allowed the detection of two bands representing the genomes of the cp and noncp viruses (for an example, see Fig. 2C, CP442 II-1, 15th passage; data not shown). Taken together, these data support the assumption that the isolated noncp BVDVs originated from cp BVDVs CP442 and CP552 by various genomic alterations.

RT-PCR analysis of recombinant noncp BVDV strains. To determine the genome organization of the isolated noncp BVDVs, RT-PCRs were performed using oligonucleotides OI BVD-7209 and OI CP7-7863-R-ApaI. RNAs previously analyzed by Northern blot hybridizations served as templates (Fig. 3A and B; also data not shown). OI BVD-7209 represents a panpestivirus primer that can anneal to the 3'-terminal parts of the NS3 genes originating from both CP7-5A and CP821,

while the antisense oligonucleotide OI CP7-7863-R-ApaI was designed to anneal to only the 5' part of the NS4B gene derived from CP7-5A (Fig. 3C and D). RT-PCR analyses performed with synthetic RNAs transcribed from pCP442 and pCP552 (or with RNA obtained from cells after infection with CP7-5A) resulted in PCR products of 826 nt (CP442) (Fig. 3A and C) or 655 nt (CP552 and CP7-5A) (Fig. 3B and D), respectively. Amplification of a second PCR product of more than 3 kb was prevented by restricting the PCR elongation time to 1 min. PCR fragments of 826 or 655 nt were also expected to be produced by PCR analysis of putative noncp BVDVs that resulted from CP442 and CP552 by homologous RNA recombination between the duplicated viral sequences flanking Nedd8*. In case of noncp BVDVs resulting from nonhomologous recombination, PCR products were expected to differ in size, as long as the 5' annealing region corresponding to primer OI BVD-7209 was maintained and the respective 3' annealing region was removed. Accordingly, the design of this RT-PCR assay should allow us to distinguish between noncp BVDVs that were generated by either homologous or nonhomologous RNA recombination. Remarkably, only for NCP552-R4, a product corresponding in size to 655 nucleotides was obtained (data not shown). In contrast, for the other 13 isolated noncp BVDVs, PCR products were obtained that were to different extents larger than expected for recombinant strains that emerged by homologous recombination (Fig. 3A and B; data not shown). Moreover, RT-PCR analysis of NCP442-R4 led to the detection of two enlarged fragments (data not shown). Taken together, the results of the PCR analyses suggested that 13 of 14 noncp BVDVs resulted from nonhomologous RNA recombination.

Nucleotide sequence analysis of recombinant noncp BVDV strains. The obtained RT-PCR products were cloned in a bacterial vector, and for each product, the nucleotide sequences of at least two different cDNA clones were determined. Nucleotide sequence analysis confirmed that NCP552-R4 resulted from homologous recombination. Moreover, each of the remaining 14 cDNA fragments derived from the other 13 noncp BVDV strains comprises a unique recombination site, resulting in fusion of sequences from the 5' recombination region (encompassing sequences from the 5'-terminal NS4A gene to Nedd8*) to sequences from the 3' recombination region (downstream of the 3'-terminal NS3^{4-end} gene) (Fig. 3C and D; also data not shown). For NCP442-R4, analysis of the two obtained PCR products (see above) resulted in the detection of two distinct recombination sites; this led to the assumption that two noncp BVDVs (NCP442-R4A and NCP442-R4B) had been simultaneously isolated. For all 15 recombinant sequences, the ORF was maintained. It cannot be excluded that additional mutations in genomic regions not investigated so far are present. When compared to CP442 and CP552, the genomes of the 15 recombinant noncp viruses are characterized by a deletion of more than 2 kb and lack in all cases at least the NS3^{4-end} gene, together with the entire cellular insertion Nedd8* or most of it (Fig. 3C and D). Taken together, the nucleotide sequence analyses are in agreement with the results obtained from Northern blot hybridizations and RT-PCR analyses. With the exception of isolate NCP552-R4 that was generated by homologous recombination, all other noncp BVDV

recombinant strains resulted from different nonhomologous RNA recombination events.

Reconstruction of NS4A/NS4B BVDV mutants and their characterization. According to the alterations in the NS4A and NS4B encoding region, the obtained noncp BVDV recombinant strains can be allocated into three groups. First, recombinant NCP552-R4 resulted from homologous recombination between the duplicated NS4A genes of CP552 and consequently harbors a chimeric NS4A gene (Fig. 3D; also data not shown). The second group is represented by NCP552-R3, which exhibits a duplication of 14 codons in the 5' part of the NS4B gene (Fig. 3D; also data not shown). The remaining 13 recombinant noncp BVDVs comprise unaltered NS4A and NS4B genes, but these two genes are separated from each other by various insertions. The latter encode different fragments of NS4A, NS4B, and, for some recombinant strains, also parts of NS2 and cell-derived Nedd8 (Fig. 3C and D; NS4B-N*, NS2-C*, NEDD8*, NS4B-N*², and NS4A-C*). With regards to the genomic region encoding NS4A to NS4B, this group of recombinant viruses resembles CP821 and CP442 (see above).

To investigate the influence of these mutations on viral viability, growth, and RNA replication efficiency, recombinant strains NCP552-R3 to NCP552-R5 were selected for further analyses. After parts of their sequences had been amplified by RT-PCR and cloned into a bacterial vector, the resulting cDNA clones were controlled by nucleotide sequencing and fragments including the respective genomic alterations (described above) were inserted into the infectious cDNA clone of noncp BVDV strain NCP7-5A (see Materials and Methods). The respective cDNA clones were linearized, and synthetic RNAs of the resulting plasmids pNCP7/552-R3 to pNCP7/552-R5 were transcribed and used for the transfection of MDBK cells. One day p.t., transfected cells were analyzed by IF analysis. For all constructs, detection of NS3 indicated viral translation (data not shown). Moreover, when supernatants obtained from transfected cells were used for the subsequent cell culture passages, IF analyses confirmed the presence of viral antigen and the production of infectious virus particles for all strains (data not shown).

In the following experiment, stocks of the obtained chimeric

strains NCP7/552-R3 to NCP7/552-R5 (Fig. 4A) were produced by infection of cells with supernatants derived from transfected cells. The infectivity titers of the obtained stocks were determined, and, in order to determine viral growth kinetics, MDBK cells were infected with each of the virus strains at a multiplicity of infection of 1. Samples were taken over a 4-day period, and respective titers were calculated by titration and subsequent evaluation by IF analyses. Taken together, the three mutant strains and the parental virus NCP7-5A reached almost identical peak titers of about 5×10^6 50% tissue culture infective doses/ml. Moreover, the growth kinetics were very similar for each of the four BVDV strains (Fig. 4B). To compare the viral RNA synthesis of the mutant viruses to that of NCP7-5A, viral RNAs were investigated by Northern blot analysis. Twenty-four hours p.i., amounts of viral RNA obtained from infections with the three reconstructed mutant viruses did not show a reduction when compared to the parental virus NCP7-5A (Fig. 4C). Finally, to investigate whether genetic reversion to the wild-type virus occurred during cell culture passages of recombinant strains NCP7/552-R3 to NCP/552-R5, cell culture supernatants derived from the experiments described above were passaged three times. Subsequent RT-PCR and nucleotide sequence analyses demonstrated the absence of genetic changes in the investigated NS4A/NS4B encoding region for all strains (data not shown).

Taken together, the results of our analyses show that pesti-viral replication efficiency is not significantly affected by different kinds of alterations in the NS4A/NS4B coding region including (i) a chimeric NS4A gene, (ii) a duplication of 14 codons in the 5' part of the NS4B gene, and (iii) a separation of the NS4A and NS4B genes by various duplicated viral sequences.

DISCUSSION

Genomic recombination has been studied for many plus-strand RNA viruses (1, 2, 14–16, 24, 25, 28, 31, 35, 39, 41, 45, 46, 50). For members of the families *Picornaviridae* and *Coronaviridae* as well as for bromoviruses, homologous RNA recombination occurs frequently. For other viruses, like alphaviruses and carmoviruses, nonhomologous recombination

FIG. 3. RT-PCR analysis and genome organization of recombinant noncp BVDVs. (A) RT-PCR analysis of RNAs obtained from MDBK cells infected with noncp BVDV strains NCP442-R1 and NCP442-R2. For comparison, synthetic CP442 RNA (CP442 s.t.), RNA from cells infected with noncp BVDV NCP7-5A, and RNA from the 5th cell culture passage of CP442 (CP442 I-1 5th p.) (see also Fig. 2) were included in this analysis. For RT-PCR, oligonucleotides OI CP7-7863-R-ApaI and OI BVD-7209 (see also panel C) were used. Note that the specific RT-PCR products obtained from the RNAs of the isolated noncp BVDVs are slightly (NCP442-R1) or considerably (NCP442-R2) larger than the ones obtained from CP442 s.t. or CP442 I-1 5th p. RNA. RNA from noninfected cells (n.i.) served as negative control. The lengths of a size standard (L) are depicted in kilobases on the right. (B) Comparative RT-PCR analysis of RNAs from MDBK cells infected with noncp BVDV strains NCP552-R2 and NCP7-5A. Note that the specific RT-PCR product obtained for NCP552-R2 is considerably larger than that derived from NCP7-5A RNA. (C) Genome organization of cp BVDV strain CP442 (top) and recombinant noncp BVDV strains NCP442-R1 to NCP442-R8 (bottom). For the region between the 5'-terminal NS3 gene and the 3'-terminal NS4B gene, an enlarged section of the genome of CP442 is displayed. The lengths of respective segments are given in nucleotides below the bars. The positions of oligonucleotides OI CP7-7863-R-ApaI and OI BVD-7209 used for RT-PCR (see also panel D) as well as the calculated size of the PCR product obtained from CP442 RNA (826 nt) are indicated. For NCP442-R1 to NCP442-R8, horizontal bold lines indicate the presence of CP442-derived sequences while "Δ" and dashed horizontal lines indicate the position and extent of various deletions, respectively. (D) Genome organization of cp BVDV strain CP552 (top) and recombinant noncp BVDV strains NCP552-R1 to NCP552-R6 (bottom). For the region between the 5'-terminal NS3 gene and the 3'-terminal NS4B gene, an enlarged section of the genome of CP552 is displayed. The calculated size of a PCR product obtained from CP552 RNA (655 nt) is indicated. For NCP552-R1 to NCP552-R6, horizontal bold lines indicate the presence of CP552-derived sequences while "Δ" and dashed horizontal lines indicate the position and extent of various deletions, respectively. Note that for all recombinant viruses, the NS3^{4-end} gene has been completely removed.

A

B

C

FIG. 4. Genome organization, growth kinetics, and Northern blot analysis of the reconstructed BVDV mutants NCP7/552-R3, -R4, and -R5. (A) Genome organization of reference strain NCP7-5A and the reconstructed noncp BVDV mutants. The lengths of the NS4A- and NS4B-specific genes and fragments are indicated in nucleotides below the bars. The calculated total length of each genome is given at the right. For all genomes, the region between the 5' NTR and the NS4A gene is partially indicated by dots. (B) Growth curves of NCP7-5A and the reconstructed noncp BVDV mutants. The titers of released virus were determined at the indicated time points over a 4-day period. TCID₅₀, 50% tissue culture infective dose. (C) Northern blot analysis of NCP7-5A and the noncp BVDV mutants. MDBK cells were infected at a multiplicity of infection of 1, and total cellular RNAs were harvested 24 h p.i. For hybridization, a radioactively labeled fragment of cDNA clone pCP7-5A corresponding to nt 6278 through 7850 was used as BVDV-specific probe. As a control, a GAPDH probe was used. Noninfected cells (n.i.) served as a negative control. The position and size of NCP7-5A RNA are given on the right.

seems to be more prevalent (for reviews, see references 27 and 30). With regards to pestiviruses, RNA recombination was first observed in connection with the molecular characterization of cp BVDV strains that were isolated from animals with mucosal disease, a lethal disease in cattle (36). Studies performed with a large number of different pestivirus isolates revealed that most cp strains evolve from persisting noncp viruses by non-homologous RNA recombination (for a review, see reference 38). Interestingly, after cell culture passages of certain genetically engineered cp BVDV strains, the emergence of noncp BVDV recombinant strains has been observed (4, 8). These recombinant noncp viruses could be detected already after the first cell culture passage of the parental cp virus. In these studies, a determination of recombination sites was not possible since homologous RNA recombination occurred between identical duplicated sequences (4, 8). In the present study, noncp recombinant viruses were derived from genetically en-

gineered cp viruses which contained duplicated viral sequences from different BVDV strains. This novel approach allowed the precise determination of the recombination sites of emerged noncp recombinant viruses. Surprisingly, sequence analyses revealed that only 1 out of 15 isolated noncp viruses arose by homologous recombination while the remaining 14 noncp viruses resulted from nonhomologous recombination. Strikingly, detection of noncp viruses was possible only after extensive cell culture passages and noncp BVDV could never be detected after the first cell culture passage of the respective parental cp virus. This is different from the earlier studies mentioned above. For RNA viruses, it is commonly accepted that genomic recombination results from template switching of the viral RNA-dependent RNA polymerase (RdRp) during replication (27, 30). In fact, in vitro experiments performed with the RdRp of several RNA viruses including BVDV demonstrated the ability of viral RdRps to efficiently switch templates during

RNA synthesis (3, 18, 26); additional reports provided convincing evidence for this mechanism (19, 24, 28, 42, 43). For template switching, sequence identity between the two recombination partners around the recombination site apparently plays an important role, especially with regards to homologous recombination (40). In addition, local sequence identity in the direct vicinity of the crossover site may also facilitate nonhomologous RNA recombination (5, 13, 33, 51). For poliovirus, the frequency of homologous recombination decreased when the degree of sequence homology between participating RNA molecules was reduced (28, 49). The present study shows that the ratio of BVDV recombinant strains resulting from homologous recombination was dramatically reduced when the sequence identity between the main part of the duplicated sequences (determined for the duplicated NS3 and NS4A genes) was reduced from 100% (7/821) to 84% (CP442 and CP552).

Interestingly, some of the noncp viruses detected by IF analyses could not be isolated but were lost during the isolation procedure that included several additional passages. This most probably was due to a disadvantage in viral replication of such recombinant strains when compared to the competing parental cp virus. It can be speculated that the almost exclusive isolation of noncp BVDV strains resulting from nonhomologous recombination is due to the fact that homologous RNA recombination would have resulted in the generation of chimeric viral NS genes; the latter might lead to impaired replication of these strains. The chimeric NS4A gene of recombinant NCP552-R4, however, did not interfere with efficient viral replication. This observation might reflect that this homologous recombinant was functionally selected. In addition, our experiments performed with reconstructed BVDV strains NCP7/552-R3 and NCP7/552-R5 revealed that the efficiency of viral growth and RNA replication was not affected. These constructs represent the two groups of isolated noncp BVDVs that originated from nonhomologous recombination, and it appears likely that these recombinant viruses were also selected according to their replication efficiency.

Taken together, the observed switch of recombinant viruses originating from homologous recombination (previous studies) to ones emerging from nonhomologous recombination (present study) can be explained by the degree of sequence identity between the RNA sequences participating in recombination. It is tempting to speculate that this switch reflects a change in the mechanism of RNA recombination. Moreover, it appears likely that a selection of the emerged noncp BVDV strains to efficiently replicating viruses had a major impact on the isolation of noncp strains.

If one considers the exceptional genome organizations of NCP7/552-R3 and NCP7/552-R5, the efficiency of viral replication is surprising. For NCP7/552-R3, a duplication of 14 codons in the 5' terminus of the NS4B gene was well tolerated and proved to be genetically stable. This gene locus represents a promising target for the insertion of foreign sequences, e.g., to determine the localization of NS4B in replication complexes of BVDV. Moreover, it appears worthwhile to investigate whether the corresponding region is suited for the tagging of NS4B from related viruses.

With regards to NCP7/552-R5, the presence of 171 nucleotides (encoding fragments of NS4A and NS4B) between the NS4A and NS4B genes did not reduce the viral growth or

efficiency of viral RNA synthesis (Fig. 4). Interestingly, an interruption of the NS4A and NS4B genes was not only observed for 12 additional recombinant viruses but already present in BVDV field isolate CP821 and, accordingly, also in CP442. Among these viruses, the respective sequence insertions between the NS4A and NS4B genes vary in origin and extent (Fig. 1 and 3). However, for the deduced viral polyprotein of all these BVDV strains, the cleavage site for viral serine protease NS3 (47, 52) between the C terminus of NS4A and the N terminus of NS4B is duplicated together with flanking sequences. Accordingly, it is reasonable to assume that both sites are efficiently processed, thereby generating authentic NS4A and NS4B and supporting the formation of a functional replication complex. In addition, a polypeptide consisting of parts from different viral proteins would be released.

Our observations expand the knowledge about the genomic region encoding components of the pestiviral replicase. For efficient pestiviral replication to occur, it is obviously not necessary to express NS3 to NS5B without interruption. We speculate that any foreign sequence inserted between the pestiviral NS4A and NS4B genes can be efficiently expressed as long as the described duplicated cleavage sites are maintained. This particular locus may also be suited for the expression of foreign sequences in the closely related hepatitis C virus system.

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