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Cytopathogenicity of a Pestivirus Correlates with a 27-Nucleotide Insertion

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Cytopathogenic (cp) bovine viral diarrhea virus (BVDV) strains are generated in cattle persistently infected with noncytopathogenic (noncp) BVDV. cp BVDV strains are considered crucial for the development of fatal mucosal disease. Comparative analysis of cp and noncp BVDV strains isolated from one animal suffering from mucosal disease revealed that the genomes of the cp BVDV strain (CP7) and the corresponding noncp BVDV strain (NCP7) are highly homologous. However, only the genome of CP7 contains an insertion of 27 nucleotides in the NS2 coding region. The inserted sequence represents a duplication of bases 4064 to 4090 of the viral genome, located between the formerly neighboring nucleotides 4353 and 4354. Parts of the viral polyproteins of CP7 and NCP7 were expressed in the T7 vaccinia virus system. These studies revealed that the insertion identified in the CP7 genome is necessary and sufficient for the induction of NS2-3 cleavage. Since the expression of NS3 is strictly correlated to cp BVDV, the insertion identified in the genome of BVDV CP7 represents most likely the relevant mutation leading to the evolvement of CP7 from NCP7.

Bovine viral diarrhea virus (BVDV), classical swine fever virus, and border disease virus represent the genus *Pestivirus* in the family *Flaviviridae*. This family also includes the genera *Flavivirus* and hepatitis C-like viruses (HCV) (52). The pestiviral genome is a single-stranded RNA molecule of usually 12.3 kb which encodes one polyprotein of about 4,000 amino acids (aa) (5, 11, 12, 28, 34). This polyprotein is co- and posttranslationally cleaved into at least 11 mature viral proteins (4, 6, 15). Whereas most of the viral structural proteins are released by host cell proteases (15, 44), the processing of nonstructural proteins is mediated mainly by a serine protease located in the NS3 (p80) region (53). The latter protease mediates the generation of its own C terminus and acts on the cleavage sites located downstream in the polyprotein. A role for the NS4A (p10) coding region as a cofactor for NS3, reminiscent to the situation found in the HCV system, has been postulated (53) but has yet to be demonstrated.

According to their effects on tissue culture cells, BVDV strains can be differentiated into cytopathogenic (cp) and non-cp (noncp) isolates (1, 50). Infection of pregnant cows with noncp BVDV strains at an early stage of gestation results in the births of persistently infected calves which exhibit an acquired immunotolerance against the respective noncp BVDV strain (3, 9, 26, 33). Such animals may spontaneously come down with the so-called mucosal disease (MD), which has a lethality rate of close to 100%. Interestingly, from each animal with MD, a cp BVDV strain, in addition to the persisting noncp BVDV strain, can be isolated (27); cp and noncp viruses isolated from one animal with MD show a high degree of serological similarity and are called a virus pair (8, 37). Recent molecular studies have provided evidence for the evolvement of cp viruses from noncp viruses by RNA recombination. The mutations defined in the genomes of cp BVDV strains include insertions of cellular sequences, sometimes together with large duplications, and genomic rearrangements combined with large duplications and deletions (31, 32, 39, 43, 49).

All these mutations affect the NS2-3 (p125) coding region of the viral genome. While NS2-3 can be detected in cells infected with cp BVDV as well as noncp BVDV, the C-terminal part of NS2-3, namely NS3 (p80), is generated only in cp-BVDV-infected cells (4, 8, 37, 38). The expression of NS3 is therefore regarded as a molecular marker for cp BVDV. In some cases, it can be directly demonstrated that the mutations identified in the genomes of cp BVDV strains are causative for the generation of NS3 (32, 48, 49).

In some cases, cp BVDV genomes do not show dramatic changes in the NS2-3 coding region (10, 35). It was interesting to investigate which mutation(s) is relevant for the generation of this type of cp BVDV strain. Since preliminary studies suggested that the genome of CP7, the cp virus of BVDV pair 7, does not contain any of the previously described mutations, we started the molecular characterization of this virus pair.

MATERIALS AND METHODS

Cells and viruses. MDBK cells were obtained from the American Type Culture Collection (Rockville, Md.). BSR cells are a BHK-21 clone kindly provided by J. Cox (Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany). Virus strains CP7 and NCP7 have been described previously (8). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The T7 vaccinia virus (vTF7-3) (16) was generously provided by B. Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

Infection of culture cells. Freeze-thaw lysates of infected cells were used for infections of culture cells, since BVDV virions are mainly cell associated and reach only low titers in the culture supernatant. Cell lysates were prepared at 48 h postinfection and stored at −70°C.

cDNA synthesis, cloning, and library screening. The methods used for the synthesis and cloning of double-stranded cDNA in Lambda ZAPII (Stratagene, Heidelberg, Germany) as well as for library screening have been described elsewhere (31). The sequences of the DNA primers, Pes9 and BVD14, used for specific priming of the cDNA have also already been published (31).

Nucleotide sequencing. A T7 sequencing kit (Pharmacia Biotech, Freiburg, Germany) was used for the analysis of double-stranded DNA (45). Truncations of cDNA clones for sequencing were carried out by using exonuclease III and DNase S1 (21). Computer-aided sequence analysis was carried out on Digital

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Microvax II with University of Wisconsin Genetics Computer Group software (13).

RNA preparation, gel electrophoresis, and Northern (RNA) hybridization. Total cellular RNA was prepared as described previously (31). Five micrograms of glyoxylated total cellular RNA was separated in phosphate-buffered 1% agarose gels containing 5.5% formaldehyde and transferred to Duralon-UV membranes (Stratagene). An RNA ladder (Bethesda Research Laboratories) served as the size standard. Labeling of cDNA probes with [^{32}P]dCTP was carried out with a nick translation kit (Amersham Buchler, Braunschweig, Germany). The cDNA fragment used as a BVDV-specific probe is derived from a cDNA clone encompassing part of the NS2-3 coding region of BVDV NCP1 (31). The procedures and conditions used for hybridization have already been published (31).

Radioimmunoprecipitation (RIP) and SDS-PAGE. MDBK cells (1.5×10^6; 3.5-cm-diameter dish) were labeled for 6 h with 0.5 mCi of [^{35}S]methionine-cysteine. The labeling medium contained no cysteine and methionine. Cell extracts were prepared under denaturing conditions (2% sodium dodecyl sulfate [SDS]). Aliquots of cell extracts were incubated with 5 μl of undiluted serum. For the formation of precipitates, cross-linked *Staphylococcus aureus* was used (25). SDS-polyacrylamide gel electrophoresis (PAGE) analysis of proteins with molecular masses of up to 50 kDa occurred on tricine gels by the method of Schägger and Jagow (47); for the separation of larger proteins, SDS gels were used by the method of Doucet and Trifaro (14). Gels were processed for fluorography by using En^{3}Hance (New England Nuclear, Boston, Mass.).

Transient expression with the T7 vaccinia virus system. BSR cells (5×10^5; 3.5-cm-diameter dish) were infected with the recombinant T7 vaccinia virus vTF7-3 (16) at a multiplicity of infection of 10 in medium lacking fetal calf serum. After 1 h of incubation at 37°C, cells were washed two times with medium lacking fetal calf serum, with subsequent transfection of 10 μg of plasmid DNA (mammalian transfection kit; Stratagene). After an incubation of 4 h at 37°C, cells were washed two times with label medium lacking cysteine and methionine and incubated in this medium for 0.5 h at 37°C. Cells were labeled in 1 ml of label medium containing 0.5 mCi of [^{35}S]methionine-cysteine for 5 to 6 h, washed with phosphate-buffered saline, and stored at −70°C.

RT-PCR. The reverse transcription-PCR (RT-PCR) procedure was carried out as previously described (32). The primers used for amplifying nucleotides 3341 to 4020 of the genome of BVDV NCP7 were derived from the previously determined genomic sequence of BVDV CP7; the cDNA sequence of the PCR fragment was determined subsequently.

The sequences of the oligonucleotide primers used are as follows: BVD34 (positive orientation), 5′-GCG TGT/C ACT TTT/C AAT/C TAT/C AC-3′; and BVD35a (negative orientation), 5′-AAA/G TAG/A TCT GTC ACA TAA CT-3′.

Construction of T7 expression plasmids. All T7 expression constructs were based on pSKT7T (7), a pBluescript SK− derivative kindly supplied by K. K. Conzelmann (Federal Research Centre for Virus Diseases of Animals). All nucleotide and amino acid positions given refer to the sequence of BVDV strain SD-1 (12). To create blunt ends, the Klenow fragment of DNA polymerase I was used.

For pC7.1, a Klenow-treated *Sal*I (pSK polylinker sequence)-*Sal*I (BVDV nucleotide position 7729) fragment of the cDNA clone pSKC7.1 was cloned together with a translational start codon into pSKT7T. The open reading frame of this construct starts with 17 vector-derived aa followed by the C-terminal region of glycoprotein E2, starting with aa 967 of the BVDV polyprotein; the BVDV polyprotein encoded by this construct ends with aa 2447 and is followed by 3 vector-derived aa and a translational stop codon.

For pN7.1, the product of the RT-PCR (see above) was cloned together with a translational start codon into pSKT7T by standard procedures. The open reading frame of this plasmid was elongated by the insertion of a *Kpn*I (nucleotide position 3815)-*Sal*I-blunted (nucleotide position 7729) fragment of pSKN7.1. By the cloning procedure, pN7.1 encodes 3 vector-derived aa at the 5′ end of the open reading frame and 3 additional aa at the 3′ end, followed by a stop codon. pN7.1 encodes aa 985 to 2447 of the polyprotein of BVDV NCP7.

By replacing a *Pfl*MI (nucleotide position 4251)-*Eco*RV (nucleotide position 4587) fragment of pC7.1 with the corresponding fragment of pN7.1, pC7.1INS− was generated; the only change in the amino acid sequence of the encompassed open reading frame is the deletion of the cp-specific insertion.

For pC7PM1, mutagenesis was carried out with a Muta-Gene Phagemid mutagenesis kit (Bio-Rad, Munich, Germany). For the generation of single-stranded DNA, a *Bam*HI (nucleotide position 5147)-*Eco*RI (nucleotide position 6258) fragment was cloned into plasmid pTZ19, which was restricted identically. An *Hpa*I (nucleotide position 5436)-*Sac*I (nucleotide position 5859) fragment was deleted from pC7.2 and substituted for with the corresponding mutagenized fragment of the pTZ intermediate. The sequence of the oligonucleotide primer used for mutagenesis is 5′-GGG TAG ACC CGC CCA TCC TT-3′ (complementary to bases 5631 to 5650 of the BVDV genomic sequence).

Generation of peptide antisera. Peptides were synthesized on a MilliGen 9050 PepSynthesizer (Millipore) by the 9-fluorenylmethoxycarbonyl-polyamide method. For the generation of antibodies, peptides were coupled to keyhole limpet hemocyanin. Glutaraldehyde was used for cross-linking (20). Immunizations of rabbits were carried out by using an equivalent of 1 mg of peptide per injection. Peptides were injected subcutaneously into rabbits with Freund adjuvant (complete for basic immunizations and incomplete for booster injections).

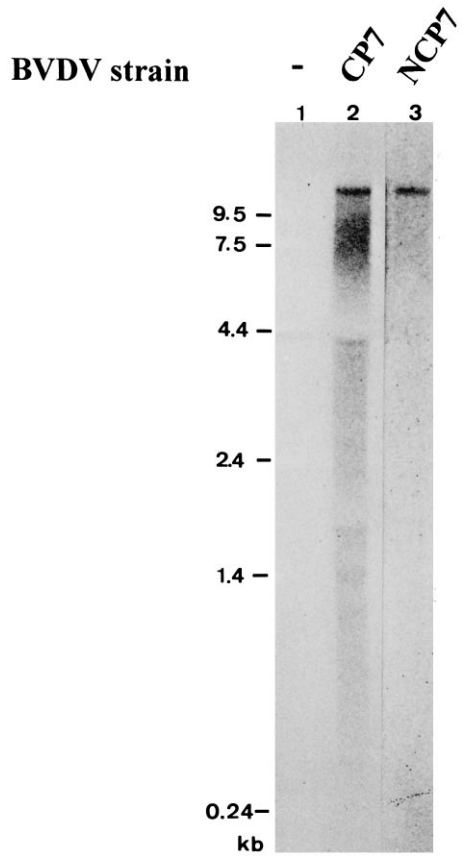

FIG. 1. Northern blot analysis. Total RNA of MDBK cells was separated by denaturing agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized against a BVDV-specific cDNA probe. Lanes: 1, RNA from MDBK cells not infected (−); 2, RNA from MDBK cells infected with BVDV CP7; 3, RNA from MDBK cells infected with BVDV NCP7.

The amino acid positions of the peptides according to the BVDV SD-1 sequence are as follows: pep2, aa 1321 to 1326, flanking the 9-aa insertion on each side by 3 aa; and pep6, aa 1571 to 1586.

Nucleotide sequence accession number. The sequence data reported here have been deposited in the GenBank and EMBL databases under accession no. U63512 and U63513.

RESULTS

Northern blot analysis. Viruses of BVDV pair 7, namely, CP7 (cp BVDV) and NCP7 (noncp BVDV), isolated from one animal which came down with MD, were analyzed. These two viruses are antigenically very similar when the reaction patterns with a panel of monoclonal antibodies are considered (8). The comparative molecular characterization was aimed at the detection of mutation(s) responsible for the cp biotype of BVDV CP7. In the genomes of other cp BVDV strains, large deletions, duplications, and/or insertions of cellular RNA sequences have been identified. Some of these changes resulted in dramatic alterations of the size of the viral genome (31, 32, 39, 49). Accordingly, the sizes of the viral genomic RNAs of CP7 and NCP7 were first compared. Total RNA of MDBK cells infected with CP7 or NCP7 was isolated and subjected to Northern blot analysis with a BVDV-specific probe (Fig. 1). Both genomic RNAs exhibited the same migration characteristics, with a length of about 12.3 kb (Fig. 1), and migrated slightly faster than did the genome of BVDV NADL (data not shown). Thus, large deletions and duplications are not present

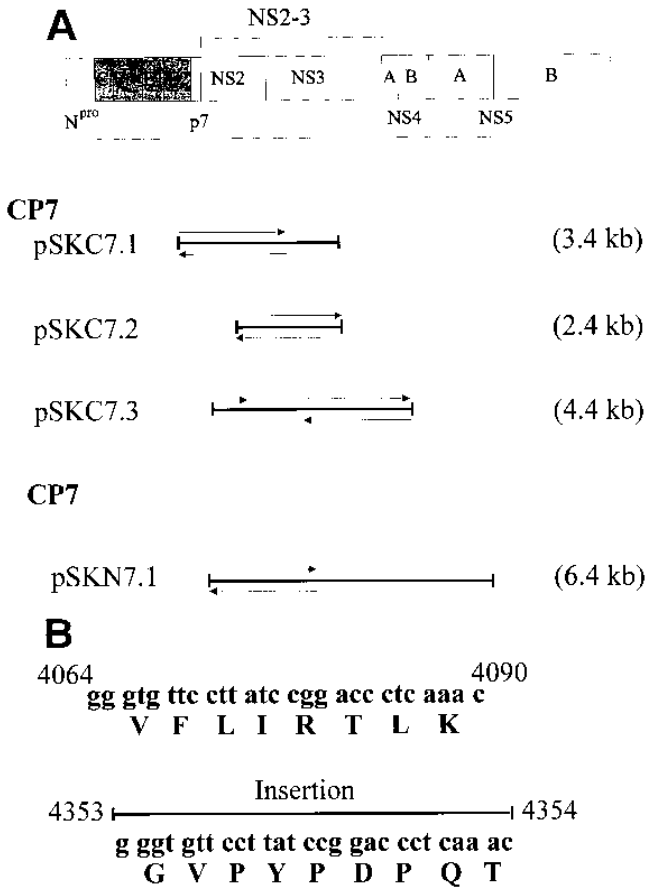

FIG. 2. Scheme of cDNA clones. (A) Polyprotein of BVDV. Lines indicate the lengths and relative genomic positions of individual cDNA clones; sequenced regions are indicated by arrows above and below the lines; the lengths of cDNA clones are given parenthetically. The genomic positions of BVDV-specific cDNA inserts are as follows: pSKC7.1, 3283 to 6685; pSKC7.2, 4674 to 7068; pSKC7.3, 4089 to 8459; and pSKN7.1, 3792 to 10216. (B) Nucleotide sequence of the BVDV CP7 genome from positions 4064 to 4090 and sequence of the 27-base insertion located between bases 4353 and 4354 (numbers refer to the BVDV SD-1 sequence [12]). Deduced amino acid sequences are given in the one-letter code.

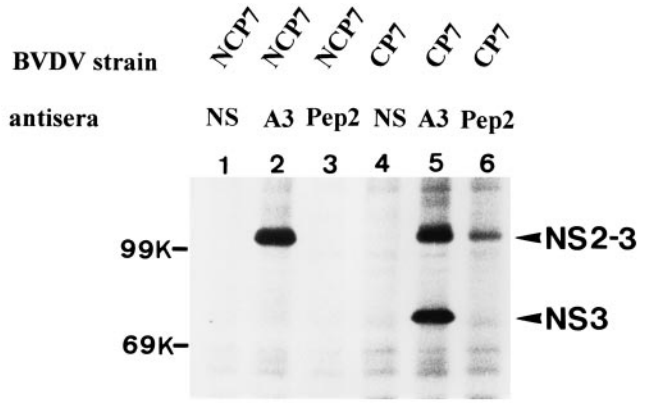

FIG. 3. RIP. SDS-PAGE analysis of proteins precipitated from MDBK cells labeled with [^{35}S]methionine-cysteine after infection with CP7 or NCP7. Numbers on the left indicate the molecular masses (in kilodaltons [K]) of marker proteins. The sera used were rabbit preimmune serum (NS), antiserum specific for NS3 (A3), and antiserum directed against the 9-aa insertion and flanking amino acids (pep2). The positions of NS2-3 and NS3 are marked by arrowheads.

in the genome of CP7. Hybridization with probes specific for the two cellular insertions identified in cp BVDV strains revealed that these cellular sequences are not present in the genomes of the viruses of pair 7 (data not shown). It can be concluded from these results that the expected mutation(s) in the genome of CP7 is basically different from the ones identified so far in the genomic RNAs of cp BVDV strains.

cDNA cloning and sequencing. In order to identify the mutation(s) specific for the CP7 genome, cDNA libraries were constructed by using as starting material total cellular RNA of CP7- and NCP7-infected MDBK cells. Since all the mutations identified so far in cp BVDV strains concern the NS2-3 coding region, the focus of analysis was on this part of the viral genome. The terminal sequences of BVDV-specific cDNA clones (Fig. 2A) were obtained. This analysis revealed that most of these clones were generated by random priming rather than specific priming. Unless indicated otherwise, the numbering of nucleotide and amino acid positions refers to the sequence of BVDV SD-1 (12). Nucleotides 3283 to 8459 of the CP7 genome and bases 3792 to 5221 of the NCP7 RNA were determined. Additionally, nucleotides 3341 to 4020 of the NCP7 genome were generated by RT-PCR. An alignment of the cDNA sequences of both viruses revealed a homology of 99.5% between both genomes (data not shown). This high degree of similarity shows that CP7 evolved from NCP7. Similar results have also been obtained for the members of other BVDV pairs (31, 32).

Surprisingly, a sequence comparison demonstrated the absence of insertions and deletions in the CP7 genome at the proposed NS2-3 processing site. However, a more comprehensive analysis of the complete NS2-3 region revealed one striking difference between the two sequences. Far upstream of the previously identified insertion sites, the CP7 genome contained an insertion of 27 bases, located between bases 4353 and 4354 in the NS2 coding region. The integration of this sequence occurred between formerly neighboring nucleotides without the deletion of viral sequences. With regard to its position in the CP7 genome, this sequence is found neither in the genome of NCP7 nor in any other pestiviral sequence published so far. A GenBank search revealed no significant homology between these 27 bases and any known cellular sequence. However, an alignment of the insertion with the obtained cDNA sequence of CP7 revealed that this insertion represents an exact duplication of the genomic sequence from nucleotides 4064 to 4090. The insertion is located about 290 nucleotides downstream of its original genomic position (Fig. 2B). Remarkably, this insertion occurred outside the original reading frame; thus, the inserted 27 bases encode different amino acid sequences in their original and new positions in the genome (Fig. 2B).

Processing of NS2-3. After the infection of MDBK cells with the viruses of pair 7, RIP was performed. Immunoprecipitation with an antiserum directed against a part of NS3 (rabbit serum A3) (51) revealed that in this case, the expression of NS3 was also linked to the cp phenotype (Fig. 3); during SDS-PAGE, NS3 of BVDV CP7 comigrated with the respective molecules of other cp BVDV strains (data not shown). In order to obtain antibodies which react only with NS2-3 of CP7, a synthetic peptide (pep2) which encompassed the insertion and 3 flanking aa was selected and used for the immunization of rabbits. The resulting antiserum precipitated NS2-3 from CP7-infected cells, but not NS2-3 from cells infected with NCP7 (Fig. 3). As expected, the anti-pep2 serum also precipitated NS2 from CP7-infected cells (Fig. 4C).

The mutation changing NCP7 to CP7 has to correlate with

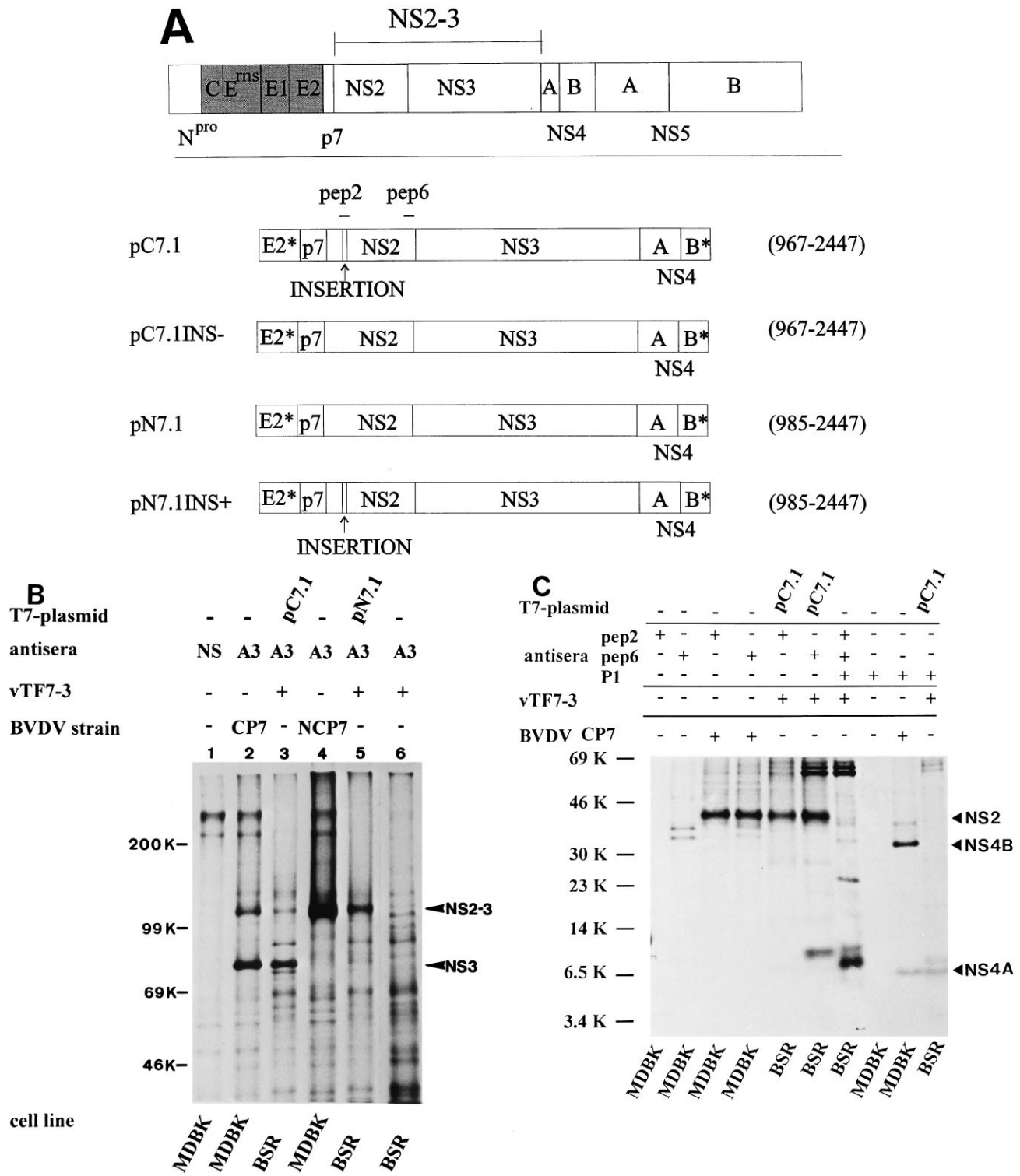

FIG. 4. (A) Scheme of T7 expression constructs. The open reading frame encoded by these constructs is under the control of a T7 RNA polymerase promoter, starts in the E2 (gp53) coding region, and encompasses genes for the recently described p7 (15), NS2-3, NS4A, and about half of the NS4B gene. The asterisks for E2* and NS4B* indicate that only parts of these proteins are encoded by both plasmids. Parenthetical numbers give the amino acid positions with respect to the SD-1 polyprotein sequence (12). pC7.1 and pC7.1INS− are derived from CP7-specific cDNA clones; pC7.1INS− is identical to pC7.1, except that the insertion is deleted. pN7.1 and pN7.1INS+ originate from cDNA clones of NCP7; pN7.1INS+ is based on pN7.1 but does contain the insertion identified in the CP7 sequence at the respective genomic positions. pep2 and pep6 indicate the relative positions of the synthetic peptides used to generate the respective antisera. (B) SDS-PAGE analysis after transient expression of pC7.1 or pN7.1 in BSR cells labeled with [^{35}S]methionine-cysteine after RIP. MDBK cells infected with BVDV CP7 or NCP7 served as controls. Numbers on the left indicate the molecular masses (in kilodaltons [K]) of marker proteins. The positions of NS2-3 and NS3 are marked by arrowheads. (C) SDS-PAGE analysis after transient expression of pC7.1 in BSR cells labeled with [^{35}S]methionine-cysteine after RIP. MDBK cells infected with BVDV CP7 served as controls. The 8-kDa protein precipitated by antiserum P1 was also detected in nontransfected T7 vaccinia virus-infected cells and is regarded as nonspecific. The positions of NS2, NS4A, and NS4B are marked by arrowheads. Numbers on the left indicate the molecular masses (in kilodaltons [K]) of marker proteins. +, presence; −, absence.

the induction of NS2-3 cleavage. All the variations identified in other cp BVDV polyproteins have been located near the assumed N terminus of NS3 (glycine 1590). In contrast, the insertion identified in the genome of CP7 is located far upstream from the corresponding genomic position. Nevertheless, it was tempting to speculate that the 9-aa insertion is involved in the generation of NS3 in CP7-infected cells. The processing of NS2-3 was further studied by transient expression of cDNA constructs using the T7 vaccinia virus system (16). All constructs were cloned downstream of a translational start codon under the control of a bacteriophage T7 RNA polymerase promoter (Fig. 4A). pC7.1 and pN7.1 contain cDNA fragments encoding parts of the polyproteins of CP7 and NCP7, respectively. These constructs encompass the genomic regions coding for the C-terminal third of E2 (gp53), NS2-3 (p125), NS4A (p10), and part of NS4B (p30) (Fig. 4A). BSR cells infected with the recombinant vaccinia virus vTF7-3 without being transfected with an expression plasmid served as controls. MDBK cells infected with BVDV CP7 or NCP7 were also included as controls. The translation products obtained after transient expression of pC7.1 or pN7.1 as well as proteins from CP7- or NCP7-infected cells are shown in Fig. 4B and C. NS3 and NS2-3 derived from BVDV-infected cells or transient expression in BSR cells were immunoprecipitated by antiserum A3 (Fig. 4B). The corresponding proteins comigrated during SDS-PAGE, indicating authentic processing in the T7 vaccinia virus system. Transient expression of pN7.1 and pC7.1 led to the generation of NS2-3 (Fig. 4B, lanes 3 and 5); in addition, the expression of a large amount of NS3 was detected after the transfection of pC7.1 (Fig. 4B, lane 3). NS2, the N-terminal processing product of NS2-3 expressed from pC7.1, could be demonstrated with antiserum pep2 (Fig. 4C). NS2 could also be precipitated with antiserum pep6 (Fig. 4C), generated in rabbits against a synthetic peptide representing aa 1571 to 1586 of the BVDV polyprotein.

In order to determine whether processing of the polyprotein downstream of NS3 takes place in an authentic way after transient expression, the generation of NS4A was investigated. Antiserum P1 was shown previously to recognize NS4A (p10) and NS4B (p30) in BVDV-infected cells (32). After precipitation from CP7-infected cells, NS4A exhibited an apparent molecular mass of 7 kDa; a corresponding polypeptide with the same migration behavior was identified after transient expression of pC7.1 (Fig. 4C). The C-terminally truncated NS4B* molecule encoded by pC7.1 could not be detected; it is possible that truncation destabilizes the NS4B* protein. Similar results were obtained for NS4A and NS4B* expressed from pN7.1 (data not shown). All processing events required for the generation of apparently authentic NS2, NS3, and NS4A could be demonstrated by using the transient T7 vaccinia virus expression system. Most importantly, the experiments showed that the information necessary for cp-specific cleavage of NS2-3 resides in the cDNA sequence included in construct pC7.1.

The insertion of 9 aa is essential and sufficient for the induction of NS2-3 cleavage. To determine the putative role of this insertion for the processing of NS2-3, pC7.1INS− was constructed. Except for a deletion encompassing the 9 aa of the insertion, the polyprotein encoded by this plasmid is identical to the one encoded by pC7.1 (Fig. 4A). After the expression of pC7.1INS−, the processing of NS2-3 no longer occurred, as judged by the absence of NS3 (Fig. 5, lane 2). Proteins with apparent molecular weights larger than that of NS2-3 were precipitated by antiserum A3 after the expression of pC7.1 or pN7.1. They represent most likely NS2-3 precursor molecules. These proteins were reactive with antiserum P1 (data not shown) and thus encompass the uncleaved C-terminal portion of the polyprotein.

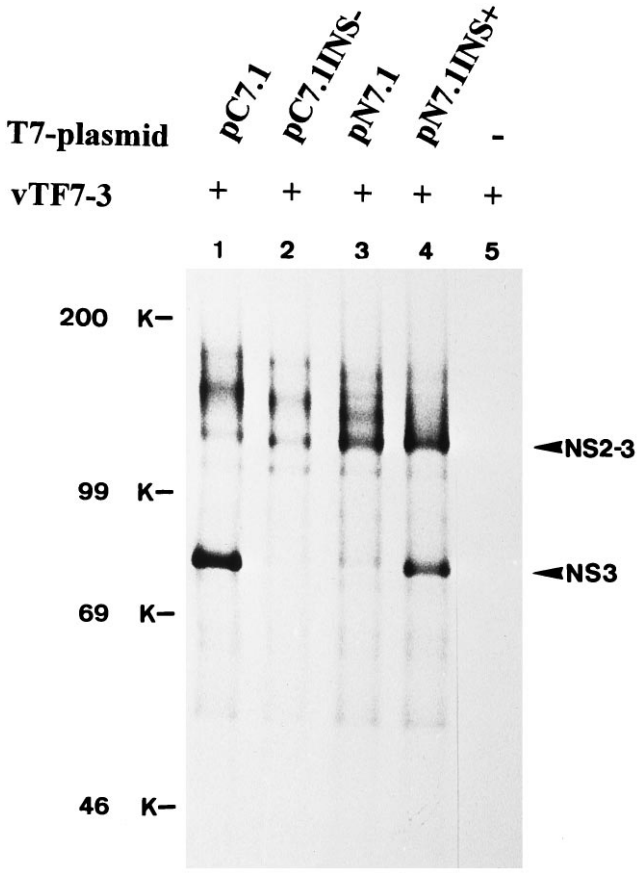

FIG. 5. SDS-PAGE analysis after transient expression in BSR cells. RIP was performed after labeling with [^{35}S]methionine-cysteine. Antiserum A3 was used for precipitation. Numbers on the left indicate the molecular masses (in kilodaltons [K]) of marker proteins. The positions of NS2-3 and NS3 are marked by arrowheads. +, presence; −, absence.

To investigate whether the insertion is sufficient for the induction of NS2-3 cleavage in the context of the NCP7 genome, pN7.1INS+ was constructed. This plasmid is based on pN7.1 and contains the 27-base insertion in the same genomic position at which it is found in the CP7 RNA. In contrast to the parental plasmid pN7.1, the expression of pN7.1INS+ resulted in the expression of large amounts of NS3, indicating the processing of NS2-3, as found for the corresponding protein encoded by pC7.1 (Fig. 5). Compared with the translation products of pC7.1, the expression of pN7.1INS+ yielded a larger amount of uncleaved NS2-3. This variation between pC7.1 and pN7.1INS+ may be caused by the different N termini of the polyproteins encoded by these constructs. Further experiments are necessary to clarify this point.

The NS2-3 serine protease. The serine protease residing in NS3 generates the C terminus of this protein and releases the viral nonstructural proteins downstream of NS3 from the precursor molecule (53). A role for the NS3 protease in the generation of its own N terminus in the polyprotein of CP7 cannot therefore be excluded at this point. Wiskerchen and Collett have shown that a mutation of the catalytic serine (serine 1752) to alanine inactivates the NS3 serine protease (53). The codon for serine 1752 was mutated to an alanine codon in plasmid pC7PM1, which is based on pC7.1. Antiserum P1 (32) precip-

itated free NS4A after the expression of pC7.1 (Fig. 6A, lane 2); in contrast, no release of NS4A was detected after the transfection of pC7PM1 in vTF7-3-infected cells (Fig. 6A), indicating that the NS3 protease encoded by pC7PM1 is inactivated by the mutation. Instead, the expression of this construct led to the generation of a high-molecular-mass (about 150 kDa) fusion protein, most likely composed of uncleaved NS2-3 and the downstream region of the polyprotein. In accordance with this assumption, this protein can be precipitated by antiserum A3 (Fig. 6B, lane 3), directed against NS3, as well as by antiserum P1 (lane 5). In addition, a protein with a molecular mass of about 92 kDa was precipitated by both sera (see below).

The question of highest interest was whether the cleavage of NS2-3 occurred despite inactivation of the NS3 serine protease. The processing of NS2-3 was studied by transient expression of pC7.1 and pC7PM1, followed by RIP with antiserum pep2. Importantly, NS2 was detected in both cases (Fig. 6A, lanes 4 and 5), demonstrating that the NS3 protease is not involved in the cleavage of NS2-3. Accordingly, the 92-kDa protein precipitated by antisera P1 and A3 after the expression of pC7PM1 most likely represents an NS3-NS4A/B fusion protein which has a calculated molecular mass of 95 kDa.

DISCUSSION

Recent studies have provided strong evidence for the evolvement of cp BVDV strains from noncp BVDV strains (31, 32, 39, 43, 49). It is generally accepted that cp BVDV strains are generated in persistently infected animals by RNA recombination; this step leads ultimately to fatal MD (50). Thus far, the expression of NS3 is the only common molecular marker for cp BVDV (4, 8, 37, 38). For several cp BVDV strains, the strategy for NS3 expression could be linked to distinct genetic changes present in the respective viral genomes (32, 48, 49). For example, a number of cp BVDV strains contain insertions of cellular ubiquitin coding sequences in their genomes (29, 30, 31, 39, 48). It has been demonstrated that ubiquitin acts as an additional processing signal in the corresponding viral polyprotein; cleavage at the respective site occurs by a cellular ubiquitin C-terminal hydrolase and leads to the release of NS3 (48). For other cp BVDV strains, the viral autoprotease N^{pro} is located within the polyprotein just upstream of NS3 (32, 49). In these cases, the viral protease mediates the generation of the N terminus of NS3. Accordingly, both host-cell-derived and virus-encoded proteases can be responsible for generation of the N terminus of NS3.

In this report, BVDV pair 7, consisting of CP7 and NCP7, has been characterized at the molecular level. The relevant mutation leading to the development of CP7 from NCP7 is most likely a 27-base insertion which is present in the NS2 coding region of CP7 but missing in the homologous part of the NCP7 genome. The insertion is of viral origin and represents a duplication of bases 4064 to 4090 inserted between the formerly neighboring nucleotides 4353 and 4354. These 27 bases encode different amino acids when the original position and the insertion are compared since the insertion occurred outside the original reading frame. This insertion is essential and sufficient for the induction of NS2-3 cleavage, resulting in the expression of NS2 and NS3. Final proof for the assumption that this insertion renders the virus cp can, however, be obtained only by manipulation of an infectious cDNA copy of the viral genome. Interestingly, the NS3 molecules of all cp BVDV strains tested so far, including CP7, exhibit the same apparent molecular weight (19) (unpublished data). This finding implies that the N termini of NS3 from all cp BVDV strains are highly

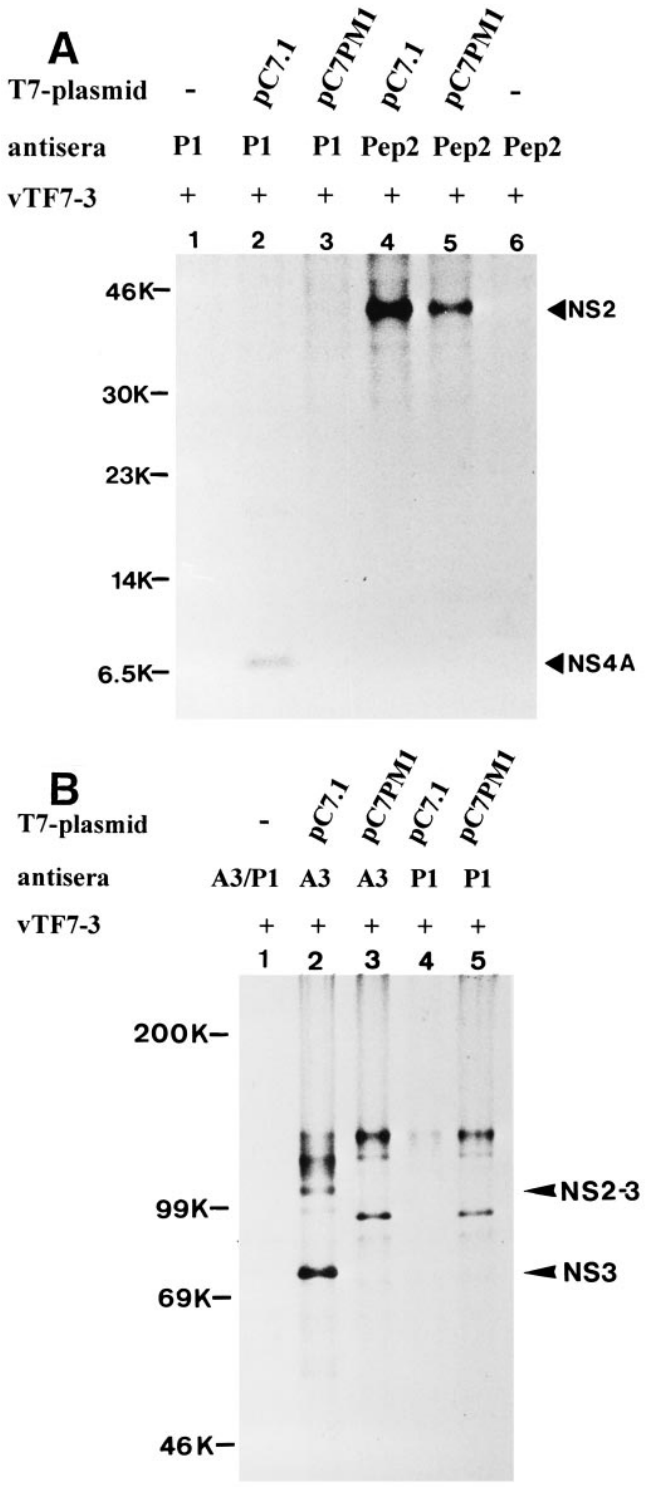

FIG. 6. SDS-PAGE analysis after transient expression of pC7.1 and pC7PM1 in BSR cells. RIP was performed after labeling with [^{35}S]methionine-cysteine. (A) For precipitation, antisera pep2 and P1 were used. Arrowheads indicate the positions of NS2 and NS4A. (B) Precipitation was carried out with antisera A3, pep2, and P1. The positions of NS2-3 and NS3 are indicated by arrowheads. Numbers on the left indicate the molecular masses (in kilodaltons [K]) of marker proteins. +, presence; −, absence.

similar and may be identical. For the cp BVDV strains mentioned above, the obtained experimental data provide evidence that the NS3 molecules start at the identical amino acid position, namely, glycine 1590 (number refers to the BVDV SD-1 sequence) (32, 48, 49). Since NS2 of strain CP7 specifically reacts with a peptide antiserum directed against aa 1571 to 1586 (pep6), the N terminus of NS3 should be located downstream of aa 1580 and thus might be close to glycine 1590. Protein sequencing has to be performed to determine the N terminus of NS3 of BVDV CP7.

As mentioned above, the polyprotein structures of some cp BVDV strains already suggested the protease responsible for generation of the N terminus of NS3. In contrast, the 9-aa insertion positioned far upstream of the cleavage site in NS2-3 of BVDV CP7 gave no hint as to the mechanism leading to NS2-3 cleavage. An obvious question is whether a virus- or a host-cell-encoded enzyme is responsible for NS2-3 cleavage. If a cellular enzyme is involved, one would assume that solely alterations in the conformation of the protein, induced by the 9-aa insertion, render NS2-3 of CP7 a suitable substrate for the respective host cell protease. On the other hand, a viral protease might be responsible for NS2-3 cleavage in CP7-infected cells. If the different genera of the family *Flaviviridae* are compared, it is surprising that noncp BVDV strains show no processing of NS2-3 since in the other two genera of this family cleavage of NS2-3 occurs in an obligatory way and is executed by viral enzymes. For members of the genus *Flavivirus*, cleavage of NS2-3 is carried out by the NS3 protease, with NS2B as an essential cofactor (41, 42). In the N-terminal region of the pestiviral NS3, a serine protease also resides (17, 53); this protease is responsible for the generation of its own C terminus and releases the nonstructural proteins downstream of NS3 from the precursor molecule. Therefore, a role for the NS3 protease in the cleavage of NS2-3 of cp BVDV, comparable to the situation described for flaviviruses, is conceivable. For cp BVDV strain NADL, a role for NS3 in NS2-3 cleavage was proposed, but the available data are controversial (36, 53). Since mutational inactivation of the NS3 serine protease of BVDV CP7 does not impair NS2-3 cleavage, another proteolytic activity is responsible for the respective cleavage in CP7-infected cells.

On the basis of studies of polyprotein processing in the HCV system, a different mechanism for the generation of NS3 in cp BVDV strains of the CP7 type can be envisioned. In the HCV system, NS2-3 is not processed by the NS3 serine protease. Instead, the respective cleavage is mediated by a putative metalloproteinase composed of NS2 and the N-terminal domain of NS3 (18, 22–24, 40, 46). It is tempting to speculate that a similar intrinsic proteolytic activity exists in the NS2-3 molecules of certain pestiviruses like cp BVDV CP7. In this context, it is interesting that some noncp classical swine fever virus strains (e.g., Alfort Tübingen) and noncp border disease virus strains (e.g., X818) show low rates of NS2-3 cleavage (2), although in their NS2 proteins no strain-specific insertions could be identified. A recent study has also demonstrated that cp BVDV strains Oregon and Singer show no insertions in their NS2 proteins and no notable amino acid substitutions in the region of the putative cleavage site, although the processing of NS2-3 takes place (35). Polyprotein processing studies need to show whether in these cases the information necessary for NS2-3 cleavage also resides in NS2. The occurrence of NS2-3 cleavage in the absence of any insertions supports the hypothesis that an intrinsic proteolytic activity is located in NS2. However, the de novo generation of an active protease by the insertion of a 9-aa sequence or even by the accumulation of point mutations is highly unlikely. Comparisons with other genera of the family *Flaviviridae* lead us to the speculation that pestiviruses once owned a protease capable of cleaving NS2-3. Accordingly, the enzyme would be present in a latent form and be reactivated even by subtle changes in NS2, such as that found in cp BVDV CP7. Only noncp pestiviruses, not cp isolates, are able to induce lifelong persistent infections in animals. This represents an obvious advantage with respect to survival and spread in the host population and may have been the driving force for a reduction in NS2-3 cleavage. NS2-3 of BVDV CP7 is an interesting candidate for further studies that aim at the identification of the relevant protease. Clarification of the mechanism responsible for the generation of NS3 in pestiviruses of the CP7 type might provide insight into a new aspect of the evolution of pestiviruses.

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